

**INVESTIGATION OF ANTIMICROBIALS FROM NATIVE
BRITISH PLANTS USED IN 10TH CENTURY
ANGLO-SAXON WOUND HEALING FORMULATIONS**

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Abstract

Chinese and Indian cultures consider their ancient herbal texts valuable resources in the search for novel compounds with potential pharmacological applications. However, despite a rich history of medicinal plant use throughout the British Isles, much of the native flora recorded in the Anglo-Saxon medical texts (the *Herbarium*, Bald's *Leechbook* and the *Lacnunga*) studied here has yet to be evaluated. A model was developed for bio-screening medicinal plants using <30 g of dried material for bioassay and structure elucidation. In this study, six native species were screened using a 96-well microdilution bioassay (200, 40 and 8 µg/mL) against representative strains of Gram-positive and Gram-negative pathogens commonly found in wounds. All plants exhibited activity against Gram-positive organisms in one or more leaf extracts (MIC₅₀ 200 µg/mL). Of the 8 extracts per plant, the 25% and 75% EtOH root extracts of *Agrimonia eupatoria* L. and *Potentilla reptans* L. were effective against *S. aureus* at the lower concentration (MIC₅₀ 8 µg/mL) and inhibited growth of Gram-negative pathogen *E. coli* (MIC₅₀ 40 µg/mL). *P. reptans* was selected for further investigation on the basis of its activity against *E. coli* and underreported phytochemistry compared to *A. eupatoria*. In the MIC assay the 75% EtOH root extract of *P. reptans* demonstrated the widest range of activity against *S. aureus* (MIC₅₀ 31.25 – full MIC of 1000 µg/mL). The *P. reptans* root decoction was the most potent extract against *E. coli* (MIC₅₀ 3.9 µg/mL) and comparable to chloramphenicol, a broad spectrum antibiotic. Principal components analysis (PCA) when overlaid with antimicrobial activity, directed the optimisation of the HPLC and LC-MS methods. Seven antimicrobial compounds were putatively identified for *P. reptans* (agrimonolide-6-*O*-glucopyranoside, chlorogenic acid, ellagic acid, epicatechin, procyanidin B, procyanidin C, and tormentic acid). This study has shown some native species in the Anglo-Saxon formulations may have been effective for treating bacterial infection in wounds and that the medical texts are a valuable source for rediscovering plants and medicinal uses lost to Western herbal practice.

Table of Contents

Chapter 1.0 Anglo-Saxon Medical Literature

1.1 Anglo-Saxon England (499-1066)	1
1.1.1 Lifestyle and Disease	2
1.1.2 The Anglo-Saxon Practitioner (<i>Laece</i>) or Medic	3
1.2 Anglo-Saxon Medical Texts	4
1.2.1 Modern English Translations	4
1.2.2 The <i>Herbarium</i>	6
1.2.3 Bald's <i>Leechbook</i>	6
1.2.4 The <i>Lacnunga</i>	8
1.2.5 Botanical Identification of the Anglo-Saxon plants	8
1.2.6 Anglo-Saxon Herbal Formulations	9
1.3 Battle Wounds and Injuries	13
1.4 Scope of Research	14

Chapter 2.0 Plant Selection Strategy

2.1 Introduction	16
2.2 Materials and Methods	19
2.2.1 Literature Search	19
2.2.2 Design of Selection Methodology	19
2.2.3 Plant Locations	20
2.2.4 Plant Collection	21
2.2.5 Drying of Plant Material	21
2.2.6 Preparation of Herbaria	21
2.3 Results	23
2.3.1 Selection Strategy	23
2.3.2 Plant Locations	26
2.3.3 Plant Collection	39
2.4 Discussion	40

Chapter 3.0 Antimicrobial Bioassays

3.1 Introduction	44
3.2 Materials and Methods	47
3.2.1 Chemicals	47
3.2.2 Extraction of Plant Material	47
3.2.3 Microbial Strains	48
3.2.4 Bacterial Cultures	48
3.2.5 Antimicrobial Assay	49
3.2.6 Statistical Analysis of Data	50
3.3 Results	51
3.3.1 Extraction of Plant Material	51
3.3.2 Antimicrobial Assay	51
3.4 Discussion	57

Chapter 4.0 Plant Metabolite Mapping	
4.1 Introduction	61
4.2 Materials and Methods	64
4.2.1 Materials	64
4.2.2 HPLC Method	64
4.2.3 Principal Components Analysis (PCA)	65
4.3 Results	66
4.3.1 HPLC Method	66
4.3.2 HPLC-PCA Metabolite Mapping	71
4.4 Discussion	74
Chapter 5.0 Phytochemistry of <i>Potentilla reptans</i>	
5.1 Introduction	76
5.2 Material and Methods	79
5.2.1 Antimicrobial Assay (MIC and MBC)	79
5.2.2 Optimised HPLC Method	79
5.2.3 Optimised HPLC-PCA	80
5.2.4 Liquid Chromatography Mass Spectrometry (LC-MS)	80
5.2.5 High Resolution Electrospray Ionisation Mass Spectrometry (HR-ESI-MS)	81
5.2.6 Identification of Unknowns	82
5.3 Results and Discussion	83
5.3.1 Antimicrobial Assay	83
5.3.2 Optimised HPLC Method	83
5.3.3 HPLC-PCA Mapping of Optimised Data	85
5.3.4 LC-MS and HR-ESI-MS Analysis	87
5.3.4.1 Compounds A,B and C	90
5.3.4.2 Compounds D and E	91
5.3.4.3 Compounds F and G	93
5.4 Conclusion	96
Chapter 6.0 Conclusions	98
6.1 Specific Future Work	102
References	103
Appendices	118

List of Tables

Table 1.1	The major Anglo-Saxon medical texts of herbal formulations compiled in 10th century England.	5
Table 1.2	Vehicles used in 10th century Anglo-Saxon formulations.	11
Table 2.1	Ethnomedical and antimicrobial records for 12 plants used in three or more 10th century medical text for treating wounds and bacterial infections.	25
Table 2.2	Phytotherapy Monograph of <i>Agrimonia eupatoria</i> L.	27
Table 2.3	Phytotherapy Monograph of <i>Arctium minus</i> (Hill) Bernh.	29
Table 2.4	Phytotherapy Monograph of <i>Betonica officinalis</i> L.	31
Table 2.5	Phytotherapy Monograph of <i>Centaurium erythraea</i> Rafn.	33
Table 2.6	Phytotherapy Monograph of <i>Plantago media</i> L.	35
Table 2.7	Phytotherapy Monograph of <i>Potentilla reptans</i> L.	37
Table 3.1	The physiological mechanisms of natural product antibiotics against Gram-positive and Gram-negative bacteria commonly found in wounds.	45
Table 3.2	P values (<0.05) for Shapiro/Wilk's test to determine normality in the distribution of plant extracts.	51
Table 3.3	IC ₅₀ values for plant extracts against Gram-positive and Gram-negative bacteria.	52
Table 4.1	HPLC-UV _{210 nm} retention times for chemical standards and corresponding peaks in complex plant mixtures.	62
Table 5.1	Main phytochemistry reported for roots in <i>Potentilla</i> species.	77
Table 5.2	The MIC ₅₀ values (µg/mL) of <i>P. reptans</i> root extracts for growth inhibition against Gram-positive and Gram-negative pathogens.	83
Table 5.3	HR-ESI-MS peak retention times, chemical formulae and putative compounds for main chromatographic peaks.	89
Appendix 2.1	Experimental codes assigned to leaf and root plant extracts using EtOH, red wine and H ₂ O.	120
Appendix 3.2	Dried yield (mg) from 2 g of leaf and root extracts.	122

List of Figures

Fig. 1.1	The English Kingdoms during 10th century (Lapidge et al., 2001)	2
Fig. 1.2	Plate IV featuring Wagbraede (<i>Plantago major</i> L.) in the Old English Illustrated Pharmacopoeia, British Library, Cotton Vitellius C. iii, fol 21v (D'Aronco and Cameron, 1998).	7
Fig. 1.3	Decorated initials for Anglo-Saxon formulations in Balds <i>Leechbook</i> , Royal 12 D XVII ff. 20v-21.	12
Fig. 1.4	Project plan to determine the antimicrobial activity of six native British plants used in 10th century Anglo-Saxon wound healing formulations	15
Fig. 2.1	The selection model designed to determine six plants for an antimicrobial study of native British flora in 10 th century Anglo-Saxon wound healing formulations.	23
Fig. 2.2	Harvesting sites for plant collected in 2010 and 2011.	39
Fig. 3.1	96-well microtitre plate layout for each plant comprising 6 aqueous/EtOH extracts at screening concentrations of 200, 40 and 8 µg/mL.	50
Fig. 3.2	<i>S. aureus</i> antimicrobial screening results for root extracts.	55
Fig. 3.3	<i>E. coli</i> antimicrobial screening results for root extracts.	56
Fig. 4.1	Biosynthetic pathways for main chemical classes of primary and secondary metabolites.	62
Fig. 4.2	HPLC-UV _{254 nm} chromatograms of root extracts (2 mg/mL).	67
Fig. 4.3	HPLC-UV _{254 nm} chromatograms of leaf and root extracts for <i>A. minus</i> , <i>A. eupatoria</i> and <i>P. reptans</i> .	70
Fig. 4.4	HPLC-PCA _{210 nm} score plot for <i>P. reptans</i> leaf and root extracts overlaid with antimicrobial activity against <i>S. aureus</i> .	72
Fig. 4.5	HPLC-PCA _{210 nm} loading plot for <i>P. reptans</i> peaks.	73
Fig. 5.1	Chemical compounds from the leaves of <i>P. reptans</i> .	78

Fig. 5.2	HPLC-UV $_{210\text{ nm}}$ chromatogram of <i>P. reptans</i> EtOH root extracts for the initial and optimised HPLC methods.	84
Fig. 5.3	Contribution line plot for <i>P. reptans</i> PC1 (optimised HPLC data).	86
Fig. 5.4	HR-MS chromatogram v positive ionisation mode for <i>P. reptans</i> 75% EtOH and root decoction.	88
Fig. 5.5	Putative compounds for <i>P. reptans</i> root decoction and 75% EtOH root extracts that matched Kew in house spectral library.	92
Fig. 5.6	Product ion fragment pattern for base peak at m/z 489.1 present in <i>P. reptans</i> root decoction and 75% EtOH extracts.	94
Fig. 5.7	Putative compound in <i>P. reptans</i> root decoction from base peak at m/z 315.122.	97
Appendix 1.1	An example of <i>A. eupatoria</i> herbarium label prepared for specimens deposited at Kew.	118
Appendix 1.2	Herbaria sheet for <i>P. reptans</i> (voucher number FMW002).	119
Appendix 3.1	Confirmation letter from Health Protection Agency for <i>E. coli</i> strain (UEL 57).	121
Appendix 3.3	<i>S. aureus</i> antimicrobial screening results for leaf extracts.	123
Appendix 3.4	<i>B. subtilis</i> antimicrobial screening results for leaf extracts.	124
Appendix 3.5	<i>E. coli</i> antimicrobial screening results for leaf extracts.	125
Appendix 3.6	<i>P. aeruginosa</i> antimicrobial screening results for leaf extracts.	126
Appendix 3.7	<i>B. subtilis</i> antimicrobial screening results for root extracts.	127
Appendix 3.8	<i>P. aeruginosa</i> antimicrobial screening results for root extracts.	128
Appendix 4.1	HPLC-UV $_{254\text{ nm}}$ chromatograms for leaf extracts (2 mg/mL).	129
Appendix 4.2	Optimised HPLC-PCA $_{210\text{ nm}}$ score plot for <i>P. reptans</i> extracts overlaid with antimicrobial activity against Gram-positive <i>S. aureus</i> .	130

Appendix 5.1	HR-ESI-MS chromatograms in negative ionisation mode for <i>P. reptans</i> .	131
Appendix 5.2	Product scan for a molecular formula of $C_{14}H_6O_8$ that may be ellagic acid.	132
Appendix 6.1	Review of plants used in 10 th century Anglo-Saxon formulations published in Drug Discovery Today.	133
Appendix 6.2	Paper titled 'Antimicrobial assays of three native British plants used in Anglo-Saxon medicine for wound healing formulations in 10 th century England', published in the Journal of Ethnopharmacology.	134
Appendix 6.3	Frances Watkins' Declaration 31 May 2013.	135

List of Abbreviations

Amu	Atomic mass unit
BHP	British Herbal Pharmacopoeia
BSBI	Botanical Society of the British Isles
Cfu	Colony forming units
CSV	Comma separated values
CV	Coefficient of variation
EtOH	Ethanol
ESCOP	European Scientific Cooperative on Phytotherapy
h	Hours
H ₂ O	Water
HR-ESI-MS	High resolution electrospray mass spectrometry
HPLC	High performance liquid chromatography
IC ₅₀	Concentration of inhibition where the response is reduced by 50 per cent
i.d.	In diameter
LC-MS	Liquid chromatography mass spectrometry
LPS	Lipopolysaccharides
mau	Milli absorbance units
MDA	Multivariate data analysis
MeOH	Methanol
mg	Milligrams
MIC	Minimum inhibitory concentration
MIC ₅₀	50% inhibitory growth of organism in a MIC assay
MIC ₉₀	90% inhibitory growth of organism in a MIC assay
min	Minutes
<i>m/z</i>	Mass-to-charge ratio
NCCLS	National Committee for Clinical Laboratory Standards
NMR	Nuclear magnetic resonance spectroscopy
PCA	Principal components analysis
PBGI	Percentage of bacterial growth inhibition
SEM	Standard error of the mean
<i>t_R</i>	HPLC retention time
µg	Micrograms
µg/mL	Micrograms per millilitre
UEL	University of East London
UV	Ultraviolet
Vol	By volume
WHM	Western Herbal Medicine

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Chapter 1.0 Anglo-Saxon Medical Literature

The pharmaceutical search for novel plant compounds has predominantly focussed on countries where the flora is highly specialised to its specific environment and mainly undiscovered, increasing the potential of finding something new and reducing the likelihood of competitors researching the same material (Schmidt et al., 2007). The British Isles shares a common biodiversity of plants with Europe, covering a relatively large land mass and consequently; native species have been neglected in the search for novel compounds with potential pharmacological applications. Chinese and Indian cultures consider their ancient medicinal texts to be valuable sources for drug discovery whereas in Britain, Anglo-Saxon medical texts have been under-researched. New translations now provide greater access to the early medieval literature for scientific investigation of medicinal formulations used in this Western herbal tradition (Watkins et al., 2011). Western herbal medicine (WHM) is a recent term to describe the use of medicinal plants mainly native to Europe within herbal practice grounded in local tradition (Nissen and Evans, 2012).

1.1 Anglo-Saxon England (499-1066)

Following the departure of the Romans from England, the Anglo-Saxons faced many battles with the Vikings and by 10th century settlements had formed around monastic sites and sea ports (Fig. 1.1). King Alfred is believed to have commissioned the Anglo-Saxon chronicle to document major battles, events and natural phenomenon from the birth of Christ; a tradition that was continued into the 12th century (Whitelock et al., 1961). When Alfred came to rule (871-99) there was a distinct lack of literacy in the land. He pioneered an educational renaissance by bringing scholars from overseas and setting up a palace school although by the 10th century, the remit of education had returned to the monasteries (Lapidge et al., 2001). The surviving 10th century medical texts are important medical records in that they were written in the vernacular; a unique practice not seen elsewhere in Europe during this period (Meaney, 2000; Payne, 1904).

1.1.1 Lifestyle and Disease

Battle wounds, amputations and burns were common place and eye, skin and urinary problems consistently feature in the medical texts suggesting deficiency in vitamin A during the winter months and also vitamin C would have resulted in bleeding gums, scurvy, ulcers and dysentery (Cayton, 1977; Hagen, 2006). Disease encompassed plague and leprosy; viral infections or '*flying venoms*' and a variety of parasites, especially '*burrowing worms*' (Cameron, 2006; Hart 2003). Illness was viewed as a range of symptoms creating an imbalance within the body and the prescribed medication having the capacity to warm or cool the body in order for the patient to regain a balance of health based on classical texts, observations and therapeutics (Meaney, 1991). The Anglo-Saxon aristocracy ate fresh meat and fish in spring/summer and salted food during winter. For the poor, the diet comprised mainly grain, of barley and wheat to provide bread and leafy vegetables and nuts when in season. The monastic communities had a varied diet and monks were taught signs so as to request food during periods of silence (Banham, 2002; Hagen, 2006).

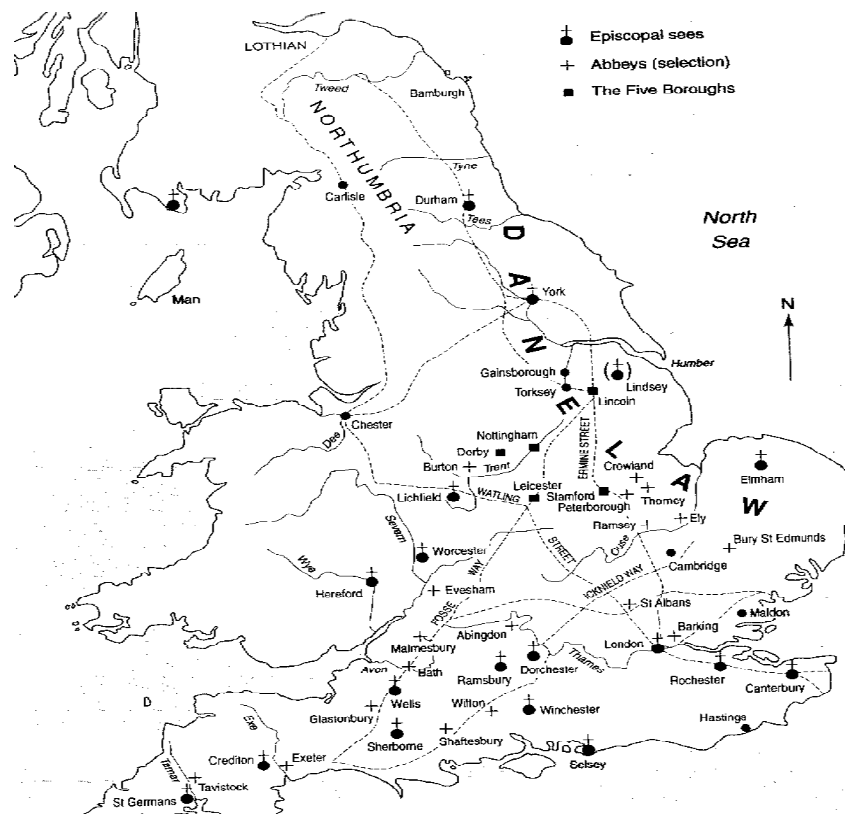


Fig. 1.1 The English Kingdoms during 10th century (Lapidge et al., 2001).

1.1.2 The Anglo-Saxon Practitioner (*Laece*) or Medic

For more than half a millennium battles, disease, droughts and famines in Anglo-Saxon England created great demands upon human health and the physicians attending the wounded and the sick. Evidence suggests multiple layers of medical practice: learned physicians or medics to attend the royal household; physicians without tonsure depicted in illustrated manuscripts in addition to practicing members of the clergy and monastic orders (Hart, 2003; Meaney, 2000) as well as folklore plant knowledge transferred by oral tradition (Allen and Hatfield, 2004). This structure of medical practice was evident in the time of Dioscorides during the 1st century and is often seen in indigenous cultures around the world (Riddle, 2011).

There are only seven medics or practitioners named in the Anglo-Saxon literature. Oxa and Dun are two named physicians in Bald's *Leechbook* although very little is known about the teaching of herbal practices at this time (Banham, 2002; Cockayne, 1865). The surviving Anglo-Saxon medical texts were part of a wider body of literature available to the Anglo-Saxon practitioner as in 'some books teach for the half dead disease...' (Cockayne, 1865, p.285). Practitioners were able to call upon Greek and Latin texts to prescribe herbal exotics for the rich that could afford to pay high prices whilst using common plants for the poor (Talbot, 1967). London was a vibrant trade centre exchanging goods with Europe, Africa and the East and exotic spices were highly valued (Hagen, 2006). The Anglo-Saxon practitioner would have required a prior knowledge of plants and herbal practices to be confident in his ability to treat disease and charge for services. As early as the 7th century Anglo-Saxon law established that should a person be injured as a result of another the perpetrator would incur the *laeceföh* or 'doctors fee' (Meaney, 2000; Pollington, 2008; Rubin, 1970).

1.2 Anglo-Saxon Medical Texts

Three of the four major early medieval texts reporting herbal formulations from 10th century England, the *Herbarium*, Bald's *Leechbook* and the *Lacnunga*, may contain insights into medicinal uses of native species yet to be evaluated (Watkins et al., 2011). These texts were compiled for medicinal use and demonstrate the breadth of medical knowledge available to those able to read in the vernacular. The compilers of the original texts were experts with a good understanding of plant names, medicinal applications and multiple languages. Bilingual glosses dedicated to plant names in Latin/Old English such as in the *Durham Herbal Glossary* and *Laud Herbal Glossary* were both in circulation in the early medieval period (D'Aronco and Cameron, 1998). There is also evidence of foreign texts being exchanged between monastic houses as physicians travelled across Europe, Africa and the Middle East (Voigts, 1979). Access to these texts was limited to readers of Old English until the 19th century when philologist Thomas Oswald Cockayne of Kings' College London, was commissioned by the British government to translate all of the known medical Anglo-Saxon manuscripts into modern English.

1.2.1 Modern English Translations

Cockayne's translation of 1864-6 is to date, the only complete English edition albeit other authors have produced more recent translations of individual texts (Cameron, 2006; Meaney, 1984, Van Arsdall, 2002). The harsh views of Charles Singer and his student, Wilfred Bonser in the middle of the last century, did much to undermine the value of the Anglo-Saxon medical literature. Bonser, said they were 'sterile formulas, which could be applied without any exercise of reasoning, alone survived for use during the Dark Ages' (Cameron, 2006; Talbot, 1967; Voigts, 1979). Around fifty years ago Anglo-Saxon historians and linguists redefined the contextual position for the original manuscripts and modern translations followed for the *Herbarium*, Bald's *Leechbook III* and the *Lacnunga*. (Cameron, 1982; Meaney, 2000; Pettit, 2001, Pollington, 2008; Van Arsdall, 2002). Today, over five hundred leaves of medical manuscripts, compiled in Old English, survive in national and private collections. Of these, three major works (Table 1.1) were compiled by physicians for physicians,

Table 1.1 The major Anglo-Saxon medical texts of herbal formulations compiled in 10th century England.

Anglo-Saxon Medical Text	Pagination	Content List	Number of Chapters	Number of Formulations	Number of Plants
The Old English <i>Herbarium</i> – a compilation of two texts: Pseudo-Appellius and Pseudo-Dioscorides (British Library, Cotton Vitellius C.III)	By plant and number of formulations	✓	185 ^{af}	605 ^c	185 ^{acf}
Bald's Leechbook I (British Library, MS Royal, 12 D XVII)	By condition head to foot	✓	138 ^{ab}	557 ^c	~196 ^a
Bald's Leechbook II (British Library, MS Royal, 12 D XVII)	By condition head to foot	✓	67 ^a	274 ^c	~129 ^a
Bald's Leechbook III (British Library, MS Royal, 12 D XVII)	By condition head to foot	✓	76 ^{ae}	149 ^c	~142 ^a
The <i>Lacnunga</i> (British Library, MS Harley 585)	By condition head to foot		191 ^{ad}	186 ^c	~250 ^e

Cockayne (1865)^a, Deegan (1988)^b, Meaney (2011)^c, Pettit (2001)^d, Pollington (2008)^e and Van Arsdall (2002)^f. The number of plant species has been calculated manually from the individual plant lists. There may be some duplication and/or unknowns and are therefore approximate. Bald's *Leechbooks* I, II and III are collectively known as Bald's *Leechbook*. Listing of disease from head to foot is Greco-Roman system of medicine (Cameron, 1982; Crawford, 2010).

describe herbal formulations for common conditions affecting human and animal health in England during 10th century (Cameron, 2006; Meaney, 2000; Voigts, 1979).

1.2.2 The *Herbarium*

The Old English translations of *Pseudo-Apuleius*, *Pseudo-Dioscorides De Herbis Femininis* and the *Curae Herbarium* are today jointly referred to as the *Herbarium*. The only surviving illustrated herbal from the 10th century (British Library: Cotton Vitellius C.iii) was compiled from an earlier Latin herbal (4-5th century) demonstrating this was an important medical text, possibly in general circulation for physicians (Meaney, 2000; Van Arsdall, 2002; Voigts, 1979). The author of this text edited and reordered the Latin literature and was selective in the final content in retaining only 29 of the 47 original formulations for *Betonica officinalis* (Voigts, 1979). The *Herbarium* lists a number of herbal formulations for each plant and for some native species specified the habitat and time of harvesting suggesting the Anglo- Saxon practitioner used an empirical approach to plant collection (Cockayne, 1864; Van Arsdall, 2002). The Old English *Herbarium* included a table of contents not seen in the Latin originals and with numerous annotations in the margins, illustrate it was designed for use as a working herbal with 101 of the 185 plants found in Bald's *Leechbook* (D'Aronco and Cameron, 1998).

1.2.3 Bald's *Leechbook*

The copy of Bald's *Leechbook* (British Library: Royal 12, D. xvii) is a unique text written in the middle of the 10th century believed to be from an earlier copy commissioned by King Alfred in the late 9th century. This gold standard literature assimilates the best of medical knowledge written in the vernacular for use by physicians to attend the royal household (Banham, 2011; Meaney, 2000; Voigts, 1979). The owner of the book, Bald, is mentioned in the preface and Cild as his scribe although it is not known if Bald was a physician and/or monk. Bald's *Leechbook* is a collection of two volumes carefully ordered by conditions starting at the head and moving down to the toes. The former is concerned with mainly external complaints and the second devoted to internal ailments. The second volume includes lengthy discussions on varying aspects of health that



Fig. 1.2 Plate IV featuring Wagbraede (*Plantago major* L.) in the Old English Illustrated copy of the *Herbarium* (D'Aronco and Cameron, 1998). © British Library, Cotton Vitellius C. iii, folio 21v (with permission of the British Library and publishers Rosenkilde & Bagger).

demonstrate an understanding of body functions including the liver (Cockayne, 1865; Cayton, 1977; Pollington, 2008). There is a reference to the quality of practitioners 'unwise leeches ween, that is loin disease, or milt wark: but it is not so...' (Cockayne, 1865, p.233) suggesting Bald's *Leechbook* was the authority on such matters. A third volume referred to as *Leechbook III* is considered to be older in origin than the other two volumes with less of a Mediterranean influence with less bloodletting and cautery and more miscellaneous incantations/ prayers (Cameron, 2006; Meaney, 2011; Pollington, 2008).

1.2.4 The *Lacnunga*

A lesser known semi-complete collection of 193 leaves commonly referred to as the *Lacnunga* (British Library, MS Harley 585) is thought to have been compiled by a practitioner for use by a practitioner although the owner and scribe are unknown. The collection includes a number of remedies from all three volumes of Bald's *Leechbook* and is therefore considered of a later date although it does not include a contents list. The collection is of a lesser quality compared to Bald's *Leechbook* and derived from a text that no longer survives. A number of folios have scribal corrections in different hands, illustrating that the texts were used and updated over time (Cockayne, 1864; Meaney, 2000; Pettit, 2001). This transfer of knowledge by copying and amending of formulations has been common practice throughout history and a large number of Old English formulae are known to have been in circulation prior to 9th century (Cameron, 1982; Meaney, 1991).

1.2.5 Botanical Identification of the Anglo-Saxon plants

The identification of plants in the texts has caused much debate. Ambiguity does arise where the Anglo-Saxons used a collective name for different species as in *docce* or dock for a group of plants with similar medicinal actions so we cannot know precisely which plant was being referred to in the formulae (Banham, 2002; Cameron, 2006; Pollington, 2008). A study of plant names from medieval manuscripts (Hunt, 1989) revealed more than 1800 vernacular names covering over 600 plant species demonstrating the difficulty in correctly assigning plant names. There is further confusion regarding some entries in the

Herbarium whereby the accompanying illustrations refer to a subsequent remedy or may be an incorrect translation of the original text (Cameron, 2006; Meaney, 2000; Voigts, 1979). Charles Singer believed the plant illustrations to be of no use and not a true likeness to the actual species, although Van Arsdall (2002) argues that the stylised illustrations depict dried specimens that would have been useful to a practitioner working with plants (Fig. 1.2). A number of entries have a definite space left for the synonym to be inserted as in ‘for a swollen stomach, take the seeds of this plant which is called *nymfeta* or ... (Van Arsdall, 2002, p.179) indicating that the scribe was unfamiliar with the plant in question and/or had other sources to consult (Cockayne, 1865, Van Arsdall, 2002). Plants mentioned in the Anglo-Saxon medical literature now known to have therapeutic properties and used in drug development have been described previously (Watkins et al., 2011).

Medicinal plants could have been identified from a number of sources: in both Bald’s *Leechbook* and the *Lacnunga* the plant names are predominantly Old English (Pettit, 2001). By contrast the illustrated copy of the *Herbarium* gives a stylised colour drawing for each plant along with a Greek or Latin name and a list of synonymies at the end of each entry including Gaulish, Etruscan, Dacian, Spanish, African and Syrian plant names ending with an Anglo-Saxon synonym if known (D’Aronco and Cameron, 1998; Meaney, 2000); a practice also found in early works of Dioscorides (Hunt, 1989; Riddle, 2011).

1.2.6 Anglo-Saxon Herbal Formulations

The classical Latin texts comprised of mainly simples whilst Bald’s *Leechbook* and the *Lacnunga* contain a combination of simple and complex mixtures perhaps demonstrating an evolutionary pattern of ethnomedica as in Traditional Chinese Medicine literature, arguably one of the oldest documented herbal traditions (Riddle, 2011). D’Aronco and Cameron (1998) state that >130 plant names in the illustrated *Herbarium* are to be found in modern Western herbal pharmacopoeias; for example *Centaureum erythraea* Rafn. and *Marrubium vulgare* L. both continue to be used in WHM (Barker, 2001; Toby et al., 2011; Williamson, 2003). Bald’s *Leechbook* and the *Lacnunga* include >20 native British tree species not referenced in the *Herbarium* and utilise a larger materia

medica with >250 medicinal plants that are also evident in the earlier pre Christian *Leechbook* III (Cockayne, 1865; Pettit, 2001).

Bald's *Leechbook* is an example of an improved text that has incorporated five formulae from the earlier *Leechbook* III and similarly there are several formulae in the later collection of *Lacnunga* that also appear in Bald's *Leechbook* (Meaney, 1991). The copying and amending of formulations is evident in later 16th and 17th century European herbals (Adams et al., 2009), '*Culpeper's Complete Herbal for the English Physician*' (1653) and more recently, '*A Modern Herbal*' (Grieve, 1931), all containing formulations similar to those in the Anglo-Saxon medical texts although the source is rarely attributed. Early medieval medical treatment was empirical, pragmatic and sometimes effective using imbalance as an explanation of illness (Cameron, 2006; Meaney, 1991; Payne, 1904). Betony or *B. officinalis* L. is the most frequently cited plant in all of the medical texts and whilst the Anglo-Saxon scribe reduced the number of entries from 47 in the Latin copy to 29 in the *Herbarium*; it is still the largest number of formulae allocated to a single plant in the Old English medical literature (Cayton, 1977; Van Arsdall, 2002; Voigts, 1979).

In *Bald's Leechbook*, the reader is encouraged to observe the patient and apply a greater or lesser amount of the formulation according to the constitution of the man, woman or child (Cockayne, 1865, p.85). Herbal formulae comprised 'simples' or single plant remedies as well as complex mixtures (Table 1.2) as herb drinks, creams, salves and poultices at the heart of this medical practice (Crawford, 2010). The whole plant or specified parts including leaves, flowers, roots, seeds and juice were prepared as infusions and decoctions, classified as 'wort' or 'spewit' drinks for internal consumption; applied topically pounded or mixed in animal grease for ointments and salves or mixed with grain and binded on as a poultice. Bald's *Leechbook* states that inflamed injuries from wounds, cuttings or blows were to be debrided and a barley poultice applied 'in the manner that leeches well know' and foul wounds to be cleaned using honey and salt mixed in a clean vessel and shaken until the consistency of a herb drink (Cockayne, 1865, p.83). For burns, salves from grease or butter were applied with one formula recommending the frequent application of an egg white to the wound (p.131). Crawford (2010) believes both

Table 1.2 Vehicles used in 10th century Anglo-Saxon formulations.

Type of Formulation	Vehicles	Example Formulation
Herbal Drinks comprising 'brewits' and 'spewits'	Water, ale, wine, milk	<i>'A wound drink: pound small the netherward and upward part of ribwort, carline thistle, and the netherward part of asthroath, put them in boiling water, rub between the hand and strain through a cloth, administer to drink', (Balds Leechbook, xxxviii)^a</i>
Washes for cleaning wounds	Water, honey, vinegar	<i>'If a cancer sore grows on a wound, take the plant, boil it gently in water, and bathe the wound with it. Then take the plant, soap and grease; pound them in vinegar. Put this on a cloth and lay it on the wound', (Herbarium, 37)^c</i>
Salves	Butter, old pig grease, chicken and fox grease, honey	<i>'If a man be smitten with wood or with stone, or if a boil bursteth on a man, for this a sound salve: cockle, 'ontre', silverweed, turnsole, pound the worts thoroughly, mingle well with butter, and prepare in the same way which before I quoth', (Balds Leechbook, xxxviii)^a</i>
Poultices	Bread, old pig grease, barley, bean, oat	<i>'If any part of the body becomes stiff, take the plant that is called lapatium or dock, some aged pig's grease and crumbs from oven-baked bread. Pound this together in the manner you would make a poultice, lay it on the sore place, and it helps wonderfully', (Herbarium, 34)^c</i> <i>'For the great oozing foot disease, which doctors call podagra. Take groundsel, that which grows in buildings, and the red wudufille, an equal amount of both; pound with the old fat of a pig; make it into a poultice', (Lacnunga, CXX)^b</i>
Steam Baths	Water	Following preparation of body salve, <i>'A bath for blotch, boil ten times the worts in a basin and separately ... of all equally much ... pour the prepared hot liquor under the stool in the bucket, let it reek on thee...', (Balds Leechbook, xxxii)^a</i>

Cockayne (1865)^a, Pettit (2001)^b and Van Arsdall (2002)^c. The references at the end of each entry detail the medical text and the numbered entry given by the translator.

Bald and Cild, owner and scribe respectively of the *Leechbook*, had sufficient knowledge of medicinal plants to consciously decide only to include weights and measures where different to the accepted preparatory method. There may have been other books for instruction in preparation of the different formulations and if so, with so little of the herbal literature surviving from this era, are now lost. D'Aronco (2007) makes the comment that three of the pre Anglo-Norman copies of *Herbarium* use capital letters for the beginning of a new chapter as well as a new remedy (p.44). Cockayne (1865) in his translation of Bald's *Leechbook*, follows the original text and repeats the frequent instructions of 'again' and 'for the same' in a single formulation although does not discuss the illuminated letters that also appear in the *Lacnunga* (Fig. 1.3). Pettit (2001) describes this collection of

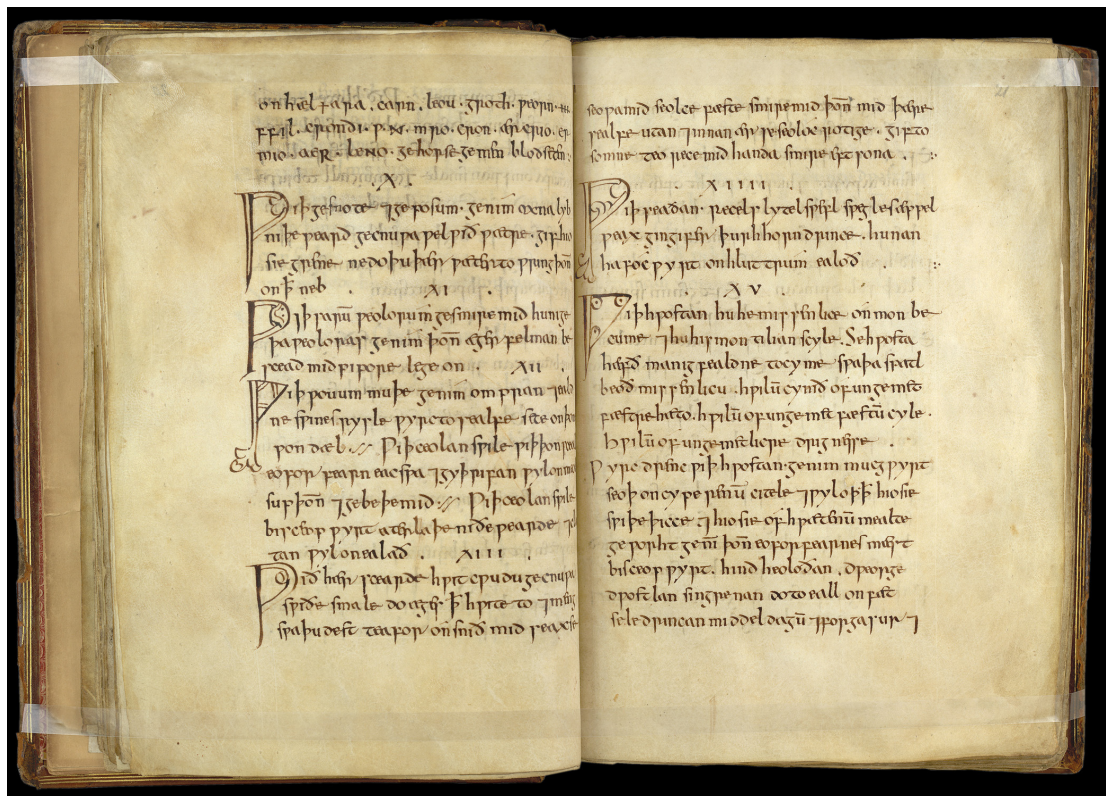


Fig. 1.3 Decorated initials in Balds *Leechbook*. © British Library (Royal 12 D. xvii, folios 20v-21).

letters as zoomorphic initials in the form of snakes, serpents and dragons or simple decorated initials used as guide letters. To date, these initials have not been considered an integral part of the formulation, perhaps defining the type of preparation. Knowing how the medication was prepared and administered would enhance current understanding of the value of historical texts in

ethnopharmacology. A coded formula would have provided the practitioner with a system to quickly recall a formulation and at the same time, prevent persons without herbal knowledge from dispensing remedies.

1.3 Battle Wounds and Injuries

Battle wounds, animal bites and burns were commonplace in Anglo-Saxon England with amputation recognised as a necessary procedure for wounds infected with gangrene (Cockayne, 1865; Van Arsdall, 2002). In the early medieval period, mortality rates on the battlefield were reported to be 42% from arrows, 67% sling shots and 100% from sword injuries (Hutley and Green, 2009). War wounds contaminated by soil borne organisms would have resulted in discharging pus, deformed scarring and slow healing as well as exposing individuals to potential bloody viruses. Seasonal and environmental variation in bacteria would have meant more faecal pathogens in summer, for example, *Enterococcus faecalis* and a greater presence of *Staphylococci* and *Streptococci* during the winter. Aerobic organisms commonly associated with wound infection include *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Collier, 2004). In the laboratory, a standard *in vitro* antimicrobial assay is based on four common wound organisms to determine whether the plant extract or compound is able to inhibit growth of Gram-positive and Gram-negative bacteria (Quave et al., 2008; Kumarasamy et al., 2003). The microdilution method (NCCLS, 2009) will provide an initial screening process to determine activity for the selected plants and ascertain whether they would have been appropriate in treating bacterial infection and wounds.

1.4 Scope of Research

This research is grounded in a 2008 UEL BSc (Hons) Herbal Medicine dissertation that compares and contrasts modern clinical use of plants named in Anglo-Saxon herbal formulations and subsequently an article was published in Journal of Herbal Medicine (Thomas, 2011). The proposed objectives herein are to:

- **Review** the 10th century Anglo-Saxon medical literature concerning the use of medicinal plants in herbal wound healing formulations.
- **Design** a selection model for conducting an *in vitro* bioassay of native British plants used specifically to treat bacterial infection and wounds (a reasonably large number of species were selected in order to minimise the risk of not finding any antimicrobial activity).
- **Evaluate** the bioactivity and phytochemistry of selected plants using chemometric techniques.
- **Identify** the active metabolites from the most potent plant.

An outline of the project plan is shown in Fig. 1.4.

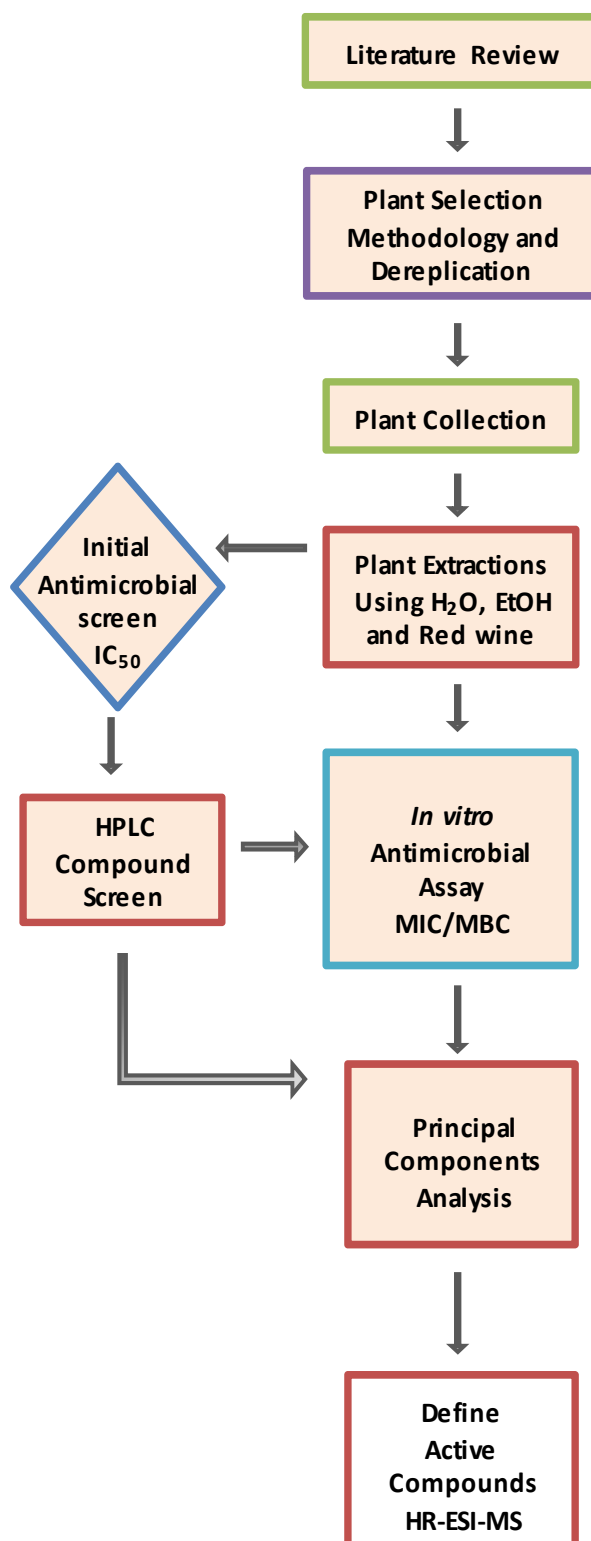


Fig. 1.4 Project plan to determine the antimicrobial activity of six native British plants used in 10th century Anglo-Saxon wound healing formulations.

Chapter 2.0 Plant Selection Strategy

2.1 Introduction

Despite a rich history of medicinal plant use throughout the British Isles, much of the native flora listed in the Anglo-Saxon medical literature has yet to be evaluated for its pharmacological and medicinal applications (Watkins et al., 2011). However, many of the underresearched medicinal plants have a long tradition of being used in Western herbal medicine (WHM) to treat bacterial infections and wounds (Culpeper, 1653; Grieve, 1931; Tobyn et al., 2011). Forbes (2003) defines a native species as a plant that arrived in the British Isles before the beginning of Neolithic farming (around 4000 BC) or since, independently of human association. A modern authority on British flora used by the Botanical Society of the British Isles (BSBI) for the identification of wild vascular plants is the *New flora of the British Isles* (Stace, 2010). Stace classifies a native species as a plant that colonised the British Isles by natural means from other native areas and an archaeophyte, as a plant mainly associated with man's activities present in the British Isles since at least medieval times.

Willow *Salix alba* L. and poppy, *Papaver somniferum* L. provided the chemical architecture for the synthetic production of aspirin and morphine respectively (Riddle, 1974). Both species are classified by Stace as archeophytes and are found in a number of Anglo-Saxon herbal preparations. One *Leechbook* formulation states 'For spleen pain pound green willow bark, boil it alone in honey, give it to him to eat, three pieces, having fasted overnight' (Pollington, 2008, p.387). A formula for *popig* or poppy in the *Herbarium* says 'For sleeplessness, take the juice of the same plant, rub it on the person and you will quickly give him sleep' (Van Arsdall, 2002, p.174). Arguably, one of the greatest contributions to drug discovery has been the alkaloid group with codeine and morphine isolated from *P. somniferum* as a result of scientific investigation of folklore traditions (Holland, 1996).

Previous pharmacological studies of native plants listed in the Anglo-Saxon medical texts suggested that some were effective and led to the

identification and isolation of natural compounds. Matricin from yarrow *Achillea millefolium* L. is a proprionic acid analogue that yields chamazulene carboxylic acid with cyclooxygenase-2- activity similar to that of ibuprofen (Watkins et al, 2011). The Anglo-Saxon practitioner employed many native species in his pharmacopoeia as well as exotic imports that would have been purchased via one of the many trading ports at London or along the English coast (Cameron, 2006; Hagen, 2006). St John's wort *Hypericum perforatum* L. is a native species that has been well studied for its pharmacological actions with hyperforin identified as the main metabolite responsible for its antibacterial properties (Saddiqe et al., 2010). However, in the *Herbarium* the plant is considered for different complaints 'pounded and drunk this plant stimulates urination... for a quartan fever, take the same plant, pounded , and give it to drink in wine' (Van Arsdall, 2002, p.216). A quatern fever is a type of malaria and according to Pollington (2008) *H. perforatum* had a reputation for purifying and strengthening other herbal formulations. Another native species, white horehound *Marrubium vulgare* L. is listed in all three of the Anglo-Saxon herbal texts for treating coughs, colds, snakebites and poisonings; a common occurrence with people collecting wild foods from the hedgerows (Hagen, 2006). One remedy for cough in Bald's *Leechbook* III specifies 'for host or cough; boil marrubium in water, a good deal of it, sweeten a little, give the man to drink a cup full' (Cockayne, 1865, p.313). Today, the plant in WHM is prescribed as a recommended daily adult dose equivalent to 3-6 g herb, primarily for treating bronchitis and asthma (Williamson, 2003). *Marrubium vulgare* has demonstrated antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) with an EtOH root extract reducing adherence of biofilms ranging from a MIC₅₀ value of 8 µg/mL to a MIC₉₀ value of 128 µg/mL (Quave et al., 2008). Another native species exhibiting antimicrobial activity is the hop *Humulus lupulus* L. derived from the Anglo-Saxon *hoppan* meaning to climb. Humulone and lupulone isolated from hops have been attributed to the antibacterial activity against *Staphylococcus* species with MIC values of 6.25 µg/mL and 3.13 µg/mL respectively (Zanoli and Zavatti, 2008). Ivy *Hedera helix* L. is an important herb in WHM, particularly in Germany, for treating respiratory conditions and, although native, is no longer used medicinally in Britain. The effectiveness of *H. helix* has been demonstrated in a post marketing study of 9,657 patients taking ivy leaf extract

in syrup for bronchitis whereby 95% showed improvement after 7 days of administering the herbal preparation (Fazio et al., 2009). Other studies for native British species include a randomised controlled study of 15 patients with infections of the lower urinary tract that were given a cup of birch or *Betula pendula* L. leaf tea to drink four times daily. After 20 days the birch leaf tea group exhibited a 39% decrease in microbial counts of urine compared to 18% in the control group (ESCOP, 2003).

2.2 Materials and Methods

2.2.1 Literature Search

An initial search of the Anglo-Saxon medical literature was conducted via the British Library online catalogue using keywords Anglo-Saxon medicine (29 hits), Anglo-Saxon herbals (4) and Anglo-Saxon medical texts (6) to find modern English translations of the surviving Anglo-Saxon medical literature. Further searches using the same keywords were undertaken in academic journal databases JSTOR, Science Direct and ISIS Web of Knowledge (Watkins et al., 2011).

The *Herbarium* was chosen as the initial data source and three translations consulted: *Leechdoms, Wortcunning, and Starcraft of Early England* (Cockayne, 1864); *Leechcraft: Early English Charms, Plantlore and Healing* (Pollington, 2008) and *Medieval Herbal Remedies: The Herbarium and Anglo-Saxon medicine* (Van Arsdall, 2002). Two translations of Bald's *Leechbook* were examined (Cockayne, 1865; Pollington, 2008) and three works reviewing the texts consulted (Cameron, 2006; Deegan, 1988; Meaney, 1984). A comprehensive translation of the *Lacnunga* by Pettit (2001) titled *Anglo-Saxon Remedies, charms and Prayers from British Library MS Harley 585* was used and supported by evidence from three other works (Cameron, 2006; Cockayne, 1866; Pollington, 2008). Both Bald's *Leechbook* and the *Lacnunga* were reviewed for plant names in single and multiple herbal formulations used to treat bacterial infections and wounds (Watkins et al., 2011).

2.2.2 Design of Selection Methodology

The broad concept of the selection strategy was based on Adams et al. (2009) survey of 16th and 17th century European herbals and using principles proposed by Cos et al. (2006) for the antimicrobial assays to determine whether the ethnomedical formulations were appropriate for the intended use. Bacterial infections and wounds were chosen as conditions that could be readily identified from the medical texts; require medical treatment and are relevant to modern health issues such as chronic leg ulcers in diabetes.

Native British plants, not subject to any conservation orders were selected if assigned both an Anglo-Saxon and Latin name in the *Herbarium*. In addition plants had to be listed for at least two external conditions whereby bacteria would have been present, including a puncture wound (battle injury, animal or insect bite), burn, ear, nose or throat infection, boil or ulcer. A study of plant names from medieval manuscripts (Hunt, 1989) was used to confirm common and vernacular names; cross referenced in *Bald's Leechbook* and the *Lacnunga* for medicinal use in single and multiple herbal formulations (Cockayne, 1865; Pettit, 2001; Pollington, 2008). The NapralertSM relational database (University of Illinois at Chicago, USA) was accessed for published research in English on ethno-medical studies, biological tests and known phytochemical compounds. A phytochemistry literature search was conducted in ISIS Web of Knowledge, Pub Med and Science Direct databases. Additional searches in antimicrobial and phytochemical publications for potential plant candidates used keywords based on Latin and/or common plant names and 'antimicrobial'. The final plant selection was based on flowering times of native British species and underreported phytochemistry and antimicrobial activity.

2.2.3 Plant Locations

Sites were chosen within a 25 miles radius of Watford, Hertfordshire, as Oxhey, a village in Watford, was first mentioned in an Anglo-Saxon Charter of 1007 (British Library: Cotton Nero, D.1, ff.149-61) and is near to where the author of this thesis resides. Areas selected for harvesting plants were typical of where an Anglo-Saxon practitioner may have collected wild specimens. Potential locations were sourced from the '*Flora of Hertfordshire*' (James, 2010) combined with an online search via Google to identify landowners of harvesting sites using keywords 'British flora' and 'Herts'. Permissions were obtained (Wildlife and Countryside Act 1981) and risk assessments authorised by University of East London (UEL) with field trips conducted in June-August 2010/2011 to confirm viable colonies of each species prior to collecting plant material for analysis.

2.2.4 Plant Collection

Botanical keys and illustrations from *The Wild Flower Key* (Rose, 2006) were used to identify specimens that were collected in flower for a true identification (Barker, 2001) and authenticated by an independent botanist, Dr. Brenda Harold, a BSBI county surveyor. Plants were selected if they were healthy in appearance and free of attack by insects or fungal contamination. Data recorded for the herbarium label included geographic location, elevation, date and time of collection (Bridson and Forman, 1999). Depending on plant size, 3-10 specimens for each species were harvested comprising whole plants gathered at random from colonies of at least eight plants. The harvesting instructions in the *Herbarium* for *B. officinalis* were observed during collection of material as in 'this plant is very wholesome and so you must gather it in the month of August without using a tool made of iron ... shake off the dirt... and dry it very thoroughly in the shade' (Van Arsdall, 2002, p.139). The plants were collected on dry August days late morning/early afternoon using a stainless steel trowel to loosen the roots, lifted by hand and placed in a paper bag ready for air drying (Barker, 2001).

2.2.5 Drying and Grinding of Plant Material

Aerial parts and roots for each species were separated and air dried in a cool dark room for five days followed by 48 h in a fan assisted oven (Gallenkamp) at 40 °C to avoid degradation by microorganisms (Houghton and Raman, 1998). The roots were brushed to remove any earth prior to drying; ground to a rough powder using an electric blender, refined using a 500 µm sieve and stored in the dark until required.

2.2.6 Preparation of Herbaria

Fresh specimens were arranged to reveal both sides of leaves, open flowers and roots; loose earth brushed from the roots, plants covered with a layer of newspaper and pressed for 2 days. The positioning of leaves, flowers and roots were checked and weight increased for 3 weeks to dry the specimens. A herbarium label was designed to include the Latin name, plant family, vernacular name, collection details, habitat/ecological notes and supplementary

data of Anglo-Saxon medicinal uses, authentication of species, collector, voucher number and references (Appendix 1.1). Specimens and label were mounted on white acid free paper in accordance with Kew Botanical Garden's guidelines (Bridson and Forman, 1999). At Kew the herbarium sheets were placed in the deep freezer for one week to decontaminate the material and then specimens were mounted and deposited in the Kew Herbarium for future reference (Appendix 1.2).

2.3 Results

2.3.1 Selection Strategy

The broad keywords identified the lack of scientific evidence on the subject of Anglo-Saxon medical literature and, that a number of translations would have to be read to appreciate the context in which the herbal formulae were used. The plant selection strategy shown in Fig. 2.1 was designed to identify six species of plants from the Anglo-Saxon literature offering the greatest potential to yield active antimicrobial compounds under scientific investigation. Six species were decided upon in order to minimise the risk of finding no activity. The *Herbarium* was the most appropriate text for the initial selection criteria in that it was grounded in an earlier 4th century Latin herbal (Cameron, 1982; Van Arsdall, 2002) and comprised mainly ‘simples’ or single plant preparations whereby outcomes could be attributed to a named plant. The pharmacopoeial text details medicinal formulations for 185 plants of which 140 are assigned both Latin and Anglo-Saxon names (Cockayne, 1864; Van Arsdall, 2002). Thirty different species were listed for the treatment of at least two external conditions: puncture wound, open wound, burn, ear, nose or throat infection, boil or ulcer.

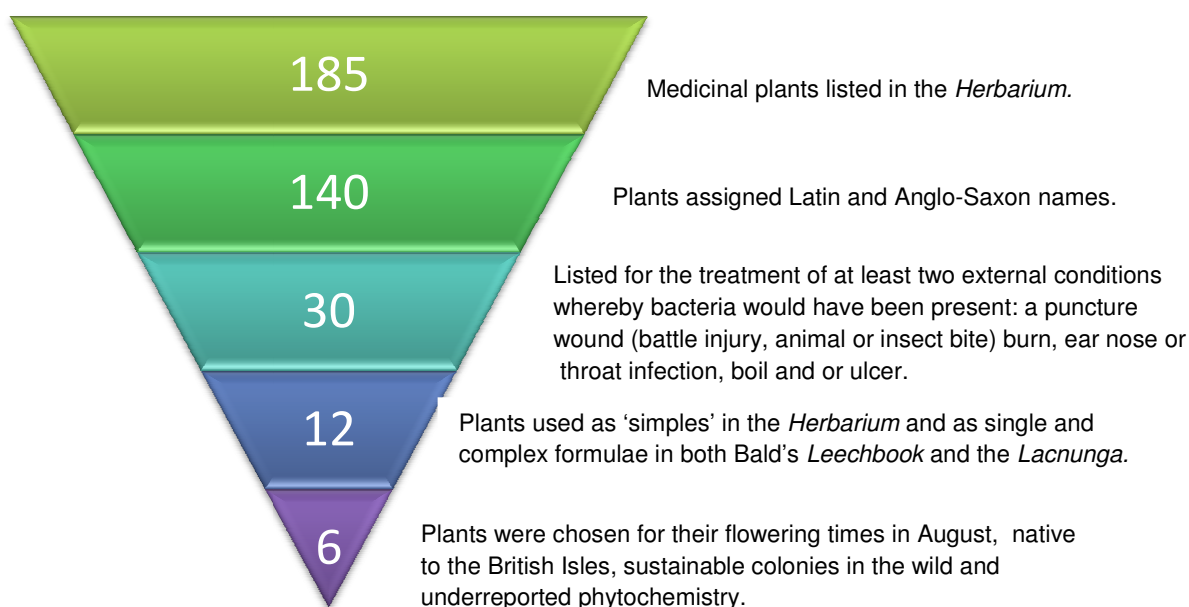


Fig. 2.1 The selection model designed to determine six plants for an antimicrobial study of native British flora used in 10th century Anglo-Saxon wound healing formulations.

Twelve of the plants listed in the *Herbarium* were used in simple and combination formulae in both *Bald's Leechbook* and *Lacnunga* (Table 2.1) demonstrating that species would have been reasonably accessible. Additional criteria excluded non native species (*Sambucus ebulus* L. and *Verbena officinalis* L.), plants not flowering in August (*Glechoma hederacea* L. and *Orchis mascula* L.) as this month was specified in the *Herbarium* for harvesting *B. officinalis* and *Potentilla reptans*. Flowering times vary according to location and year and in 2010, *Galium aparine* L. had finished flowering in Hertfordshire by the beginning of July and was therefore excluded whereas *P. media* was still flowering in August. Lastly, *Teucrium chamaedrys* L. was excluded on the basis of it having >100 reported compounds. The study set out to investigate plants with underreported phytochemistry and thus *Plantago media* L. was chosen as a substitute plant for *P. major* L. and was referenced by Cockayne (1866, p. 347) and *A. minus* (Hill) Bernh., as it is used interchangeably with *Arctium lappa* L. in WHM.

Table 2.1 Ethnomedical and antimicrobial records for 12 plants used in three or more 10th century medical texts for treating wounds and bacterial infections. Source: NAPRALERT database (1975-2003) and photochemical literature search (adapted from Watkins et al., 2012).

Plant Name	Plant Family	Status of species	Flowering Time	Ethnomedical Records	Antimicrobial Assays	Reported Compounds	Additional References
<i>Agrimonia eupatoria</i> L.	Rosaceae	Native	Jun-Sep	23	3	55	Copland et al., 2003
<i>Arctium minus</i> (Hill) Bernh.	Asteraceae	Native	Jul-Sep	43	4	26	Sanders et al, 1945; Moskalenko, 1986
<i>Betonica officinalis</i> L.	Lamiaceae	Native	Jun-Sep	11	1	57	Grujic-Jovanovic et al., 2004
<i>Centaurium erythraea</i> Rafn.	Gentianaceae	Native	Jun-Oct	30	2	81	Kumarasamy et al., 2002
<i>Galium aparine</i> L.	Rubiaceae	Native	Jun-Aug**	10	5	69	Related species <i>G. mexicanum</i> Bolivar et al., 2011
<i>Glechoma hederacea</i> L.	Lamiaceae	Native	Mar-May*	28	6	70	Kumarasamy et al., 2002
<i>Orchis mascula</i> L.	Orchidaceae	Native	Apr-Jun*	12	0	8	Bulpitt, 2005
<i>Plantago media</i> L.	Plantaginaceae	Native	May-Aug	9	1	23	Related species <i>P. lanceolata</i> L. ESCOP, 2003
<i>Potentilla reptans</i> L.	Rosaceae	Native	Jun-Sep	4	1	8	Redzic et al., 2009;
<i>Sambucus ebulus</i> L.	Caprifoliaceae	Archaeophyte*	Jul-Aug	43	4	60	Tomczyk and Latte, 2009
<i>Teucrium chamaedrys</i> L.	Lamiaceae	Native	Jun-Sep	50	1	108*	Suntar et al., 2010
<i>Verbena officinalis</i> L.	Verbenaceae	Archaeophyte*	May-Aug	73	9	140	Related species <i>Teucrium arduini</i> L. Samec et al., 2010
							Williamson, 2003; Tobyn et al., 2011

Taxonomy according to Stace (2010) and flowering times by Rose (2006). Antimicrobial assays using Gram-positive (*S. aureus*) and Gram-negative (*E. coli* or *P. aeruginosa*) pathogens commonly found in wounds. * denotes reason for exclusion. ** In 2010, *G. aparine* had finished flowering by beginning of July.

A phytotherapy monograph was prepared for each selected plant as shown in Tables 2.2-2.7.

2.3.2 Plant Locations

The BSBI hectad maps (10 x 10 km) were used to determine nationwide distribution of each species. Harvest locations and voucher numbers are given in Fig. 2.7. and permission to collect whole specimens was obtained from: Allan Crawley, Berry Bushes Farm, Kings Langley, Hertfordshire (*Agrimonia eupatoria* L.; *Arctium minus* (Hill) Bernh. and *Potentilla reptans* L.); Paul Thrush, Herts and Middlesex Wildlife Trust (*Centaurea erythraea* Rafn. from Alpine Meadow near Berkhamstead) and Nancy Reed, Berkshire, Buckingham and Oxford Wildlife Trust (*Plantago media* L. from College Lakes near Tring). Lisa Rowley, Wildlife Trust for Bedfordshire, Cambridgeshire, Northamptonshire and Peterborough (*Betonica officinalis* L. from Boddington Meadows in Northampton) and Weston Parish Council gave permission to also collect plants from Weston, Herts.

Table 2.2 A Phytotherapy Monograph of *Agrimonia eupatoria* L.

The name of *Agrimonia eupatoria* is derived from *Argemone*, a word given by the ancient Greeks to plants that were healing to the eyes and *Eupatoria*, after Mithridates Eupatorthe, King of Pontus (120-63 B.C.) who was renowned for his herbal formulations (Grieve, 1931).



Anglo-Saxon Name: Gar-Clive (Hunt, 1989), garclife or 'spear-cleavers' to describe the small burrs that stick to clothing or animal fur (Pollington, 2008).

English Synonyms: Common Agrimony, Cocklebur and Sticklewort.

Parts Used: Fresh and dried flowering tops.

Plant Family: Rosaceae.

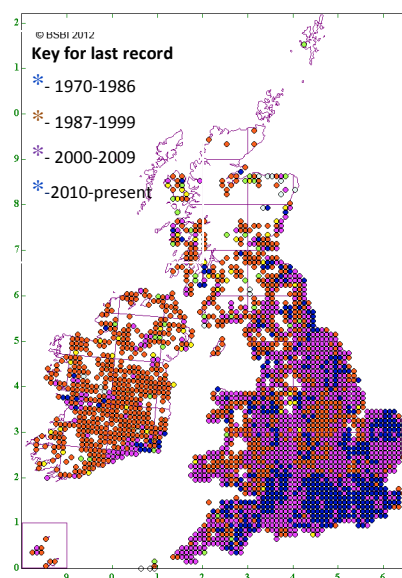
Description: *A. eupatoria* is a hairy herbaceous perennial with erect stems growing up to 60 cm that can be reddish and fragrant when bruised; pinnate leaves with smaller leaflets between the main pairs and flowers in long spikes each comprising 5 yellow petals and 5 sepals.

Source: www.british-wild-flowers.co.uk

Flowering from June to September, the fruit is a hard case covered in hooked spines containing two seeds (Stace, 2010). The plant has a frequent distribution in the British Isles except northern Scotland and is found in hedgerows, calcareous grassy pastures and scrubland.

Flowering from June to September, the fruit is a hard case covered in hooked spines containing two seeds (Stace, 2010). The plant has a frequent distribution in the British Isles except northern Scotland and is found in hedgerows, calcareous grassy pastures and scrubland.

Constituents: The roots and leaves mainly comprise condensed tannins (3-11%) proanthocyanidins and lesser amounts of hydrolysable tannins catechins, ellagitannins and gallotannins (ESCOP, 2009). Flavonoid glycosides of quercetin, luteolin, apigenin and kaempferol; triterpenoids euscaphic acid, tormentic acid and ursolic acid. Others compounds include polysaccharides, phenolic acids *p*-hydroxybenzoic, protocatechuic, Vanillic and salicylic acid and steroid β -sitosterol (ESCOP, 2009; Tobyn et al., 2011; Williamson, 2003).



Source: www.bsbi.org.uk

Anglo-Saxon Formulations: Eight single formulations are listed in the *Herbarium* and all parts of the plant used (Van Arsdall, 2002, p.164). Fresh or dried leaves were crushed as a topical application to treat sore eyes whilst the plant was pounded in vinegar and laid upon warts. However, 9 g of the herb in two cups of wine was administered as an internal 'brewit' for snakebite and a decoction of the roots given as a drink for soreness of the abdomen and spleen (Cockayne, 1864, p.131). In *Lacnunga*, a complex formula for loin pain reads 'fennel-seed, fresh leaves of betony, the lower part of agrimony; grind to a powder; macerate with sweetened ale; make tepid; give to drink hot in a standing position; let him stand for a good while' (Pettit, 2001, p.57).

Current Herbal Use: *A. eupatoria* is used as a household medicinal herb and in Western herbal practice for treating diarrhoea, irritable bowel disorders (IBD), upper respiratory tract and urinary tract infections. An infusion is applied as a topical antiseptic for mild skin inflammations and a gargle for throat complaints. The recommended daily adult dose is 1-4 g of the dried herb as an infusion, 1-3 mL of fluid extract (1:1 in 25% EtOH) or 5-10 mL of tincture (1:5 in 45% alcohol) three times per day. (BHP, 1996; ESCOP, 2009; Tobyn et al., 2011).

Pharmacological Properties: In another related species, isolated agrimol derivatives from *Agrimonia pilosa* L. have been reported to inhibit the growth of *S. aureus* with a MIC range of 3.13 -50 µg/mL (Yamaki et al., 1994). In an herbal screening for antibacterial activity against *Helicobacter pylori* and *Campylobacter jejuni*, the most common cause of enteric infection, an EtOH extract of *A. eupatoria* exhibited significant inhibition of *H. pylori* at 97 ± 5% and of *C. jejuni* at 96 ± 30% inhibition (Cwikla et al., 2010). Copland et al., (2003) reported an *A. eupatoria* seed hexane extract to have a MIC value of 750 µg/mL against *Bacillus subtilis* but no activity against Gram-negative pathogens albeit it is the herb and not the seed that is used in Western herbal practice.

Clinical Studies: In a patient study (n=20) an agrimony infusion was given 3-4 times per day to treat cutaneous porphyrias, a blistering and fragility of sun exposed skin. After 15 days all the patients exhibited substantial improvement in the skin conditions (ESCOP, 2009).

Toxicity: No known side effects or toxicity have been documented however large doses may lead to nausea and constipation (Tobyn et al., 2011).

Contraindications: Limit use during pregnancy and lactation (Barnes et al., 2002).

Table 2.3 A Phytotherapy Monograph of *Arctium minus* (Hill) Bernh.

The name *Arctium* comes from the Greek word *arktos* for a bear which is reflective of the furry burrs on the plant that cling to passers-by and *minus* from the Latin meaning lesser (Grieve, 1931).



Anglo-Saxon Name: Personica, Boete (Van Arsdall, 2002), A closely related species *Arctium lappa* is referred to *herrif* or *aireve* from the Anglo-Saxon *reafe*, meaning a thief and *reafian* to seize. There is some confusion with foxesclife and eaweyrt assigned to multiple species (Pettit, 2001).

English Synonyms: Lesser Burdock, Burdock.

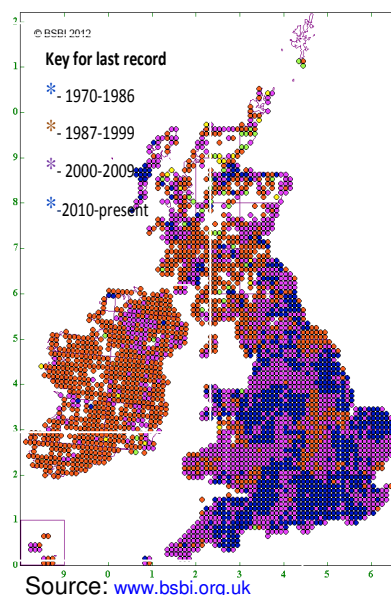
Parts Used: Dried aerial parts and roots in the first year.

Plant Family: Asteraceae.

Source: www.british-wild-flowers.co.uk

Description: *Arctium minus* is a biennial similar in appearance and structure to *A. lappa* although smaller and forming a rosette in the first year. In the second year strong furrowed stems grow to 60-130 cm, spreading branches with hollow leaf-stalks and large lower leaves broadly oval to heart shaped. Flower heads are purple with hooked bracts forming burrs appearing in July to September (Barker, 2001, Rose, 2006). In the British Isles the plant is restricted to lowland central and southern England and borders of Wales and is occasionally found in open woods, grassy waysides and scrubland (Barker, 2001). Medicinally the plant is used interchangeably with *A. lappa* (Grieve, 1931).

Constituents: The leaves comprise sesquiterpenes and sesquiterpene lactones; flavonoids luteolin, quercetin, quercetrin and rutin (Tobyn et al., 2011); phenolic acids caffeic and chlorogenic, cynarin and hydroxycinnamoylquinic acid and in the roots, polyacetylenes, germacrolide, arctic acid and up to 50% inulin, a storage polysaccharide (Barker, 2001; Williamson, 2003).



Anglo-Saxon Formulations: Six single and two complex formulae are given in the *Herbarium* (Van Arsdall, 2002, p.167) the latter two believed to have originated from Dioscorides materia medica written in the 1st century (Riddle, 2011). All parts of the plant were used including the fresh juice and seeds and in the case of fever, leaves were bound to the person (Cockayne, 1864; Pollington, 2008). 'For fresh wounds that are still wet, take equal amounts of the roots of the same plant and hawthorn leaves; pound them together and lay this on the wounds'. 'For persons coughing up blood and phlegm, four pennies weight (modern equivalent of 6.2 g) of seeds and pine nut were made into a dumpling and given to eat' (Van Arsdall, 2002, p.167).

Current Herbal Use: In many of the herbals *A. minus* is used interchangeably with *A. lappa* and the latter is most often cited in the scientific literature. *Arctium lappa* has a long history of being used externally to treat chronic skin conditions including eczema, acne and psoriasis as well as internal and external use to treat impetigo, boils, cystitis, rheumatism and gout (Tobyn et al., 2011). The recommended daily dose for an adult is 2-6 g of the infusion of the leaf or light decoction of root, 1-5 mL of tincture (1:5 in 25% alcohol) (Barker, 2001). In addition the British Herbal Pharmacopoeia (1996) recommends a daily adult dose of 2-8 mL for a liquid extract (1:1 in 25% alcohol), 8-12 mL for a tincture (1:10 in 45% alcohol) and a root decoction of 1:20 not to exceed 500 mL.

Pharmacological Properties: There was only one report found for *A. minus* in the literature for antimicrobial activity. Sanders et al. (1945) report an aqueous juice of *A. minus* aerial parts being active against *B. subtilis* with an inhibitory zone of 15-25 mm compared to an inhibitory zone against *E. coli* of 15-20 mm. In another antimicrobial disc diffusion assay, a 70% EtOH infusion of *A. lappa* flower at 400 µg/disc inhibited growth of *S. aureus* (10-15 mm zone) and *B. subtilis* (15-20 mm) with positive controls of streptomycin and chloramphenicol (Moskalenko, 1986). The antimicrobial activity against Gram-positive bacteria has been attributed to the polyacetylenes and the bitter artctiopicrin (Barnes et al., 2002).

Clinical Studies: No published data (ESCOP, 2003).

Toxicity: No known side effects.

Contraindications: None reported.

Table 2.4 A Phytotherapy Monograph of *Betonica officinalis* L.

This plant has been reassigned from *Stachys officinalis* to its original Linnaen name of *Betonica officinalis* (Stace, 2010) named after the pre-Roman Catholics from Vettones in Lusitania (Barker, 2001).



Anglo-Saxon Name: Biscopwyrth (Cockayne, 1864; Hunt, 1989).

English Synonyms: Betony, Wood Betony, Bishopswort.

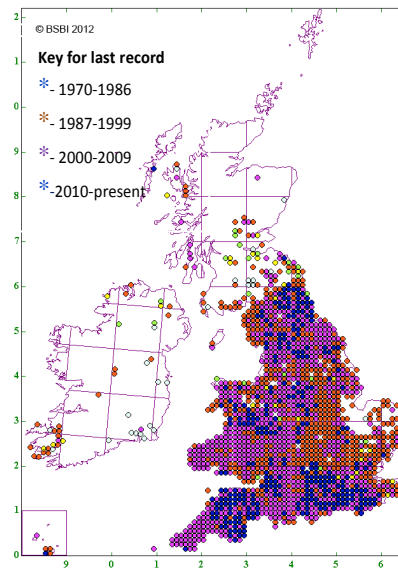
Parts Used: Aerial parts should be gathered during flowering and used fresh (Barker, 2001). Dried leaves are used as well as the whole plant.

Plant Family: Lamiaceae.

Description: A sub genus of *Stachys* now classified as *Betonica* with five species growing wild in Britain

Source: www.british-wild-flowers.co.uk

(Stace, 2010). This plant is a sparsely hairy perennial herb growing 10-60 cm; basal rosette of long-stalked, oblong leaves in a few distant pairs all coarsely toothed and blunt. *Betonica officinalis* has red-purple flowers between June and September (Rose, 2006). The plant thrives in impoverished pastures in England and Wales, except East Anglia; in open woods, hedge banks, heaths and grasslands on acidic and calcareous soils. However, in southern England it has declined by 46% since the county flora records of 2005 and is now not common anywhere in Hertfordshire (James, 2010).



Source: www.bsbi.org.uk

Constituents: The leaves contain alkaloids stachydrine and betonicine; diterpenes betonicosides A-D and betaconolide; flavonoids *p*-coumarolyglucosides, phenolic acids caffeic acid, verbascoside, chlorogenic acid, apigenin glycoside; up to 6% gallic acid and 0.02% essential oil (Marin et al., 2004; Tobyn et al., 2011; Vundac et al., 2006; Williamson, 2003).

Anglo-Saxon Applications: The most frequently cited plant in the *Herbarium* with 29 formulations this was edited down from an initial list of 47 formulae (Voigts, 1979) for a wide range of conditions: nightmares, shattered skull, ear, eye and throat pain, toothache, nose bleeds, stomach pain, constipation, boils, fatigue, nausea, drinking poison, animal bites, fever and gout. The whole plant was used although 'for earache, take the leaves of this same plant when it is greenest, gently boil them in water, and press the juice out, and after it has stood for a time, warm it up again and use a piece of wool to drip it into the ear'. A decoction of the plant in aged wine and reduced by two thirds is given for toothache whereas the plant is ground and applied directly to the site of an animal bite. (Van Arsdall, 2002, p.141). In Bald's *Leechbook* an instruction for a complex mixture says 'for mist of eyes again, juice of betony beaten with its roots and wrung, and juice of yarrow and of celandine, equally much of all, mingle together, apply to the eye' (Cockayne, 1865, p.31). Three European herbals during 16th century mention *B. officinalis* to relieve pain in polyarthritis, gout, hip pain and lameness by making a broth of the roots or the flowers in sugar (Adams et al., 2009).

Current Herbal Use: *Betonica officinalis* is used in WHM to treat tension headache, vertigo, anxiety, hysteria, neuralgia and herbalists have prescribed it to assist in withdrawal from benzodiazepines (Williamson, 2003). Leclerc recommends it against sores and varicose ulcers (Barker, 2001). The British Herbal Pharmacopoeia (1996) recommends an adult dose of 2-4 g of the dried herb or as an herbal infusion; 2-4 mL liquid extract (1:1 in 25% alcohol) or 2-6 mL tincture (1:5 in 35% alcohol) three times per day. Barker suggests slightly less as an infusion or 1-10 mL of tincture (1:5 in 25% alcohol).

Pharmacological Properties: In an antibacterial study of essential oils from six *Stachys* species *B. officinalis* inhibited growth of both *B. subtilis* and *E. coli* with a MIC value of 100 µg/mL (Grujic-Jovanovic et al., 2004). Similar activity was seen in another study of eight Greek species with the essential oil of *Stachys scardica*, inhibiting growth of both *B. subtilis* and *E. coli* with a MIC value of 100 µg/mL compared to the positive control Streptomycin, MIC range 10-20 µg/mL (Skaltsa et al., 2003).

Clinical Studies: No published data relating to wound healing (Tobyn et al., 2011).

Toxicity: None reported.

Contraindications: No side effects reported.

Table 2.5 A Phytotherapy Monograph of *Centaurium erythraea* Rafn.

The plant name is derived from the Greek centaur Chiron, who was recognised for his knowledge and application of medicinal plants and the word *erythros* meaning red (Grieve, 1931).



Anglo-Saxon Name: Feferfuge, FelTerre, Feverwort and Curmealle seo lœsse (Cockayne, 1864; Hunt 1989; Van Arsdall, 2002).

English Synonyms: Common Centaury, Lesser Centaury.

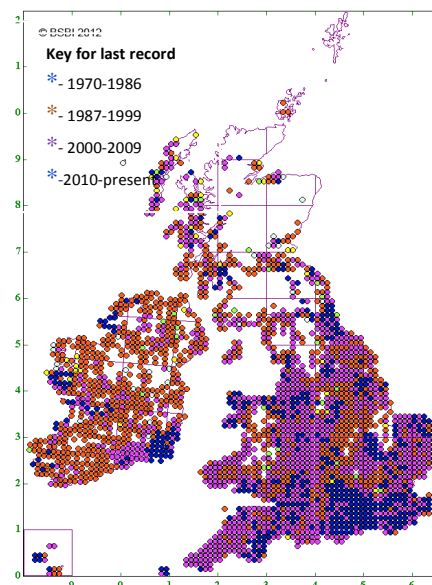
Parts Used: Dried flowering aerial parts.

Plant Family: Gentianaceae.

Description: Very large genus of almost 1000 species around the world with only *C. erythraea* and

Source: www.british-wild-flowers.co.uk

Blackstonia perfoliata L. common in the British Isles (Barker, 2001). An erect biennial growing to 50 cm with one or more stems from a basal rosette and a few elliptical leaves. The pink rose coloured tubular petals are longer than the narrow pointed sepals arranged in dense forking cymes on top of stem. This plant usually flowers between July and August although in some years it can continue into October (Barker, 2001; Rose, 2006). The plant is common throughout the British Isles although scarcer in Scotland. It is found in rough grasslands, dunes, clearings in woods on neutral or slightly calcareous soils and is often dwarfed in exposed places (James, 2010; Stace, 2010).



Source: www.bsbi.org.uk

Constituents: The bitter tasting secoiridoid glycosides in the leaves are mainly swertiamarin. Other iridoids include centauroside, secologanin, 6'-*m*-hydroxy-benzoyl-loganin, dihydrocornin, gentioflavoside and the secoiridoid alkaloid gentianine. Minor constituents include phenolic acids *p*-hydroxybenzoic, Vanillic, syringic, *p*-coumaric, ferulic and caffeic; phytosterols, β -sitosterol and stigmasterol and triterpenoids (Barker, 2001; ESCOP, 2003; Toby et al., 2011).

Anglo-Saxon Formulations: There are six formulations in the *Herbarium* for snakebite, eye pain, poor vision, 'anyone dangerously ill' possibly a specific medical condition such as fever; nerve spasms, ingested poison and for the removal of worms that 'irritate around the anus' (Van Arsdall, 2002, p.166). All parts of the plant are used and often as a decoction simmered in wine and left to stand, or for snakebite the dried plant is powdered, or the fresh plant bruised and drunk in aged wine (Cockayne, 1864). Other Anglo-Saxon uses include fevers perhaps explaining its name of feverwort and as a topical application to chronic wounds (Grieve, 1931; Toby et al., 2011).

Current Herbal Use: The main therapeutic indications are inflammatory conditions of the upper digestive tract, dyspepsia, liver and gallbladder complaints, anorexia, and as an appetite stimulant. Toby et al., (2011) recommend the root for external use of chronic wounds with a decoction of the aerial parts in flower for acute wounds, bites and stings. It is also used for arthritis and muscular joint pain as well as a preventative treatment in the form of a herbal tea for irritable bowel (IBD). The recommended daily adult dosage is 1-4 g of dried herb in 150 mL infusion or decoction of water or 2-4 mL of a liquid extract (1:1 in 25% EtOH) three times per day (Barker, 2001; ESCOP, 2003).

Pharmacological Properties: Methanol seed extracts of *C. erythraea* have shown to inhibit growth of *S. aureus* with a MIC value of 100 µg/mL and against *S. aureus* (MRSA) a MIC value of 1000 µg/mL (Kumarasamy et al., 2002). In a subsequent study, swertiamarin and sweroside were isolated from the aerial parts and both compounds inhibited growth of *B. subtilis* and *E. coli* at 0.534 and 0.558 µM respectively (Kumarasamy et al., 2003). In an air pouch granuloma assay, an aqueous extract of *C. erythraea* in 2.5 and 5 % creams was shown to inhibit inflammation by 19 and 42% respectively (Adams et al., 2009) whilst another *in vivo* study, a dry EtOH extract given orally to rats at 100 mg/kg body weight inhibited carageenan-induced paw oedema by 40% (Capasso et al., 1983).

Clinical Studies: No published clinical data (Toby et al., 2011).

Toxicity: No known side effects.

Contraindications: *C. erythraea* is contraindicated for people with peptic ulcers and should not be used during pregnancy and/or lactation (Barnes et al., 2002; ESCOP, 2003).

Table 2.6 A Phytotherapy Monograph of *Plantago media* L.

The plant name is derived from the Latin word '*planta*' meaning sole of the foot and '*ago*' meaning like. In the United States it is commonly known as '*white man's foot*' believed to have grown wherever the settlers went (Grieve, 1931).



Anglo-Saxon Name: Arnoglossa and Wegbraede (Hunt, 1989; Van Arsdall, 2002).

English Synonyms: Hoary Plantain.

Parts Used: Fresh and dried leaves and the seed.

Plant Family: Plantaginaceae.

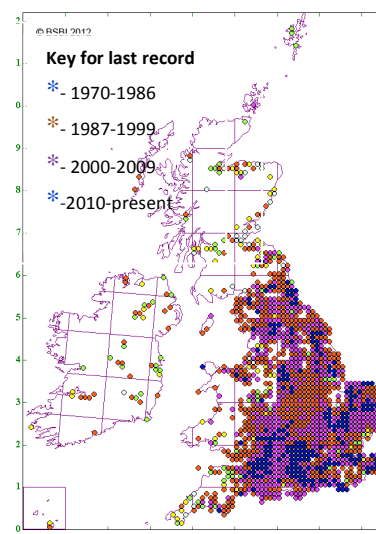
Description: A diverse genus of 250 species three of which have been used medicinally in Britain: *P. major*, *P. lanceolata* and *P. media* with the medicinal virtues of the latter considered being interchangeable with *P.*

Source: www.british-wild-flowers.co.uk

major (Barker, 2001;

Grieve, 1931; Van Arsdall, 2002). Downy and greyish basal rosette with blade leaf tapering to short stalk growing from a very short rhizome with long yellowish roots with many fine hairs and stem growing to 2-6 cm; scented flower spike with lilac anthers pollinated by insects. Harvest whilst in flower preferably in May to June although can flower until August (Barker, 2001). In England *P. media* is common in chalk and calcareous boulder clay soils of unimproved grassland, churchyards and mown road verges (Stace, 2010).

Constituents: The leaves of *P. media* comprise aucubin and plantamajoside both characteristic of the *Plantago* species (Ronstead et al., 2000). Additionally *P. lanceolata* leaves comprise mucilage polysaccharides, iridoid glycosides aucubin and catalpol, phenylethanoids verbascoside and plantamajoside (Adams et al., 2009; ESCOP, 2003). Other constituents reported for *P. major* include traces of alkaloids; flavonoids apigenin, baicalein, scutellarein; triterpenes, steroids, tannins, Vitamin C, protocatechuic and fatty acids (BHP, 1996; Williamson, 2003).



Source: www.bsbi.org.uk

Anglo-Saxon Formulations: Twenty one single formulations are given in the *Herbarium* ranging from headaches to wounds and animal bites. All parts of the plant used including juice 'wrung from the plant' for treating worms. For wounds and hot inflammations powdered seed was applied topically whilst for snakebite the whole plant was crushed into wine and eaten (Van Arsdall, 2002, p.144). In Bald's *Leechbook* for the same plant 'drip into the ear juice of ribwort [*P. lanceolata*] and oil made lukewarm, mingled together, it wonderfully healeth' (Cockayne, 1865, p.41) whilst for *P. major* in the *Lacnunga* 'let one take greater plantain; put it in wine; drink the juice, and let one eat the plants; then it will be good for every internal infirmity' (Pettit, 2001, p.79).

Current Herbal Use: *P. media* is not used in WHM. However, the leaves and seed of *P. major* are used in the treatment of blepharitis and conjunctivitis, coughs, fungal skin infections, wounds, haemorrhoids and ulcers. By contrast *P. lanceolata* is used to treat pulmonary conditions including chronic bronchitis and has a long history for healing skin tumours (Barker, 2001) and according to Weiss (2001), is the superior species. The recommend daily adult dose for *P. major* is 2-5 mL three times per day and for *P. lanceolata*, a daily adult dose of 3-6 g or equivalent preparations (BHP, 1996; ESCOP, 2003).

Pharmacological Properties: There was no reported pharmacological activity for *P. media*. However in another species, 50% EtOH leaf extracts of *P. major* inhibited growth of *S. aureus*, *B. subtilis*, *P. aeruginosa* and *E. coli* with an inhibition zones of 10-15mm compared to positive control, gentimicin with inhibition zone >25mm (Samuelsén, 2000). Aucubigenin, the aglycone of aucubin has been shown to be responsible for the antibacterial activity (ESCOP, 2003).

Clinical Studies: A syrup of *P. lanceolata* leaves was given to 593 patients suffering with acute bronchitis and post-infectious dry cough, and after 10 days the symptom score declined by >65% with physician evaluations being 25.9% excellent and 61.8% moderate outcomes (ESCOP, 2003).

Toxicity: None reported.

Contraindications: May cause a contact allergic reaction and in excess may cause a laxative and hypertensive effect so should therefore not be used during pregnancy (Barnes et al., 2002) although the ESCOP monograph states no contraindications known (2003).

Table 2.7 A Phytotherapy Monograph of *Potentilla reptans* L.

Potentilla is derived from the Latin *potens* meaning *powerful* and *reptans* to creep or crawl as is the habit of the plant (Tobyn et al., 2011).



Anglo-Saxon Name: Fifleafe, pentafileon, pentafofium, filofolium (Hunt, 1989).

English Synonyms: Creeping Cinquefoil, Five Fingers, Five-Leaf-Grass.

Parts Used: Fresh and dried leaves; dried rhizome and the whole plant.

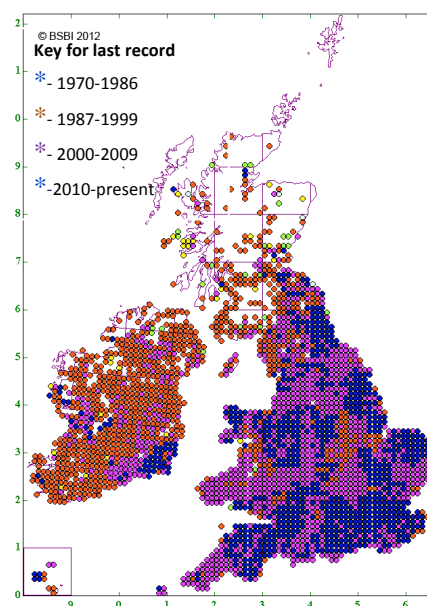
Plant Family: Rosaceae.

Description: There are more than 500 species worldwide. *Potentilla reptans* has slender creeping

Source: www.british-wild-flowers.co.uk

stems with five and sometimes seven leaflets. The stem roots at nodes as it creeps and may be up to 1 m long. Small yellow flowers growing individually on long stalks with 5 petals, 5 sepals and numerous stamen. The plant flowers from June to September and the rhizomes are best harvested during the autumn but may be used at any time (Stace, 2010; Barker, 2001). It is a very common plant of hedge banks, roadsides, open grasslands and sand-dunes in the British Isles although not so common in Scotland.

Constituents: *Potentilla reptans* has to date only eight reported compounds in the leaves (Tomczyk and Latte, 2009; Napralert, 2003): ascorbic acid, kaemperol-3-*O*-glucoronide, quercetin-3- *O*- β -D-glucuronpyranoside, quercetin-3-7-diglucuronide, ellagic acid, *p*-coumaric acid, caffeic acid and ferulic acid. In other species triterpenoid saponins including euscaphic acid and tormentic acid glycosides; flavonoids with 15-20% type B proanthocyanidins and hydrolysable tannins ellagitannins including pedunculagin, agrimoniin and laevigatin; coumarins including umbelliferone, esculetin and scopoletin; carotenoids and sterols isolated from *P. erecta* (Bazylko et al., 2013; Tomczyk and Latte., 2009; Tobyn et al., 2011).



Source: www.bsbi.org.uk

Anglo-Saxon Formulations: There are nine single formulations listed in the *Herbarium* using the whole plant, roots or juice. For a bloody nose the instruction is 'give him cinquefoil to drink in wine and smear the head with it; then the bleeding will stop quickly' (Van Arsdall, 2002, p.145). An aqueous root decoction is given as a drink for aching joints, sore stomach, aching mouth, tongue and throat; for a burn the plant is used topically whereas a poultice is made by simmering the whole plant in wine and unsalted old pig grease to 'stop an ulcerous sore from spreading' (Van Arsdall, 2002, p.145). In Bald's *Leechbook* a simple formula reads 'for the bite of snake again, cinquefoil wrung and mingled with wine is good to drink' (Cockayne, 1865, p.111).

Current Herbal Use: *Potentilla reptans* is not used by Western medical herbalists although *P. erecta* L. is used to treat diarrhoea, relieve pain and soothe bleeding gums and in Turkey, *P. anserina* L. is used to treat ulcerative colitis and as a lotion for haemorrhoids (Tomczyk and Latte, 2009). The recommended daily adult dose for *P. erecta* is 2-4 g dried rhizome as an infusion; 2-4 mL of liquid extract (1:1 in 25% alcohol) or 2-4 mL of tincture (1:5 in 45% alcohol) three times a day and up to 10 mL as a lotion or mouthwash for bleeding gums. (Barker, 2001, BHP, 1996; Williamson, 2003).

Pharmacological Properties: In an antimicrobial study of *Potentilla* species from the Dinaric Alps the aqueous root decoction of *P. reptans* exhibited activity against Gram-positive and Gram-negative bacteria including *S. aureus* (inhibitory zone of 10.3-12 mm), *B. subtilis* (7-15 mm zone) and *E. coli* (4.3-7.2 mm zone) compared to penicillin, the positive control, demonstrating a 25 mm zone (Redzic et al., 2009). In a survey of nine *Potentilla* species excluding *P. reptans*, aqueous infusions of aerial parts inhibited *S. aureus* (ATC6538) within a MIC range of 12.5 - >100 mg/mL and in the same study, *P. argenta* L. was the most active against Gram-negative bacteria, inhibiting *E. coli* (ATCC 25922) at 50 mg/mL and *P. aeruginosa* (ATC 27853) at >100 mg/mL (Tomczyk et al., 2008; Tomczyk et al., 2007).

Clinical Studies: No published data for *P. reptans* although in another species, *Potentilla erecta* L. rhizome was shown to significantly reduce diarrhoea with no clinical side effects in a randomised controlled study of 40 children (Subbotina et al., 2003).

Toxicity: None reported.

Contraindications: Caution is advised regarding long term use because of the high tannin content and Tobyn et al., (2011) recommend *A. eupatoria* as an alternative, being less astringent.

2.3.3 Plant Collection

Specimens were collected in July and August for two consecutive years with all plants harvested in 2010 and 2011, except *B. officinalis* that was only collected in 2011. Each species was harvested from different colonies within the given locations in Hertfordshire, Bedfordshire and Buckinghamshire (Fig. 2.2). The size of each species determined the number of specimens collected ranging from three *A. minus* to ten *C. erythraea* plants. On the day of collection *P. reptans*, *B. officinalis* and *C. erythraea* were all in flower; *A. eupatoria* and *A. minus* had flowers and fruits on the same plant whilst *P. media* had few flowers and only part of the stalks intact. For *A. minus* only one whole plant was harvested as one tap root was ample and for *P. reptans* only mature plants with black tap roots were collected (Barker, 2001). The thin sinewy roots of *C. erythraea* and high moisture content of *P. media* leaves meant that repeat visits were necessary in both years to gather sufficient material for analysis. Plant colonies for all species in 2011 were less abundant compared to the previous year and only *P. media* was prolific in its environment.

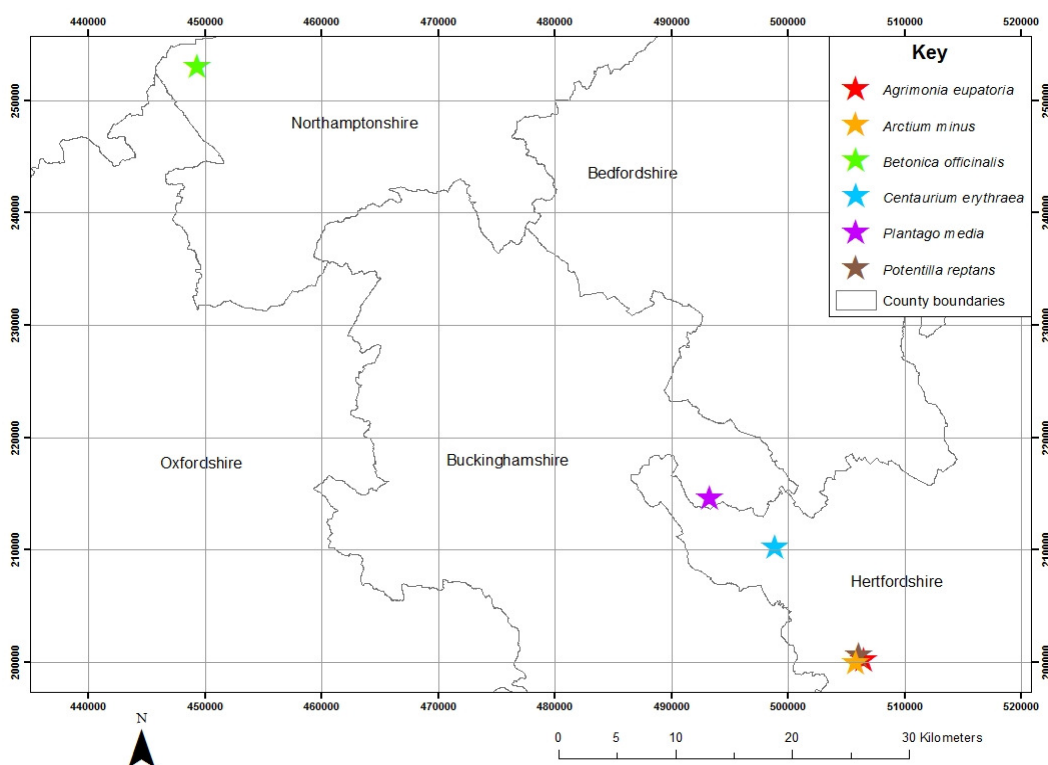


Fig. 2.2 Harvesting sites for plants collected in 2010 and 2011. Voucher specimens deposited at Royal Botanic Gardens, Kew and location references: ★ *A. eupatoria* FMW001 (TL0653000296), ★ *A. minus* FMW003 (TQ0583599987), ★ *B. officinalis* FMW003 (SP4938053120), ★ *C. erythraea* FMW005 (SP9887310299), ★ *P. media* FMW004 (SP9328914640) and ★ *P. reptans* FMW002 (TL0611800660).

2.4 Discussion

This research has been based on modern English translations of the Anglo-Saxon medical literature as the author does not read the vernacular (Watkins et al., 2012). The plant selection strategy is a robust model that could be further utilised to investigate the 10th century texts for additional conditions relevant to allied health and, other ancient medical texts awaiting scientific scrutiny. Battle wounds and burns in Anglo-Saxon England were common place (Cameron 2006; Van Arsdall, 2002) and mortality rates high from sword injuries, sling shot and arrow wounds as well as individuals being exposed to bloody lacerations on the battlefield (Hutley and Green, 2009). Bacterial infection and wounds were specifically chosen as descriptors for conditions readily identified in the medical texts with minimum ambiguity. They are also relevant to global health issues such as antibiotic resistance and increasing lifestyle disease as in chronic foot ulcers in diabetes (Coenye and Nelis, 2010; Hancock, 2005).

The selected plants were known to the Anglo-Saxon practitioner with each species allocated an Old English name and some more popular than others. *Betonica officinalis* is the most highly cited plant in the *Herbarium* (29 formulations) and, the most referenced medicinal plant in the 10th century medical literature (Deegan, 1988; Voigts, 1979) whereas in WHM, the plant is used for nervous headache, nerve pain and anxiety (Williamson, 2003). With the advent of the printing press in the 15th century and many new herbals published, elements of traditional herbal medicine were lost over time. For example, in the *Herbarium* the fresh juice of *A. eupatoria* was specified for treating fresh wounds; a practice that continued to the 17th century although now lost to traditional folklore and WHM (Allen and Hatfield, 2004; Barker, 2001; Williamson, 2003). *Centaurium erythraea* is used for a different range of therapeutic treatments in WHM compared to 10th century formulae. Toby et al. (2011) recommend herbal practitioners now consider using the root to treat chronic wounds, as described by Dioscorides, in antiquity. Some Anglo-Saxon medicinal formulae remain as with *P. major* and *P. lanceolata* to treat upper respiratory tract infections and external skin conditions (Adams et al., 2009). The lesser known *P. media* is referenced in Bald's *Leechbook* and listed in the medicinal flora (Barker, 2001; Cockayne, 1865, p.293) as a substitute plant for

P. major. The Anglo-Saxon practitioner would have substituted species according to the local terrain; *P. major* grows well in clay, whereas in sandy soils *P. media* would be most abundant (Watkins et al., 2011). *Arctium minus*, *P. media* and *P. reptans* have been used interchangeably for *A. lappa*, *P. major* and *P. erecta* respectively. All six species are listed in the medicinal flora (Barker, 2001) with only the latter three being commercially available from UK herbal manufacturers as dried herbs and tinctures.

Little is known about the phytochemistry and pharmacological actions of the substitute plants although *A. minus* is often grouped with *A. lappa* and, in herbal practice, is considered to be comparable even though the plants are genetically distinct (Stace, 2010). By contrast *P. reptans* has been superseded by *Potentilla erecta* L.; it may be that the former was more abundant in southern England with the latter preferring acid soils of northern and Western parts of the country (Allen and Hatfield, 2004). This correlates with an entry in *Culpeper's Complete Herbal and English Physician* (1653) that was compiled in London and includes *P. reptans* but not *P. erecta*. The phytochemistry for *A. eupatoria*, *B. officinalis* and *C. erythraea* is well reported in the literature albeit evidence of pharmacological actions and in particular, significant antibacterial activity, was lacking for all plants (Cwikla et al., 2010; Daglia, 2011; Skaltsa et al., 2003). In some cases, the phytochemical literature focussed on parts of the plant that have no traditional herbal application as in essential oil of *B. officinalis* and seeds for both *C. erythraea* and *A. eupatoria* (Grujic-Jovanovic et al., 2004; Kumarasamy et al., 2002).

Plants synthesise a significant diversity of secondary metabolites in response to microbial infection, insect and animal predators. Many of these constituents have been reported as effective antimicrobial agents including alkaloids, saponins, tannins and resins (Cowan, 1999; Cushnie and Lamb, 2005; Lucas, 1998; Gibbons, 2008). Tannins, the most abundant secondary metabolites made by plants, are widely distributed in the woody parts as well as the leaves. Tannins have the ability to react with proteins to form water insoluble polymers and are used in the tanning industry to transform animal hides into leather. Plants with a large amount of tannins are generally avoided by herbivores owing to the astringent taste and less protein being accessible to

the digestive juices (Barbehenn and Constabel, 2011; Daglia, 2011; Harborne and Williams, 2000). The antibacterial properties of tannins are well known and prominent amounts reported in the leaves of *B. officinalis* (6% gallic acid), *A. eupatoria* (>7% catechin and epicatechins), *Rosa damascena* L. (8% gallic acid equivalents) and for *P. reptans* 15-20% proanthocyanidin and hydrolysable tannins in the leaves and roots (ESCOP. 2009; Ozturk et al., 2009; Toby et al., 2011). Polyacetylenes have been reported for *A. lappa* and *Plantago* species with a wide range of biological functions including antimicrobial, antifungal and anti-inflammatory activity (Adams et al., 2009; Cowan, 1999). Verbascoside, a caffeic glycoside ester present in the leaves and roots of *Betonica*, *Plantago* and *Potentilla* species, is reported to have *in vitro* antimicrobial activity against Gram-positive and Gram-negative bacteria and *in vivo* activity inhibiting arachidonic acid induced mouse ear oedema (Adams et al., 2009; Fons et al., 1998; Tomczyk and Latte, 2009).

The Anglo-Saxon authors were aware that the medicinal properties of plants vary during the growth cycle and, where considered important, instructed how and when to collect the plants (Cameron, 2006; Cockayne, 1864; Van Arsdall, 2002). In an antimicrobial study of *Hypericum perforatum* the aerial parts collected in August inhibited growth of *S. aureus* (15 mm zone) and *E. coli* and *P. aeruginosa* (6 mm zone) compared to inactive specimens harvested in July (Borchardt et al., 2008). A phytochemical study by Southwell and Bourke (2001) confirmed that hypericin, the active principle of hypericum, increased fourfold when flowering of the plant had nearly finished, compared to 648-981 pmm in the preceding two weeks. In another study, aucubin an iridoid glycoside with anti-inflammatory activity was found to have a higher concentration in the leaves of *P. major* (1.3%) when harvested in June (Samulsen, 2000).

For millennia many native British plants have been used medicinally and some continue to be prescribed by practitioners of WHM whilst others are no longer used as a result of exotic imports, changing uses of landscapes, over harvesting or plant toxicity (Watkins et al., 2011). Wild plants were used in this study to:

- mimic the activity of an Anglo-Saxon practitioner collecting local material
- be reasonably sure that the plants had not been fertilised or genetically enhanced from cultivated sources
- perform analysis using minimal amounts of plant material compared to classical phytochemical methods that typically use 1-2 kg of plant material for an initial phytochemistry screening (Houghton and Rahman, 1998).

There was a noticeable difference in the abundance of species at the locations visited in 2010 and 2011 showing that plants continually adapt and respond to the environmental conditions. *Betonica officinalis*, favours impoverished hay meadows and has with evolving agricultural methods, declined significantly in southern England during the last 25 years (James, 2010). By contrast, *P. major* an example of the *Plantago* species, is common globally and cultivated as a medicinal horticultural crop in Brazil (Zubair et al., 2011).

The author previously reported three native British species *A. millefolium*, *H. perforatum* and *M. vulgare* all listed in Anglo-Saxon formulations, are well researched with *in vitro* and *in vivo* studies, revealing bioactive metabolites that underlie the uses in the medieval medical texts (Watkins et al., 2011). Whilst the antimicrobial literature is limited for the plants in this study, many of the secondary metabolites reported would be beneficial in treating bacterial infections and wounds; flavonoids, kaempferol and quercetin glycosides exhibit antibacterial, anti-inflammatory and potent radical scavenging activity as well as inhibition of fibroblast growth (Daglia, 2011; Harborne and Williams, 2000). Procyanidin-enriched fractions from *Plantago ovata* Forskal have also shown to stimulate the proliferation of keratinocytes in the wound healing process (Houghton et al., 2005). In this study, aqueous, EtOH and wine formulations for six native species will be screened for *in vitro* antimicrobial activity to determine whether they would have been appropriate in the 10th century wound healing formulations.

Chapter 3.0 Antimicrobial Bioassays

3.1 Introduction

In ethnopharmacological studies, complex plant mixtures and isolated pure compounds are initially screened *in vitro* (Houghton et al, 2005). Wound healing assays have been developed for antimicrobial, anti-inflammatory, antioxidant, fibroblast proliferation and collagen lattice formation. These methods can be performed in a relatively short period in the laboratory. In terms of screening plants with little or no known phytochemistry, *in vitro* assays are more cost efficient and ethical than *in vivo* studies at the preliminary stage of investigation (Adams et al., 2009; Annan and Houghton, 2008; Cos et al., 2006).

Wound healing progresses through three distinct stages: haemostasis or the staunching of blood flow; inflammation including pain and swelling soon after injury and regeneration or remodelling, taking up to 2-3 weeks for complete healing to occur (Majno and Joris, 1996; Suntar et al., 2010). Wound infection is a clinical diagnosis that includes pain, redness, splitting of tissue, discharge and sinus or abscess formation (Hutley and Green, 2009). By contrast, a clinical infection is defined as an internal or external site whereby the pathogens reach a concentration in excess of 10^5 /mL and overcome the host's immune system (Black and Costerton, 2010; Siddiqui and Bernstein, 2010).

The discovery of penicillin by Alexander Fleming in 1928 (Rishton, 2008) led to a better understanding of infectious disease and more effective treatment for wounds. The main classes of antibiotics used in the West and the mechanistic actions against Gram-positive and Gram-negative pathogens are shown in Table 3.1. A variety of broad-spectrum antibiotics, derived from microbes, were widely used until the 1990s, resulting in increased bacterial resistance and, more recently, multi-drug resistance (Gibbons, 2008; Theuretzbacher, 2009). A drug causing bacteriostatic action involves intracellular disruption of the RNA and/or DNA preventing cell reproduction whilst not necessarily killing the microbial cell (Kohanski et al, 2010).

Table 3.1 The physiological mechanisms of natural product antibiotics against Gram-positive and Gram-negative bacteria commonly found in wounds (adapted from Kohanski et al; 2010)

Mode of Action	Antibiotic Class	Drug	Microorganism Susceptibility	Primary Target	Reference
Cell wall synthesis inhibitor	β -lactams	Penicillin	Gram-positive and Gram-negative	Penicillin-binding proteins	Kohanski et al. (2010)
	Glycopeptides	Vanomycin	Gram-positive especially <i>S. aureus</i>	Peptidoglycan units	Kohanski et al. (2010)
	Lipopeptides	Daptomycin	Gram-positive	Cell membrane	Hancock (2005)
Protein synthesis inhibitor	Aminoglycosides	Gentamicin	Gram-positive and Gram-negative	Binds to the 16S rRNA of the 30S ribosome subunit - protein mistranslation	Kohanski et al. (2010)
	Macrolides	Erythromycin	Gram-positive and Gram-negative	50S ribosomal subunit	Dewick (2009)
	Phenicol	Chloramphenicol	Gram-positive and Gram-negative	50S ribosomal subunit	Dewick (2009)
	Streptogramins	Streptogramin A and Streptogramin B		50S ribosomal subunit	Kohanski et al. (2010)
	Tetracyclines	Tetracycline	Gram-positive and Gram-negative	30S ribosome	Dewick (2009)
DNA synthesis inhibitor	Sulphonamides	Gantrisin	Gram-positive and Gram-negative	Inhibits folic acid metabolism	Roche Laboratories (1997)
RNA synthesis inhibitor	Rifamycins	Rifamycin	Gram-positive and Gram-negative	DNA-dependent RNA polymerase	Kohanski et al. (2010)

By contrast, bactericidal action is defined as death of the microbial cell via degradation of the cell wall. The drug or pure compound also needs to be selective in its action by only attacking the microbial cell and having minimal effect on host cells at the therapeutic dose, for example, macrolides and aminoglycosides (Hancock, 2005). Gram-positive *S. aureus* is largely associated with skin infections and drug action against this organism is predominantly bactericidal by inhibiting cell wall synthesis as in β -lactam penicillin (Dewick, 2009; Hancock, 2005). Gram-negative pathogens are more complex in structure having an outer cell wall thereby decreasing permeability of the drug and efflux pumps on the cell membrane, capable of rendering the drug inactive (Gibbons, 2008; Kuete et al., 2008). Deep wounds are polymicrobial, prone to chronic infection, and potentially provide an entry point for bacteria to enter the host and produce a systemic infection (Houghton et al., 2005).

Many indigenous cultures use plants topically and systemically to treat bacterial infection and wounds for example *Echinacea purpurea* L. In WHM this species is used externally to treat superficial wounds and internally as an adjuvant for recurrent respiratory and urinary tract infections (ESCOP, 2003; Gibbons, 2008; Savoia, 2012). Heinrich et al. (2012) describe lemon balm *Melissa officinalis* and garlic *Allium sativa* as broad spectrum antibiotics inhibiting both Gram-positive and Gram-negative bacteria. In the Anglo-Saxon medical texts *A. minus* is used in a similar manner: the juice of the leaves mixed with wine as a herbal wound drink; the plant boiled and the liquid used as an antiseptic wash with the residue pounded in grease and applied as a poultice (Van Arsdall, 2002, p.167).

Ethnomedical knowledge can be a useful tool in identifying plants that may yield new leads in drug development (Adams et al., 2009; Gibbons, 2008) and the Anglo-Saxon medical texts may yet prove to be a valuable source of indigenous plant knowledge for further investigation. The aim of this chapter is to experimentally determine *in vitro* whether the selected plants (*A. eupatoria*, *A. minus*, *B. officinalis*, *C. erythraea*, *P. media* and *P. reptans*) would have been appropriate for the Anglo-Saxon treatment of bacterial infection and wounds.

3.2 Materials and Methods

3.2.1 Chemicals

Chloramphenicol (Sigma Aldrich), dimethyl sulphoxide (DMSO), formic acid (50 %), HPLC grade EtOH and MeOH were obtained from Fisher Scientific UK Ltd and red wine (Cabernet Sauvignon, 2007; Barkan Classic, barcode: 7290000023809). Nutrient broth (CM0001) and nutrient agar (CM0003) were purchased from Oxoid Limited; distilled water purified using a reverse osmosis system (Purelab Option S).

3.2.2 Extraction of Plant Material

The basic concept of the methodology followed antimicrobial protocols proposed by Cos et al. (2006) and Rios and Recio (2005) both widely cited in the literature. The plants were divided into two experimental groups determined by availability of material and an experimental code assigned to each extract (Appendix 2.1). Aerial parts and roots for each species were separated and air dried in a cool dark room for five days followed by 48 h in a fan assisted oven (Gallenkamp) at 40 °C to avoid degradation by microorganisms. The material was ground to a rough powder using an electric blender, refined using a 500 µm sieve and stored in the dark in sealed glass jars for further use (Houghton and Rahman, 1998).

Grounded aerial parts and roots (2 g) were separately macerated for 24 h in 20 mL cold solvent (25% and 75% EtOH in H₂O, red wine) making four EtOH and two wine extractions for each plant. For the hot extracts additional H₂O was used to accommodate the expansion in plant material; the infusion was prepared with 40 mL boiling H₂O to 2 g of dried aerial parts and left to infuse for 10 min with container covered to prevent vapour loss. For the decoction, 100 mL H₂O was added to 2 g of roots, boiled then simmered for 20 min and reduced to a third (Van Arsdall, 2002). The supernatant for each crude extract was filtered (Whatman No. 11), evaporated to 5 mL using reduced pressure under vacuum (Buchi Syncore) at 45 °C, transferred to a drying block (Techne Dri-block) at 40 °C for 1-2 days. Stock solutions (20 mg/mL) comprised 60 mg of crude extract dissolved in 3mL DMSO (100%) using an ultrasonic

water bath (QHKerry) for 30 min and stored at 4 °C for further use (Cos et al., 2006).

3.2.3 Microbial strains

The four microorganisms selected for the *in vitro* bioassays were representative of those commonly used in the antimicrobial screening of natural products (Kumarasamy et al., 2003; Moskalenko, 1986; Quave et al., 2008). The four bacterial strains were Gram-positive *Staphylococcus aureus* (NCTC 7447), *Bacillus subtilis* (NCTC 3610), Gram-negative *Escherichia coli* (UEL 57) and *Pseudomonas aeruginosa* (NCIB 8295). The *E. coli* isolate was independently serotyped by the Gastrointestinal Infections Unit, Health Protection Agency, Colindale as *E. coli* as rough serotype, H49 (Appendix 3.1). A rough strain is determined by the loss of the O-Antigen and shorter core oligosaccharides whilst retaining the lipid portion in the outer membrane of gram-negative bacteria (Triantafilou et al., 2000).

3.2.4 Bacterial Cultures

Four to five colonies were transferred from nutrient agar plates to 5 mL sterile nutrient broth and incubated at 37 °C overnight. The bacterial concentration for each pathogen was standardised using a spectrometer (Jenway 6305) with optical density readings of 0.1 at 600 nm. The spectrometer was calibrated with 1 mL of sterile nutrient broth and following absorbance reading, cultures diluted with sterile nutrient broth to an inoculum density of 1.5×10^6 colony forming units (CFUs) (Kuate et al., 2009). The standardised inoculum was seeded in sterile nutrient broth 15 min before filling the 96-well microtitre plate (Cos et al., 2006). A loop of overnight culture was streaked on to an agar plate (Oxoid) and incubated at 37 °C for 18 h for all microorganisms to verify no contamination in the bacterial cultures.

Viable colonies were maintained of each species by preparing new agar plates every 2-3 weeks and stored at 4 °C until required. Reserve supplies of bacterial cultures comprised 4.5 mL of inoculum added to 1.5 mL glycerol in saline solution (40%) and were stored at minus 20 °C and minus 80 °C for future use. Growth curves were conducted for each typed culture in a round bottomed

96-well microtitre plate; 195 μ L nutrient broth and 5 μ L of standardised inoculum for each microorganism was placed in quadruplicate cells, incubated at 37 °C with optical density readings of 600 nm recorded at 0 min and subsequently every 60 min for 18 h. Bacterial growth was calculated plotting hourly absorbance readings over time and experiment repeated on three independent days (n=3). An antibiotic susceptibility test (MASTRING-S™) was performed using 8 impregnated tips to confirm the selected Gram-positive and Gram-negative bacteria (M13 and M27 respectively) were not resistant to reference antibiotic, chloramphenicol. Nutrient agar plates were streaked with overnight culture, antibiotic tips applied to the media and plates incubated at 37 °C overnight and zone of inhibition measured.

3.2.5 Antimicrobial Assay

The microdilution method was used for the *in vitro* antimicrobial screen, prepared in round bottomed 96-well microtitre plates and conducted according to NCCLS (2009) protocol. Wells were filled with 100 μ L plant extract at final concentrations of 200, 40 and 8 μ g/mL, 95 μ L nutrient broth and 5 μ L inoculums at the standardised concentration of 1.5×10^6 CFUs (an optical density reading of 0.1 at 600 nm). Sterile nutrient broth was seeded (5 % inoculums) and placed on a vibrating plate for 15 min before use (Cos et al., 2006; Rios and Reico, 2005). Chloramphenicol was used as the reference antibiotic and positive control (Houghton et al., 2005), nutrient broth with DMSO (1% final concentration) as the negative control, nutrient broth seeded with inoculums for bacterial growth control, and nutrient broth only wells to ensure no contamination was present.

One 96-well microtitre plate was used for each plant per organism and the layout is shown in Fig. 3.1. The wine extracts were prepared on a separate microtitre plate with a red wine control, making a total of twenty plates per experiment. Each plant extract and control was performed in quadruplicate and repeated on three independent days (n=3). Each plate was assigned a unique code that was carried through to file name when exporting pre and post incubation absorbance data into Microsoft Excel software. Bacterial growth was determined by taking optical density readings at 600 nm at 0 h (t_1) and again at

18 h (t_2) after incubating at 37°C without agitation. The plate reader was programmed to shake each microtitre plate for 10 seconds prior to each reading. The percentage of bacterial growth inhibition (PBGI) was calculated as follows:

$$\text{PBGI} = 100 - (t_2 - t_1) / \text{Mean growth of control cells} \times 100).$$

	A	B	C	D	E	F	G	H
	200 µg/mL	40 µg/mL	8 µg/mL	200 µg/mL	40 µg/mL	8 µg/mL		
1	1	1	1	2	2	2	7	9
2	1	1	1	2	2	2	7	9
3	1	1	1	2	2	2	7	9
4	1	1	1	2	2	2	7	9
5	3	3	3	4	4	4	7	9
6	3	3	3	4	4	4	7	9
7	3	3	3	4	4	4	7	9
8	3	3	3	4	4	4	7	9
9	5	5	5	6	6	6	8	10
10	5	5	5	6	6	6	8	10
11	5	5	5	6	6	6	8	10
12	5	5	5	6	6	6	8	10

Fig. 3.1 96-well microtitre plate layout for each plant comprising 6 aqueous/EtOH extracts at screening concentrations of 200, 40 and 8 µg/mL. A separate plate was prepared for wine extracts. **Key** Infusion (1), Decoction (2), Leaf 25% EtOH (3), Root 25% EtOH (4), Leaf 75% EtOH (5), Root 75% EtOH (6), Nutrient Broth (7), Chloramphenicol (8), DMSO at 1% (9) and blank cells (10). Plate layout adapted from Cos et al., (2006).

3.2.6 Statistical Analysis of Data

A control group was created for each organism comprising 12 wells (4 from each experiment) to confirm any significant difference between the intra and interday experimental data. The Shapiro-Wilk test was used to assess the normality of the data (Field, 2005; Razali and Wah, 2011). One way Analysis of Variance (ANOVA) was used to establish any statistically significant differences between plant extracts and also the control groups for each organism ($p < 0.05$) using Microsoft Excel 2007 software. The MIC_{50} and MIC_{90} values were determined as 50% and 90% of the inhibition of bacterial growth expressed as \pm standard error of the mean (SEM) of three independent bioassays ($n=3$).

3.3 Results

3.3.1 Extraction of Plant Material

A total of 50 aqueous, EtOH and red wine extracts were prepared from the six selected plants including a red wine control. Ground material (2 g) was used to prepare each extract with the largest leaf yield being *A. minus* infusion (530 mg) compared to *A. eupatoria* 25% EtOH extract (104 mg). For the roots, *P. reptans* decoction produced the largest yield (1232 mg) and *C. erythraea* 75% EtOH extract the smallest yield (42 mg) (Appendix 3.2).

3.3.2 Antimicrobial Assay

The antimicrobial assay was an initial screen of the six selected plants at 200, 40 and 8 µg/mL. The full MIC and MBC activity for the most potent extracts will be discussed in Chapter 5. The Shapiro-Wilk test (Table 3.2) confirmed that there was no significant difference in the normality of the activity after removing plant extracts identified as outliers (*B. officinalis* 75% EtOH leaf against *S. aureus*, root wine against *B. subtilis* and both 75% EtOH roots of *B. officinalis* and *P. media* against *P. aeruginosa*).

Table 3.2 P values (p < 0.05) for Shapiro Wilk's test to determine normality in the distribution of plant extracts inhibiting growth of Gram-positive and Gram-negative wound pathogens.

Plant	<i>S. aureus</i>	<i>B. subtilis</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
<i>A. eupatoria</i>	0.371	0.473	0.242	0.274
<i>A. minus</i>	0.116	0.544	0.712	0.745
<i>P. reptans</i>	0.792	0.351	0.453	0.184
<i>B. officinalis</i>	0.307	0.852	0.769	0.081
<i>C. erythraea</i>	0.855	0.804	0.064	0.411
<i>P. media</i>	0.689	0.865	0.499	0.535

A summary of IC₅₀ values from the initial antimicrobial screening of all six plants against Gram-positive and Gram-negative bacterial commonly found in wounds is shown in Table 3.3.

Table 3.3 Summary of IC₅₀ values for the antimicrobial screening of selected plants at 200, 40 and 8 µg/mL against Gram-positive and Gram-negative wound pathogens – there were no IC₅₀ values for any extracts against *P. aeruginosa*.

Plant extracts	<i>S. aureus</i>	<i>B. subtilis</i>	<i>E. coli</i>
<u>Agrimonia eupatoria</u>			
Leaf Infusion	200	-	200
Leaf Wine	40	-	-
Leaf 25% EtOH	40	-	-
Leaf 75% EtOH	40	-	-
Root Decoction	8	40	40
Root 25% EtOH	40	200	200
Root 75% EtOH	40	200	-
<u>Arctium minus</u>			
Leaf 25% EtOH	200	200	-
Root Decoction	200	-	-
Root Wine	-	200	-
Root 25% EtOH	40	200	-
Root 75% EtOH	200	200	-
<u>Betonica officinalis</u>			
Leaf Wine	-	200	-
Leaf 25% EtOH	-	40	-
Leaf 75% EtOH	-	200	-
Root Decoction	200	200	-
Root 25% EtOH	-	8	-
<u>Centaurium erythraea</u>			
Leaf Infusion	-	200	-
Leaf Wine	-	200	-
Leaf 25% EtOH	-	40	-
<u>Plantago media</u>			
Leaf Infusion	-	8	-
Leaf 25% EtOH	-	40	-
Root Decoction	-	8	-
Root 75% EtOH	-	8	-
<u>Potentilla reptans</u>			
Leaf Infusion	200	-	-
Leaf 25% EtOH	200	40	-
Root Decoction	200	40	40
Root Wine	-	200	-
Root 25% EtOH	40	200	200
Root 75% EtOH	8	-	-

All plants demonstrated greater activity against the Gram-positives than Gram-negative pathogens. The leaf and root wine extracts exhibited the weakest activity with 4 plants having MIC₅₀ values of 200 µg/mL against *B. subtilis* (*B. officinalis*, *C. erythraea*, *A. minus* and *P. reptans*). Only *A. eupatoria* leaf wine extract inhibited growth of *S. aureus* at the lower concentration of 40 µg/mL. None of the wine extracts exhibited MIC values against the Gram-negative bacteria.

The aqueous and EtOH leaf extracts demonstrated greater antimicrobial activity inhibiting Gram-positive bacteria in three or more samples with *C. erythraea* and *P. media* infusions the most potent against *B. subtilis* (MIC₅₀ 8 µg/mL) and against *S. aureus*, *A. eupatoria* 25% and 75% EtOH leaf extracts (MIC₅₀ 40 µg/mL) as shown in Appendices 3.3 and 3.4. Against Gram-negative pathogens *A. eupatoria* infusion was the only leaf extract to inhibit *E. coli* (MIC₅₀ 200 µg/mL (Appendix 3.5). None of the leaf extracts inhibited growth of *P. aeruginosa* (Appendix 3.6).

The roots were more active than the leaves against Gram-positive bacteria with all aqueous and EtOH extracts (except *C. erythraea*) exhibiting MIC₅₀ values in two or more extracts at 40 µg/mL (Fig. 3.2). One *B. officinalis* and two *P. media* EtOH root extracts demonstrated MIC₅₀ values of 8 µg/mL against *B. subtilis* (Appendix 3.7). The most potent root extracts to inhibit growth of *S. aureus* were *A. eupatoria* decoction and *P. reptans* 75% EtOH root (MIC₅₀ 8 µg/mL) with both plants exhibiting activity in a dose dependent manner (Fig. 3.2). The root decoctions for the same two plants were the only mixtures to demonstrate MIC₅₀ value of 40 µg/mL against *E. coli* (Fig. 3.3). The *P. reptans* root decoction also exhibited 48% inhibition of *E. coli* at 8 µg/mL whereas the only root extract to inhibit >40% growth of *P. aeruginosa* was *A. eupatoria* 25% EtOH root (Appendix 3.8).

The *A. eupatoria* 75% EtOH root extract demonstrated the greatest range of inhibitory growth against *S. aureus* with a MIC₉₀ at 200 µg/mL to a MIC₅₀ at 40 µg/mL. The most potent preparations inhibiting *S. aureus* were *A. eupatoria* decoction and *P. reptans* 75% EtOH root extracts (MIC₅₀ 8 µg/mL) in a dose dependent manner. The only plants to inhibit growth of Gram-negative

E. coli were *A. eupatoria* and *P. reptans* (MIC₅₀ 40 µg/mL). A one way analysis of variance (ANOVA) showed there was no statistical difference between the activity of the two plants ($p = 0.5324$).

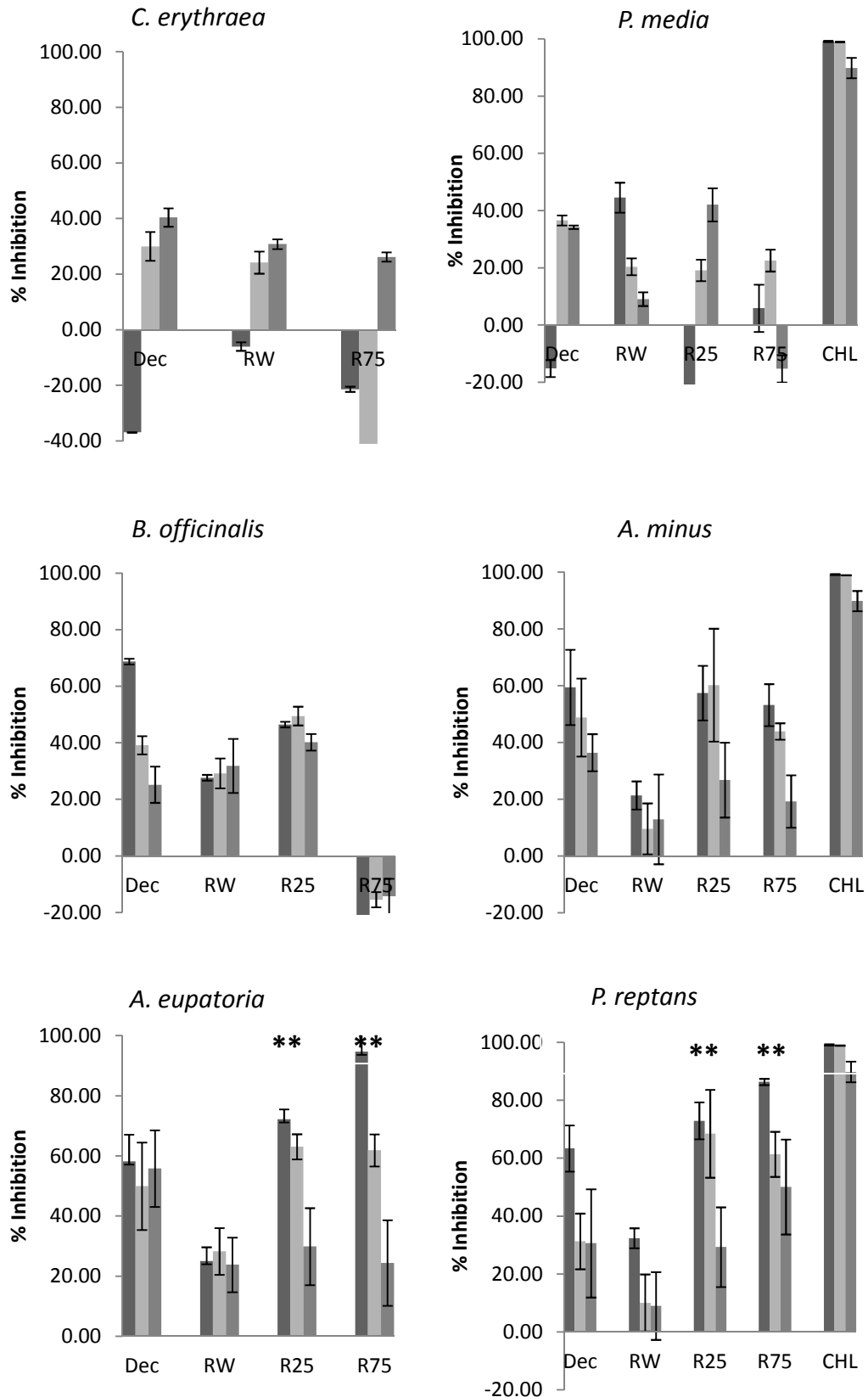


Fig. 3.2 *S. aureus* antimicrobial screening results for root extracts (from left to right) at 200, 40 and 8 µg/mL. **Key:** **Dec** Roots in boiling H₂O; **RW** Red wine; **R25** 25% EtOH; **R75** 75% EtOH and **CHL** Chloramphenicol (positive control). ** denotes significant inhibition (p<0.01).

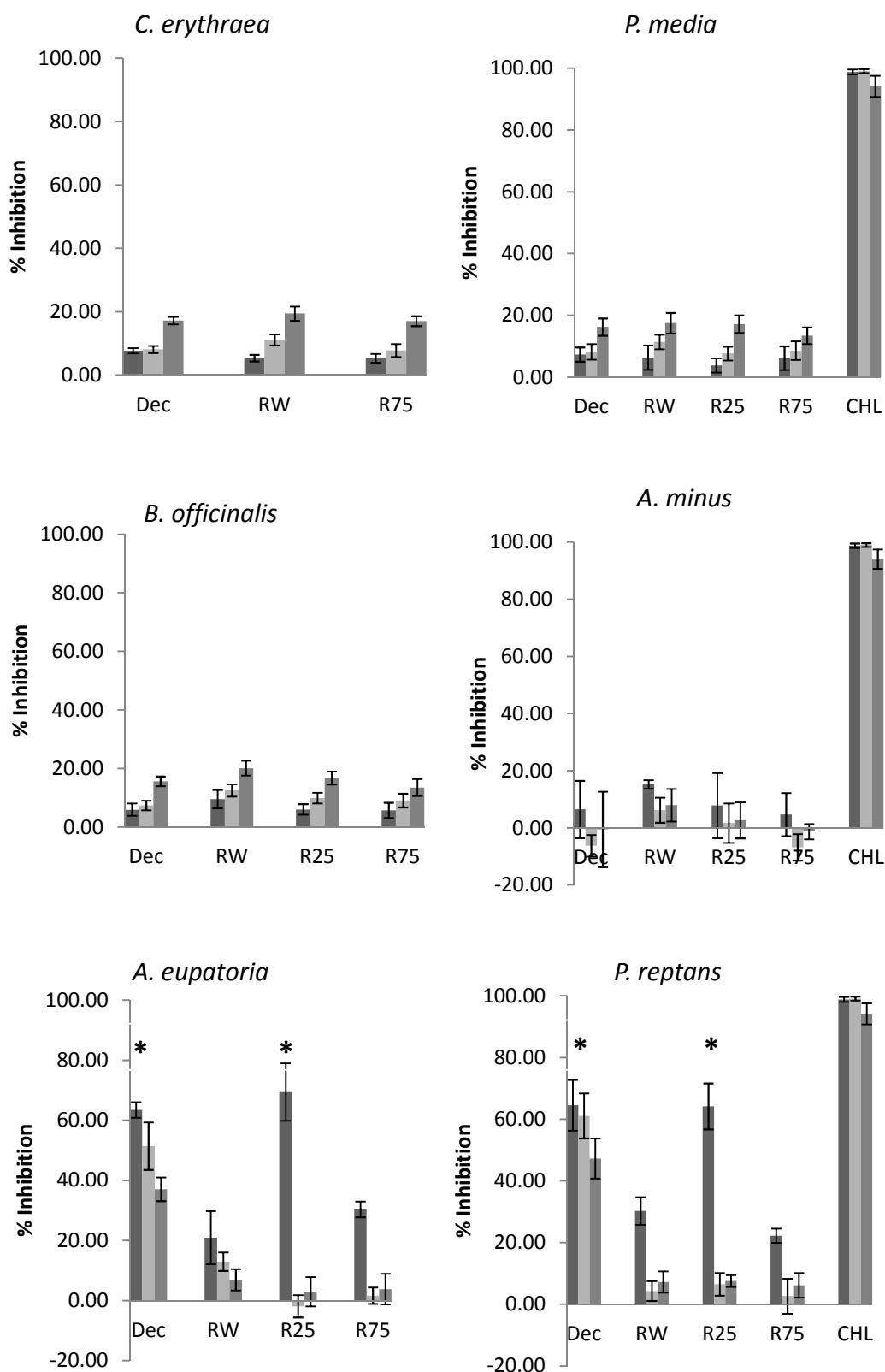


Fig. 3.3 *E. coli* antimicrobial screening results for root extracts (from left to right) at 200, 40 and 8 $\mu\text{g/mL}$. **Key:** **Dec** Roots in boiling H₂O; **RW** Red wine; **R25** 25% EtOH; **R75** 75% EtOH and **CHL** Chloramphenicol (positive control). * denotes significant inhibition (p < 0.05).

3.4 Discussion

Many indigenous cultures use plants to treat bacterial infection topically and systemically (Gibbons, 2008) although for the plants in this study, there was limited or no evidence of significant *in vitro* antimicrobial activity against wound pathogens. Relevant pharmacological studies were found for some plants including anti-inflammatory and analgesic activity (Adams et al., 2009; Capasso et al., 1983; ESCOP, 2003).

The phytochemical literature reported on parts of the plant that have no traditional use in WHM as in *C. erythraea* and *A. eupatoria* seeds and the essential oil of *B. officinalis* (Copland et al, 2003; Kumarasamy et al., 2002; Skaltsa et al., 2003). The following of classic phytochemical methods would have entailed decrepitating compounds from the plant extracts that may have antimicrobial activity, for example, chlorophylls and tannins, prior to the *in vitro* screening (Houghton and Rahman, 1998; Harborne, 1998). However, in this instance the hypothesis set out to establish whether the plants used in 10th century Anglo-Saxon formulations may have been appropriate in treating wounds and therefore no constituents were removed prior to screening activity. There is evidence of water soluble chlorophyll extracts being used successfully to treat chronic wounds not responding to orthodox treatment (Mowbray, 1957).

It is estimated that 400-500 mg of pure compound is required to conduct comprehensive *in vitro* and *in vivo* studies which equates to 100 kg of dried plant material (Machesney et al., 2007). This is not a viable option when collecting specimens from the wild for species that may or may not be active. The microdilution assay is a quantitative method used to determine *in vitro* activity for liquid extracts (Cos et al., 2006) and combined with chemo metrics is a more economical method for identifying compounds that significantly contribute to the antimicrobial activity (Roost et al., 2004). The model designed for this study used <30 g of dried material for both the *in vitro* bioassay and chemical analysis of each species. The hot extracts were more dilute extractions (1:15 for the roots and 1:20 for the leaves) yet exhibited greater antimicrobial activity than the cold extracts (1:10 for roots and leaves).

The UEL 57 teaching strain of *E. coli* was independently confirmed as a rough strain meaning that the lip polysaccharides (LPS) have lost part or all of the O-polysaccharides resulting in the outer membrane having greater permeability that could reduce the effectiveness of the organism resistance to many antibiotics. In this study, the *E. coli* strain was only susceptible to the most potent extracts of *A. eupatoria* and *P. reptans* demonstrating that loss of the O-chain in this species did not make the organism susceptible to all plant extracts or to the antibiotic control, Chloramphenicol in the Mastering test.

The aqueous infusions, decoctions and red wine extracts were chosen to reflect the liquid solvents used in Anglo-Saxon wound healing formulations (Van Arsdall, 2002). The lyophilise compounds extracted by the animal fat formulations could have been tested using hexane but were not part of this project. The 25% EtOH were representative of solvents commonly used in WHM (Bone, 2003; Mills and Bone, 2007) and the 75% EtOH extracts as examples of solvents used in classic phytochemical analysis (Harborne, 1998). Anglo-Saxon formulae specify various vehicles for both topical and internal 'brewits' (Table 1.2) including honey, vinegar, wine, dairy products and animal grease (Cameron 2006; Pettit, 2001; Van Arsdall, 2002). Phytochemical analysis would be conducted on specific samples with the underlying premise that the active metabolites would be present, albeit in very small quantities. The full bodied cabernet sauvignon was chosen as an example of an 'old wine' specified in the texts for both systemic and topical preparations (Van Arsdall, 2002) and at 13% vol, may explain why the wine extracts were less active than the 25% EtOH mixtures.

There is evidence of wine being imported from Europe during the Anglo-Saxon era as well as being produced locally with more than 38 vineyards recorded in the Domesday Book in 1086 (Voigts, 1979). Ale and prepared fruit wines including blackberry, elderberry, raspberry and mulberry were common drinks. Dioscorides in the 1st century recommended British mead being superior to other European sources (Hagen, 2006; Riddle, 2011). The strength of wine during 10th century is not known although it must have contained >10% alcohol in order not to become acetic acid during transit and according to Hagen (2006) a fruit wine could have reached 18-20% vol. The health benefits of red wine

today are attributed to the many polyphenols including gallic acid and ellagic acid known to inactivate bacteria by cell wall synthesis (Annan and Houghton, 2008; Daglia, 2011; Garcia-Ruiz et al., 2011). Supercritical extracts from Merlot pomace demonstrated activity against *S. aureus* (MIC 625 \pm 375 to 750 \pm 250 μ g/mL) and to a lesser extent, activity against Gram-negative pathogens *E. coli* and *P. aeruginosa* with MIC values of 1000 μ g/mL (Oliveira et al., 2013). All plants in this study exhibited MIC₅₀ values in either leaf or root wine extracts at 200 μ g/mL against *B. subtilis* (except *P. media*) and only *A. eupatoria* leaf wine extract exhibited activity against *S. aureus* at 40 μ g/mL.

In an *in vitro* bioassay of *Plantago major*, a species in the same genus as *Plantago media*, aqueous extracts of the aerial parts inhibited 78% of *B. subtilis* at 400 μ g/mL whereas a hexane extract inhibited the same organism at 50 μ g/mL (Velasco-Lezama et al., 2006). By contrast, the leaf infusion of *P. media* in this study was more active against *B. subtilis* (MIC₅₀ 8 μ g/mL). The *A. eupatoria* 25% EtOH was the most potent leaf extract inhibiting growth of *S. aureus* (MIC₅₀ 40 μ g/mL) compared to a hexane extract of *A. eupatoria* seeds reported to have a MIC value of 750 μ g/mL (Copland et al., 2003). Leaf extracts for *A. minus*, *C. erythraea* and *P. media* against Gram-positive bacteria exhibited growth in the wells that was greater than the control cells. Sanders et al. (1945) commented on a similar observation in *A. minus* samples which they attributed to the plant extract providing growth factors or similar compounds. Another theory is that the bacterial cell “became enlarged” as a result of one or more plant compounds crossing the cell membrane; one of the known mechanisms of emodin is the “ability to elongate” the bacterial cell (Izhaki, 2002). It is not possible to determine the antimicrobial mechanisms of the plant extracts in this study as the active metabolites are unknown.

Plant roots have greater applications in Traditional Chinese and African medicines with the use of leaves more prominent in WHM; perhaps reflective of availability in the local environment? An EtOH root extract of white horehound *Marrubium vulgare* was shown to be a potent inhibitor of MRSA with IC₅₀ values of 32 μ g/mL (Quave et al., 2008). Only one antimicrobial study was found in the literature for roots of the selected plants; an agar disc diffusion assay for *P. reptans* aqueous decoction that inhibited *S. aureus* (10-12 mm zone of

inhibition) and *E. coli* (4-7 mm zone) (Redzic et al., 2009). In this study, the most potent root extracts were the decoctions and 75% EtOH mixtures of *A. eupatoria* and *P. reptans* with activity against Gram-positive (MIC_{50} 8 $\mu\text{g/mL}$) and Gram-negative pathogens (MIC_{50} 40 $\mu\text{g/mL}$) although the roots of *A. eupatoria* are not used in WHM (ESCOP, 2009; Tobyn et al., 2011).

Antimicrobial activity for crude plant extracts is considered to be significant at concentrations below 100 $\mu\text{g/mL}$ (Cos e al., 2006; Rios and Recio, 2005) although in the literature many plant extracts are reported as being active at much higher concentrations for example, a MeOH extract of *Hypericum perforatum* was considered active against *S. aureus* with a MIC of 1250 $\mu\text{g/mL}$ (Saddiqe et al., 2010). Many authors have cited a MIC based system by Aligiannis et al. (2001) with levels of up to five times greater than those of Cos et al. (2006), thereby creating confusion in the literature as to what is considered a strong, moderate or weak inhibitor (Roersch, 2012). Whilst none of the extracts in this study exhibited full MIC values at the screening concentrations, all of the plants (except *A. minus*) exhibited MIC_{50} values of 8 $\mu\text{g/mL}$ in one or more extracts against Gram-positive bacteria, suggesting they are worthy of further investigation. However, plants active against Gram-positive and Gram-negative pathogens are more likely to combat infection and assist in permanent wound healing (Hutley and Green, 2009). Both *A. eupatoria* and *P. reptans* root extracts were active against *S. aureus* (MIC_{50} 8 $\mu\text{g/mL}$) and against *E. coli* (MIC_{50} 40 $\mu\text{g/mL}$) and whilst they were the most potent overall in the antimicrobial screening, a one way ANOVA showed there was no significant difference between the two plants. Therefore all plants were analysed using HPLC and multivariate analysis before deciding which extracts would be investigated for the active antimicrobial metabolites.

Chapter 4.0 Plant Metabolite Mapping

4.1 Introduction

Biosynthetic pathways are fundamental to metabolism and plants within the same taxonomic family often have related therapeutic actions. Thousands of secondary metabolites are produced from a small quantity of primary metabolites, of which a large number are defence mechanisms used to deter microbial and fungal invaders as well as insects and herbivores (Dewick, 2009; Harborne and Williamson, 2000). For example, cocoa *Theobroma cacao* L. has been shown to produce a broad range of antimicrobial compounds including sulphur when under attack from a vascular wilt virus (Dixon, 2001). Surprisingly, this diversity arises from a distinctly small number of chemical classes mainly terpenoid, phenylpropanoid, flavonoid and alkaloid/polyketides outlined in Fig. 4.1 (Marienhagen and Bott, 2013).

The terpenoids are of pharmacological importance even though they are only present in relatively small quantities. These compounds are synthesised via the mevalonate pathway. Phenylpropanoids are derived from the shikimate pathway with hydroxycinnamic acids having many therapeutic actions. For example, the analgesic and anti-inflammatory properties of caffeic acid result from inhibition of DOPA-decarboxylase and 5-lipoxygenase enzymes. Caffeine and berberine, the most widely distributed nitrogen containing alkaloids, occur in the leaves, roots, fruits and seeds of flowering plants (Dewick, 2009; Ganora, 2009; Pengally, 2004). Flavonoids are produced via the shikimate and acetate pathways with varying degrees of solubility ranging from highly polar tannins to non polar quercetin and kaempferol. These compounds are plant pigments that protect tissue against UV radiation and are anti-inflammatory, antioxidant as well as enzyme inhibitors (Harborne, 1998).

The use of hyphenated analytical instruments including high performance liquid chromatography (HPLC) and high resolution mass spectrometry (HR-MS) form the basis for non targeted analysis to identify peaks in plant extracts of unknown phytochemistry (Kite et al., 2007). This is fundamental where availability of plant material is insufficient for traditional phytochemical methods

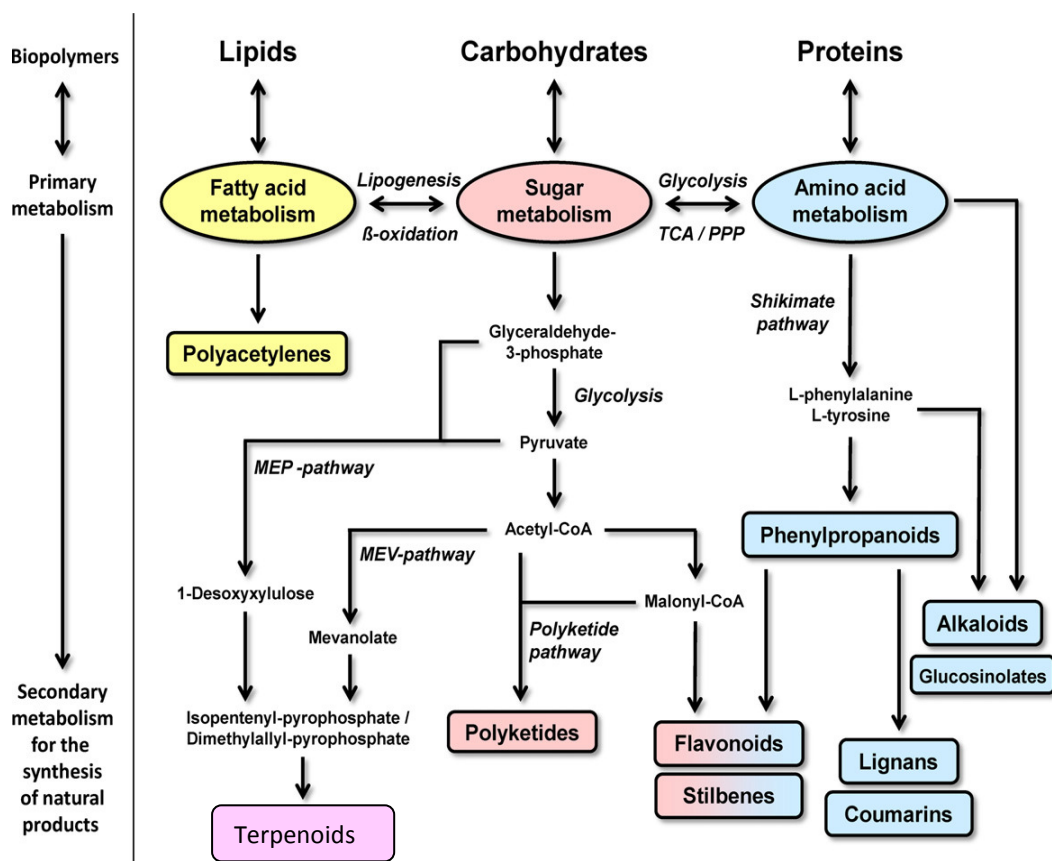


Fig. 4.1 Biosynthetic pathways for main chemical classes of primary and secondary plant metabolites (with permission and adapted from Marienhagen and Bott, 2013).

such as isolation and fractionation of the active constituents (Houghton and Raman, 1998). The presence of thousands of metabolites in a plant extract makes the spectral data too complex to analyse visually and, whilst some peaks may be matched to available reference standards, the activity could be from an unknown compound and require further analysis (Kueger, 2012).

Chemometrics as defined in the 1970's, is a branch of chemistry that applies a combination of mathematical models to interrogate and extract meaningful data from complex experimental data for example, HPLC, LC-MS and NMR (Roos et al., 2004; Wold, 1995). More recently, the term metabolomics has been used to describe qualitative and quantitative studies of all metabolites present in a given biological system including plants (Heinrich et al., 2012). Metabolomics has a wide range of applications including the quality control of phytomedicines; defining chemical variations in plants grown at different locations and, in botany, used to confirm taxonomic markers for different species (Kueger et al., 2012; Yuliana et al., 2011). Roos et al., (2004)

have shown there is a specific correlation between the spectral pattern of St. John's Wort *Hypericum perforatum* extracts and the corresponding IC₅₀ activity in both *in vitro* and *in vivo* studies. The mapping of pharmacological activity onto chemical features using multivariate data analysis (MDA) to determine the active principles in a phytochemical formulation can also be applied to analysing unknowns in complex plant mixtures (Bailey, 2004; Gao et al., 2010; Holmes et al., 2006). Herbal fingerprinting as applied to analytical data such as HPLC and NMR can be interpreted with principal component analysis (PCA), an unsupervised pattern recognition method.

PCA uses linear transformation to reduce large and complex datasets to a minimal number of new dimensions that show the dominant patterns in a data matrix (Tistaert et al., 2011; Wold, 1995). The peak retention time on the X axis and UV absorbance units on the Y axis data is transformed to a new set of variables called principal components (PCs) that are orthogonal to each other, with PC1 explaining the greatest variance in the dataset and successive PCs for the remaining variances (Eriksson et al., 1999; Holmes et al., 2006). Score plots are used to visually determine similarities of samples by clustering and differences, by sample separation whether chemical class and/or solubility of the plant extracts. The score plots overlaid with bioactivity, may indicate which principal components are relevant to the activity of the herbal extracts (Gao et al., 2010; Roos et al., 2004; Wold, 1995). The loading plots derive the weighting or value of the contributing variables (HPLC t_R), with the data points furthest from the origin in the quadrant of interest considered significant to the bioactivity (Gao et al., 2010; Holmes et al., 2006; Liu et al., 2012; Wold, 1995).

The aim of this chapter is to obtain a HPLC chemical fingerprint for all 50 plant extracts and, using PCA modelling, elucidate which sample might be selected for further investigation and identify the active antimicrobial compounds.

4.2 Materials and Methods

4.2.1 Materials

DMSO, HPLC grade MeOH, ascorbic acid, caffeic acid, ellagic acid, esculin, trans-ferulic acid (99%), gallic acid, kaempferol, naringenin, *p*-coumaric acid, phenol, quercetin, quinic acid, rutin, rosmarinic acid and vanillic acid were purchased from Sigma Aldrich; formic acid (50%) from Fluka; scopoletin from Acros and β -sitosterol from Calbiochem. Distilled water was obtained using a reverse osmosis system (Purelab Option S).

4.2.2 HPLC Method

The reversed phase HPLC assay utilised an internal Medicines Research Group (MRG) method for uncharacterised herbal extracts. The HPLC system (Agilent 1200 series) comprised a Quaternary pump (G1311A), degasser (G13221), autosampler (G1329A), column oven (G1316A) and diode-array detector (G1316A). Ultraviolet (UV) data were acquired from 200-360 nm with 210, 254 and 320 nm wavelengths selected for monitoring. A Zorbax Eclipse C18 analytical column (150 x 4.6 mm i.d.; 5 μ m) was maintained in the column oven at 25°C using a flow rate of 1 mL/min. The starting mobile phase comprised aqueous 0.01% formic acid in distilled water (A) and MeOH (B) with initial conditions set at 25% B with a linear gradient to 90% B at 30 min and retained for 2 min. The solvent was returned to 25% B at 34 min and the column equilibrated for 6 min giving a total gradient cycle of 40 min.

Two hundred microlitres of stock solution of plant extract (20 mg/mL in DMSO) was centrifuged for 5 min (13,000 rpm) and 100 μ L transferred to HPLC vial and diluted tenfold in 900 μ L starting mobile phase (Sanchez-Medina et al., 2007). Solvent controls were prepared for DMSO (10%), MeOH and red wine. Internal reference standards (2 mg) were dissolved in 1 mL MeOH, placed in a sonicating water bath for 20 min and centrifuged for 5 min (13,000 rpm). The sample injection was 20 μ L ($n=3$) resulting in 40 μ g total on the column and samples were stored at 4°C for further use.

The HPLC chromatogram data was acquired using Agilent 1200 Chemstation software (Rev B.04) resulting in 6000 discrete regions. Data points

were acquired between 0.00 and 40.00 min at 210, 254 and 320 nm. The HPLC chromatogram data for all six plants including peak height and retention time (t_R) were exported into Microsoft[®] Excel 2003 as comma separated value (.CSV) files and subsequently combined into one master Excel file per species at each of the selected wavelengths. Quality of the HPLC data was confirmed using the coefficient of variation (CV) at 210 nm for t_R and area of main peak in each plant extract.

4.2.3 Principal Components Analysis (PCA)

The methodology used in this study was developed by Gao et al. (2010) who used PCA to confirm active metabolites in a traditional herbal preparation of *Scutellaria baicalensis* L. prescribed in China as an adjuvant for the treatment of lung cancer. Multivariate data analysis was performed using SIMCA-P+, version 12.00 software (Umetrics, Sweden). The Excel HPLC files were imported into SIMCA-P+, data transposed with Variable and Primary Id tags assigned. Unsupervised models for each species were constructed for principal components to determine the main variance in each data set; score plots for similarities and differences between the plant extracts and loading plots to determine retention times (t_R) for the potentially active compounds. Following analysis of raw and normalised datasets, the former was used to avoid impacting clarity of the HPLC peak t_R in the loading plots (Gao et al., 2010). The full acquisition data set (0-40 min) was analysed prior to removing background noise when the column was equilibrated during the last 6 min. This resulted in 4500 data points retained for further analysis with a total of 72 samples for each plant (8 extracts x 3 replicates at each of the three selected wavelengths).

A principal components model was created for each species with PC1 attributable for the main variance in the data set and score plots generated for each species. Hotelling's model (95% confidence) was applied to all data sets to determine outliers (Field, 2005). Score plots generated for each plant were superimposed with antimicrobial activity at each of the screening concentrations of 200, 40 and 8 µg/mL against the Gram-positive and Gram-negative bacteria. The loading plots show the correlation between variables and were used to determine which peak or peaks t_R significantly contributed to the antimicrobial activity (Roos et al., 2004).

4.3 Results

4.3.1 HPLC Method

The HPLC diode array chromatograms for the root extracts (Fig. 4.2) are shown at 254 nm with polar peaks eluting in the first 10 min and non polar peaks towards the end of the gradient run. This wavelength was used to visually compare HPLC peaks for plant metabolites as the solvent peak was not present. Greater intensity for metabolites was at 210 nm with polar compounds co-eluting in the first 5 min followed by a series of less polar peaks at 10-20 min with a series of smaller non polar peaks in the subsequent 15 min. At 320 nm the y-axis response was poor for all plants.

A number of reference compounds gave the same peak t_R for some plant extracts indicating a potential match for these metabolites (Table 4.1). These included caffeic acid at 6.25 min (*A. minus*, *B. officinalis* and *C. erythraea*), ferulic acid at 10.32 min (*A. minus*), rutin at 11.98 min (*C. erythraea*) and kaempferol at 19.21 min (*A. eupatoria*). These known antimicrobial compounds are ubiquitous in plants and are reported in the monographs in Chapter 2, Tables 2.2-2.7. The most dilute samples were the leaf and root red wine extracts with many peaks observed at 320 nm albeit in small quantities.

The leaf extracts exhibited a series of highly polar HPLC peaks in the first 5 min and a subsequent range of lesser polar peaks between 8-13 min (Appendix 4.1). The 25% EtOH leaf extracts were generally more active than the 75% EtOH leaf samples and at 254 nm there were major peaks at 5.00 min (*B. officinalis*) and 8.88 min (*B. officinalis* and *Plantago media*) that did not match any of the reference compounds although had a similar retention time to the *p*-coumaric acid standard. There was a greater concentration of peaks in the root extracts with more non polar peaks between 15 and 25 min (Fig.. 4.2). The root extracts were the most active against both Gram-positive and Gram-negative organisms (*A. eupatoria* and *P. reptans*). Overall, the hot root decoctions contained a greater intensity of HPLC peaks than the leaf infusions. This was reflective of the extraction method with the leaves (1:20) steeped for 15 min compared to the roots (1:15) being boiled and simmered for 20 min.

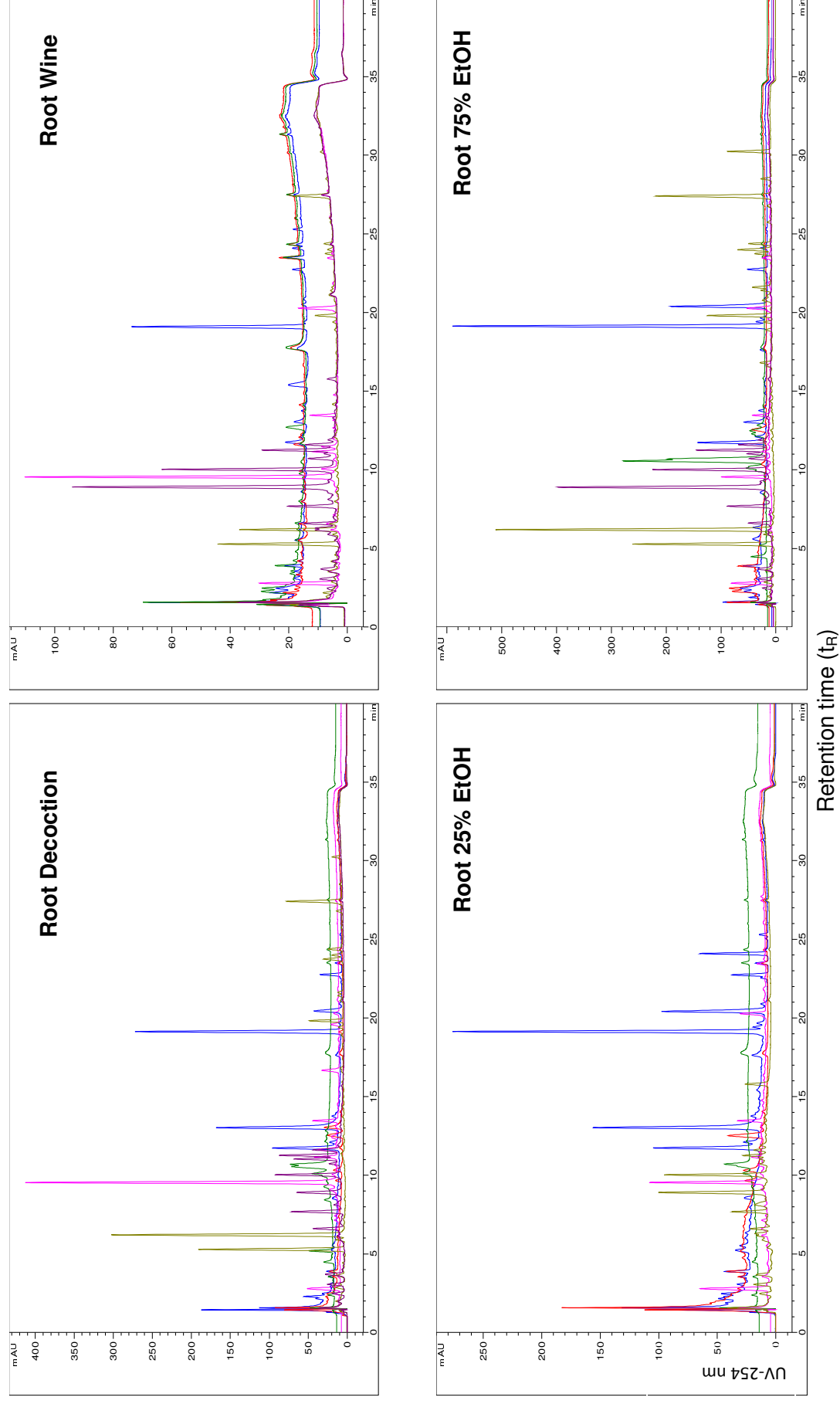


Fig. 4.2 HPLC-UV_{254 nm} chromatograms for root extracts (2 mg/mL). **Key:** Blue (*A. eupatoria*), Red (*P. reptans*), Green (*A. minus*), Pink (*B. officinalis*), Gold (*C. erythraea*) Purple (*P. media*). There is no 25% EtOH root extract for *C. erythraea* as insufficient plant material.

Table 4.1 HPLC-UV_{210 nm} retention times for chemical standards and corresponding peaks in complex plant mixtures.

No.	Compound	Chemical Formula	Molecular Weight (g/mol)	HPLC tR (min)	Matching Plant tR
1	Quinic acid	C ₇ H ₁₂ O ₆	192.17	1.44	A. eupatoria, C. erythraea, P. reptans
2	Ascorbic acid	C ₈ H ₈ O ₆	176.12	1.48	P. reptans,
3	Gallic acid	C ₇ H ₆ O ₅	170.12	2.16	P. reptans, B. officinalis
4	Esculin	C ₁₅ H ₁₆ O ₉	340.28	3.43	C. erythraea
5	Caffeic acid	C ₉ H ₈ O ₄	180.16	6.25	A. minus, B. officinalis
6	p-coumaric acid	C ₉ H ₈ O ₃	164.16	8.88	B. officinalis, P. media
7	Rutin	C ₂₇ H ₃₀ O ₁₆	609.56	11.98	A. eupatoria, C. erythraea, P. media, P. reptans
8	Ellagic acid	C ₁₄ H ₆ O ₈	302.19	12.38	P. reptans
9	Quercetin	C ₁₅ H ₁₀ O ₇	302.24	16.59	A. eupatoria, A. minus
10	Kampferol	C ₁₅ H ₁₀ O ₆	286.24	19.21	A. eupatoria, C. erythraea
11	B-sitosterol	C ₂₉ H ₅₀ O	414.71	30.03	C. erythraea, P. reptans

Whilst all plants exhibited MIC₅₀ values in one or more extracts, the three most active plants were *A. minus*, *A. eupatoria* and *P. reptans* (Fig. 4.3). The HPLC-UV_{254 nm} chromatogram for *A. minus* shows the main peak at 10.5 min. This may correlate to the ferulic acid standard and was present in both leaves and roots. The *A. eupatoria* extracts showed a series of highly polar peaks in the first 5 min followed by a second series at 10-15 min and a third series in the subsequent 10 min. The early eluting polar peaks in the first 2 min that may be quinic acid or gallic acid whilst the main peak at 19.5 min correlated to the t_R of kaempferol standard. By contrast, the *P. reptans* leaf and root extracts showed a major peak in the first 2 min followed by a series of smaller peaks eluting during the first 15 min, a smaller range in the subsequent 10 min with β -sitosterol (t_R 30.03 min) confirmed by matching t_R of reference standard. The y axis showed the peaks in the *P. reptans* extracts were noticeably more dilute compared to *A. minus* and *A. eupatoria* extracts (Fig. 4.3).

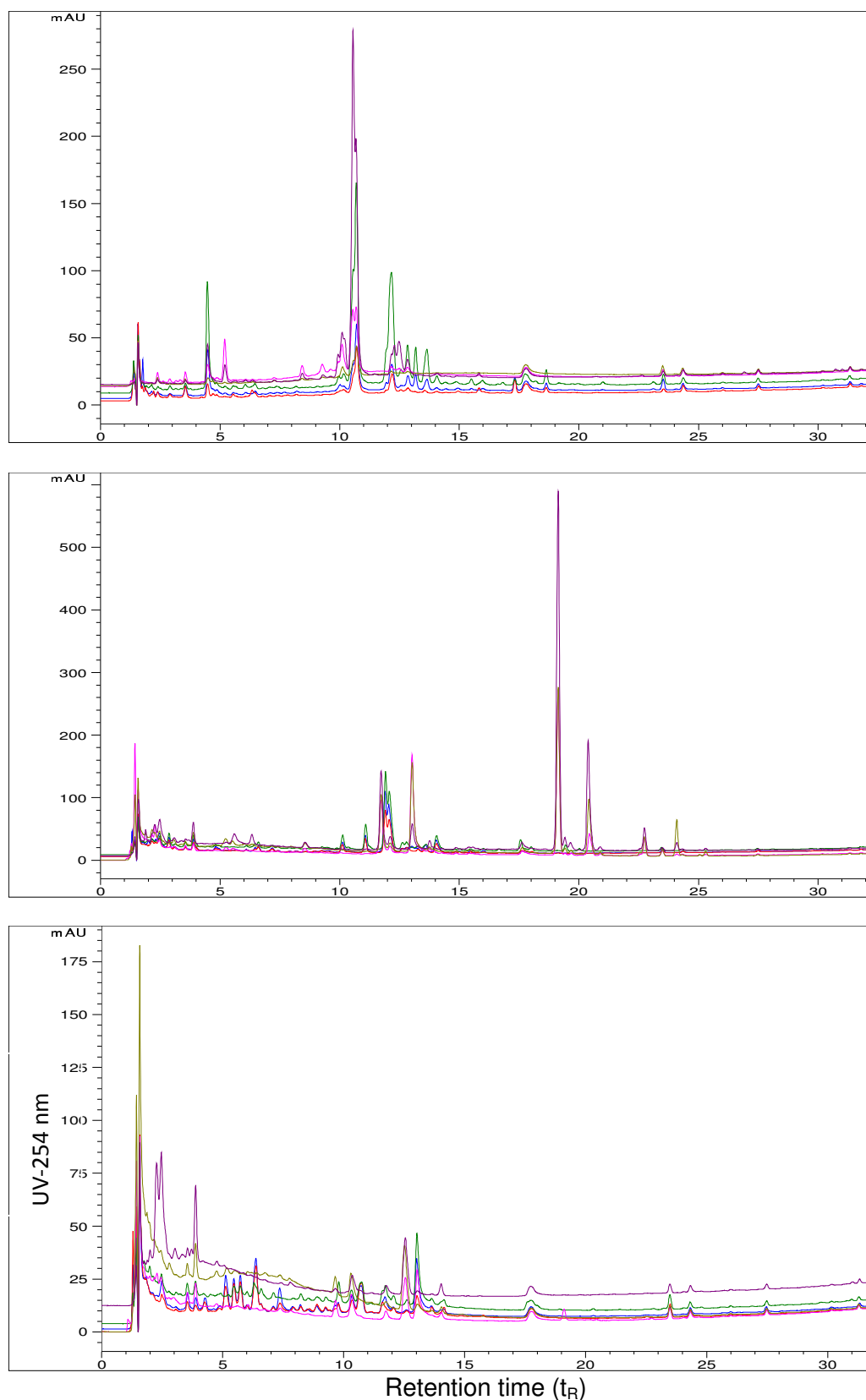


Fig. 4.3 HPLC-UV_{254 nm} chromatograms of leaf and root extracts for *A. minus* (top), *A. eupatoria* (middle) and *P. reptans* (bottom). **Key:** leaf infusion (blue), 25% EtOH leaf (red), 75% EtOH leaf (pink), root decoction (green), 25% EtOH root (gold) and 75% EtOH root (purple).

4.3.2 HPLC-PCA Metabolite Mapping

The HPLC-PCA $_{210\text{ nm}}$ scores plot for principal component 1 (PC1) versus principal component 2 (PC2) represents the distribution of samples in multivariate space, and is used to discern differences and similarities between plant extracts and each species. The 210 nm model expresses a good fit for all plants with PC1 and PC2 accounting for > 80% of variance in each data set (except *B. officinalis*) and, for *P. media* and *P. reptans* models >90% of the total variance. Each set of coloured triangles in Fig. 4.4 represent an individual plant extract for *P. reptans* and its replicates (n=3).

When the antimicrobial activity against *S. aureus* was mapped on to the score plot, the most potent extracts (75% EtOH root and root decoction) appeared in the top left hand quadrant. PC1 for *A. eupatoria* accounts for 70% of the variance versus PC2 of 11% (data not shown) and for *P. reptans*, 78% of the variance is due to PC1 and 12% to PC2 (Fig. 4.4), demonstrating there is no significant difference between the two plants. The contribution or loading plot has been shown to discriminate peak retention times that significantly contribute to the antimicrobial activity (Roos et al., 2004). The loading plot for *A. eupatoria* indicated activity may be related to a peak with t_R between 19.05 and 20.34 min, and this correlated to the HPLC t_R for the kaempferol reference standard (data not shown). The contribution line plot for *P. reptans* in Fig. 4.5 illustrates the two peaks identified in loading plot that were farthest from the origin and therefore considered significant contributors to the activity of the 75% EtOH extract. This activity was associated with HPLC peaks around 2.29 and 21.86 min. The initial retention time may be gallic acid or a derivative at 2.16 min whilst none of the reference standards matched the later retention time. Only two plants, *A. eupatoria* and *P. reptans*, demonstrated MIC₅₀ values against Gram-positive *S. aureus* (MIC₅₀ 8 µg/mL) and Gram-negative *E. coli* (MIC₅₀ 40 µg/mL). Score plots for *A. eupatoria* and *P. reptans* overlaid with activity against *E. coli* show the most active extracts positioned in the bottom left and right hand segments indicating there may be more than one active compound present.

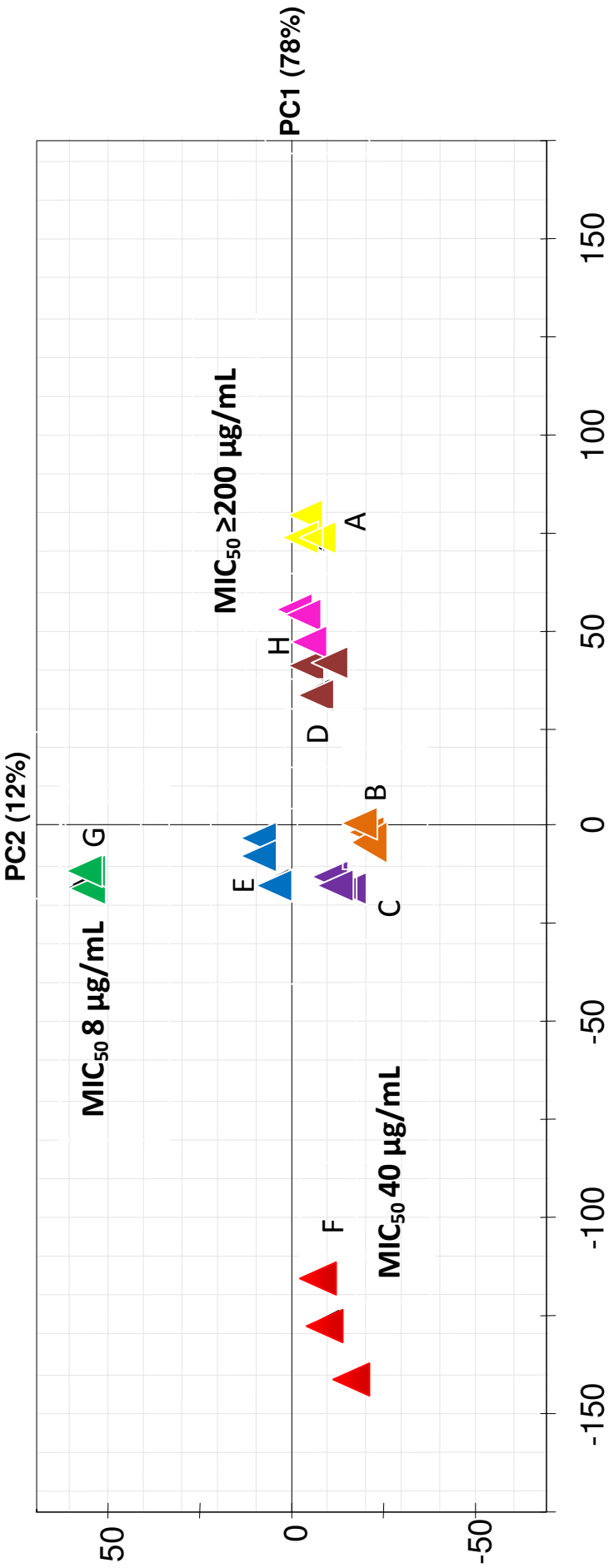


Fig. 4.4 Unsupervised HPLC-PCA_{210 nm} score plot for *P. reptans* leaf and root extracts overlaid with antimicrobial activity against *S. aureus* at three screening concentrations of 200 (A, B, C, D, E and H) 40 (F) and 8 µg/mL (G). A coloured triangle represents one replicate for each plant extract (n=3). **Key:** **A** Leaf Infusion; **B** Leaf 25% EtOH; **C** Leaf 75% EtOH; **D** Leaf Wine; **E** Root Decoction; **F** Root 25% EtOH; **G** Root 75% EtOH and **H** Root Wine extract.

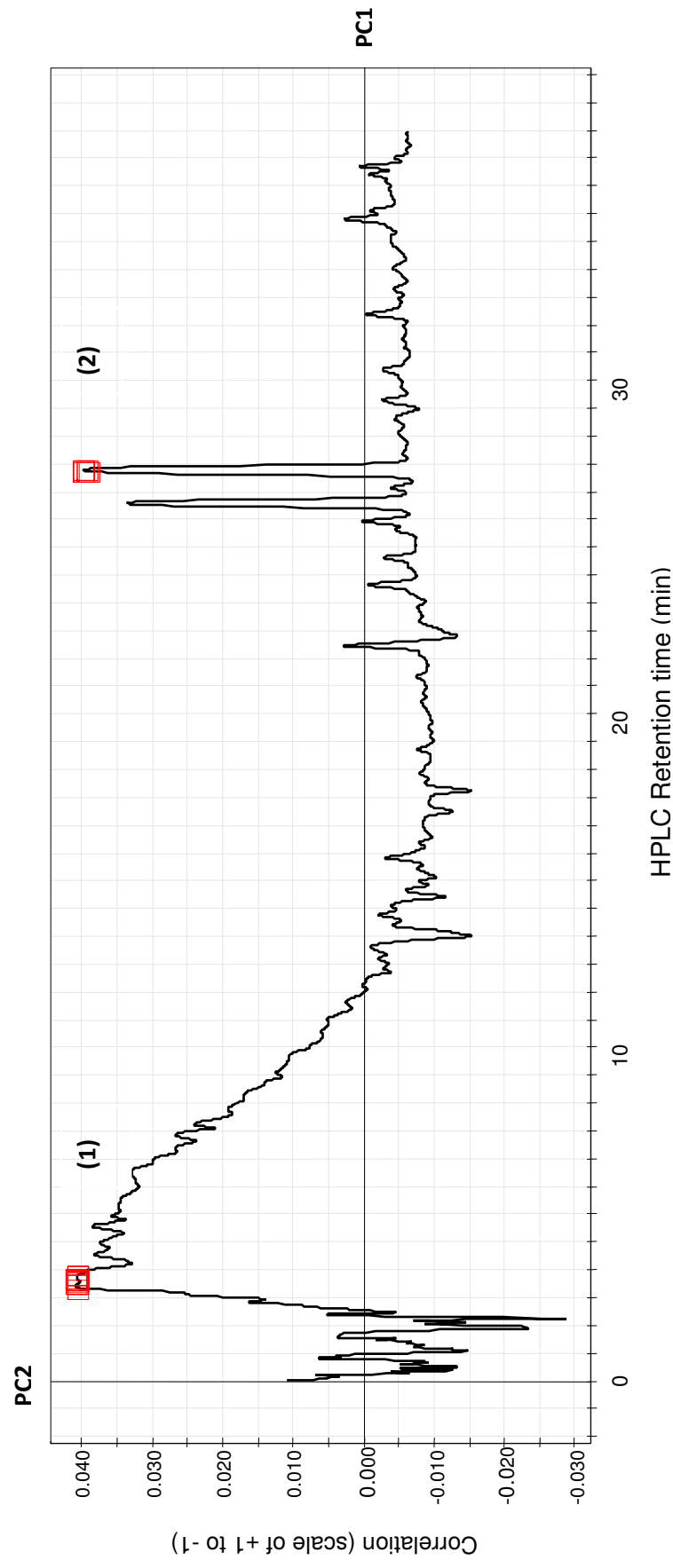


Fig. 4.5 HPLC-PCA $_{210\text{ nm}}$ contribution line plot for *P. reptans* showed peak t_R at 2.29 min (1) and 21.86 min (2) from the loading plot that may significantly contribute to the antimicrobial activity (Roos et al., 2004).

4.4 Discussion

Compounds without chromophores such as mono and disaccharides as well as co-eluting metabolites, would not be detected in the HPLC chromatograms and therefore not detected in the PCA analyses, even though they may have been present in the extracts tested for antimicrobial activity (Harborne, 1998; Kite et al., 2013). The PCA modelling of plant extracts and antimicrobial activity allowed us to determine which samples were selected for further investigation, and how best to optimise the LC-MS method for profiling the active metabolites. The metabolite profile of the most potent extracts will be further explored in Chapter 5 using liquid chromatography mass spectrometry (LC-MS) and high resolution mass spectrometry (HR-MS).

There was no prior separation of plant samples by traditional methods for example, hexane to extract the lipophilic compounds, as one of the key aims was to determine whether the aqueous and EtOH extractions used in 10th century Anglo-Saxon formulations would have been appropriate for the intended use. Multiple compounds may elute with the same retention time under any conditions so whilst some peaks matched the internal standards, further HPLC analysis is required to spiking the extracts with pure reference compounds to show an increase in peak intensity under the same conditions. The hot plant extracts prepared in this study (1:15 for the root decoctions and 1:20 for leaf infusions) required more solvent than the cold extracts. The dry plant material needed more solvent as it expanded from the heat and, as a result, the extracts were dilute solutions compared to a commercial herbal EtOH tincture (1:3) used in WHM; suggesting greater activity would have been achieved with more concentrated extracts.

The PCA analyses were performed using the original HPLC data, without normalisation, in order to preserve locating the peaks significantly contributing to the antimicrobial activity (Gao et al., 2010; Roos et al., 2004). HPLC-UV_{210 nm} peaks were more concentrated in the hot infusions and decoctions compared to the cold EtOH extracts as seen in Fig. 4.2. Gao et al., (2010) developed a PCA model to confirm activity of known phytomarkers in *Scutellaria baicalensis* L. root used in China as an adjuvant to orthodox chemotherapy for lung cancer. By contrast, this study utilised PCA to correlate unknown plant chemistry as

detected by the HPLC-UV $_{210\text{ nm}}$ chromatograms with bioactivity from the antimicrobial screening for all six plants. The HPLC-PCA $_{210\text{ nm}}$ score plots when overlaid with activity against *S. aureus* showed the most active extracts for the majority of plants to be in the top left and lower right quadrants indicating that the active compounds would be from a selective range of chemical compounds. The two exceptions were the *C. erythraea* root decoction positioned in the bottom right quadrant and *A. minus* EtOH leaf and root extracts in the top and bottom right hand quadrants respectively indicating a different class of compounds being responsible for the activity (data not shown).

In Chapter 3 it was found there was no significant difference between the antimicrobial activity of *A. eupatoria* and *P. reptans* against gram-positive *S. aureus* and gram-negative pathogen *E. coli*. Here in Chapter 4, the HPLC-UV $_{210\text{ nm}}$ chromatograms show on the y axis that the peaks in the *P. reptans* extracts are more dilute than the corresponding *A. eupatoria* samples, suggesting the isolated compounds of *P. reptans* would be more potent (Fig.4.3). The contribution line plot indicated specific HPLC peaks found furthest from the origin in the loading plot that may be attributable to this antimicrobial activity (Fig.4.5). Ultimately, *P. reptans* was chosen for further investigation based it being more active than *A. eupatoria* against *E. coli* and it only having 8 reported compounds for the leaves (Tomczyk and Latte, 2009).

Chapter 5.0 Phytochemistry of *Potentilla reptans*

5.1 Introduction

Potentilla reptans was a common component in Anglo-Saxon wound healing formulations (Cockayne, 1865; Van Arsdall, 2002). In *Bald's Leechbook* the plant was considered to be interchangeable with *Potentilla aserina* L. although the leaves of both plants are visually very different (Stace, 2010). Use of *P. reptans* continued in herbal practice until 17th century (Culpeper, 1653) and thereafter was replaced with *Potentilla tormentilla* L., the preferred plant used in current Western herbal practice (Barker, 2001; Tobyn et al., 2011). In Turkey, *P. reptans* is used medicinally to treat heartburn and abdominal pain. In an *in vivo* study the leaves were found to be effective in treating ethanol induced peptic ulcers (Gurbuz et al., 2005; Tomczyk and Latte, 2009) whereas the Chippewa in North America steep the root of *Potentilla arguta* L. to stop bleeding after bloodletting, for headache, cuts, diarrhoea, inflammation and as a tonic (McCutcheon et al., 1995).

A phytochemical and pharmacological review of the *Potentilla* species (Tomczyk and Latte, 2009), identified a total of 67 compounds previously reported for the roots and rhizomes of 11 species. Forty three compounds have been structurally elucidated for *Potentilla erecta* L., compared to 143 compounds identified for the aerial parts of more than 20 species, of which 57 were flavonoids including many *O*-glycosides (28) and *O*-glucuronides (6). The main chemical classes in the *Potentilla* species are phenolic compounds comprising condensed and hydrolysable tannins, triterpenoids and coumarins in both leaves and roots with flavonoids primarily isolated from the aerial parts of the plant (Table 5.1). Many species have been reported having antimicrobial activity and there is a long tradition for within the genus for it being used to treat diarrhoea and dysentery (Tobyn et al., 2011; Tomczyk and Latte, 2009). There is little known about the phytochemistry of *P. reptans* with only eight compounds isolated from the aerial parts for example: isoquercitrin, miquelianin, kaempferol, ellagic acid, caffeic acid, *p*-coumaric acid, ferulic acid and isosalipurposide (Tomczyk and Latte, 2009). The pharmacological activity reported for these compounds is relevant to wound healing with IC₅₀ values in antioxidant, radical scavenging and antihemorrhagic assays (Fig. 5.1).

Table 5.1 Main phytochemistry reported for roots in *Potentilla* species (adapted from Tomczyk and Latte, 2009).

Compounds	<i>Potentilla</i> species	References
<u>Flavonoids</u>		
Kaempferol	<i>Potentilla argentea</i> L., <i>Potentilla recta</i> L., <i>Potentilla discolor</i> L.	(Tomczyk, 2006; Tomczyk, 2011; Song et al., 2013)
<u>Hydrolysable Tannins</u>		
Agrimoniin	<i>P. recta</i> ,	Bazylo et al. (2013)
Ellagic acid	<i>P. anserina</i> , <i>P. argentea</i> , <i>P. discolor</i> , <i>P. recta</i> , <i>Potentilla multifida</i> L., <i>Potentilla chinensis</i> L.	(Tomczyk, 2006; Tomczyk, 2011; Xue et al., 2006)
<u>Condensed Tannins</u>		
Catechin	<i>P. erecta</i> , <i>P. anserina</i> , <i>P. alba</i>	Tomczyk and Latte, (2009)
Epicatechin	<i>Potentilla alba</i> L., <i>P. erecta</i>	(Oszmianski et al., 2007)
Procyanidins	<i>P. alba</i> , <i>P. erecta</i>	(Oszmianski et al., 2007; Tomczyk and Latte, 2009)
<u>Triterpenoids</u>		
Ursolic acid	<i>P. anserine</i>	Li et al. (2003)
Tormentic acid	<i>P. erecta</i>	Csuk et al. (2012)
Euscaphic acid	<i>P. anserine</i>	Li et al. (2003)
Tormentoside	<i>P. anserina</i> , <i>P. erecta</i>	(Csuk et al., 2012; Jang et al., 2007)
<u>Organic acids</u>		
Gallic acid	<i>P. discolor</i>	(Song et al., 2013; Xue et al., 2006)
<i>p</i> -coumaric acid	<i>P. alba</i>	Oszmianski et al., (2007)
<u>Others</u>		
β -sitosterol	<i>P. argentea</i> , <i>P. anserina</i>	(Li et al., 2003; Tomczyk, 2006)

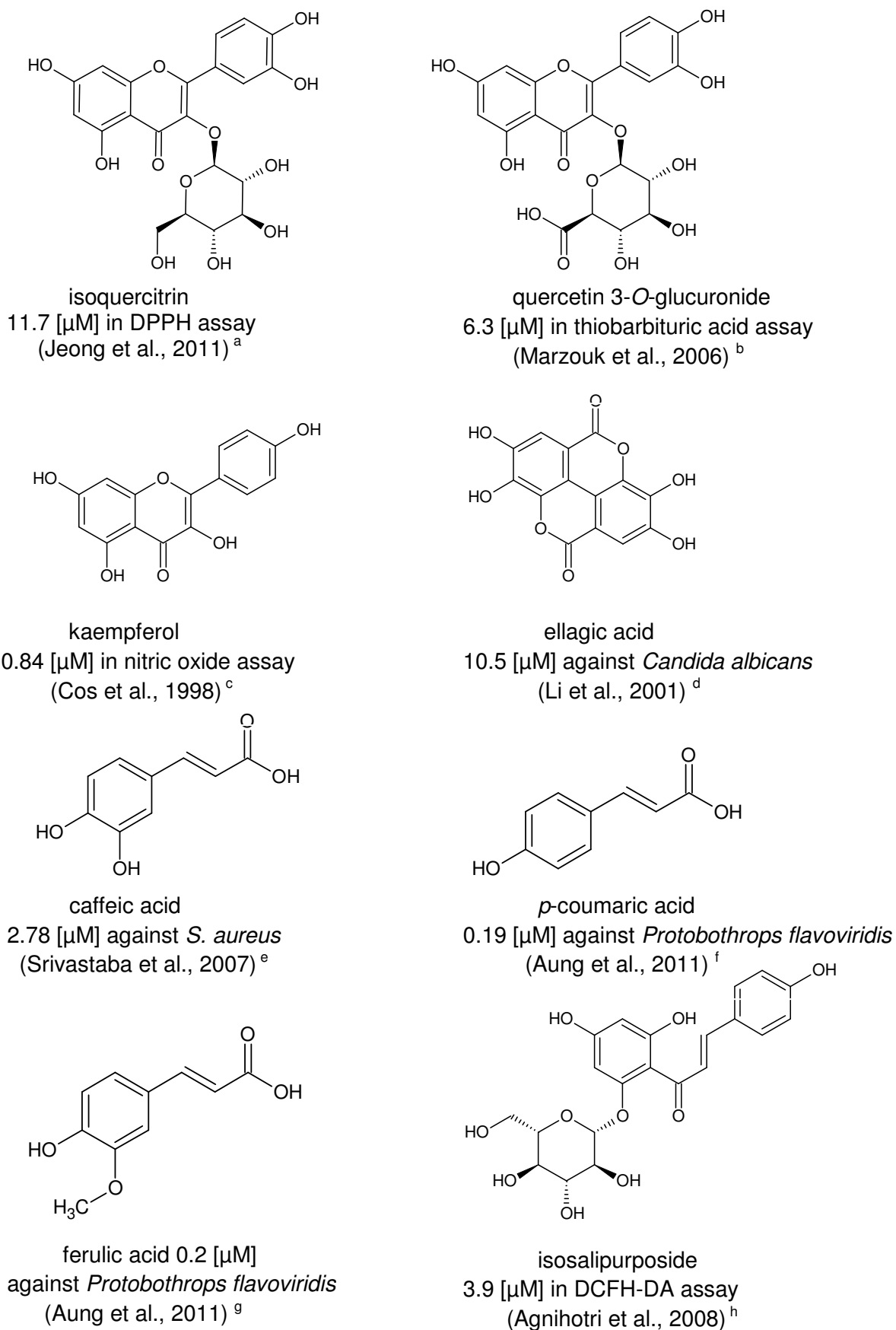


Fig. 5.1 IC₅₀ values for compounds isolated from *P. reptans* leaves. Chemical structures reported by Saddique et al., (2010)^a, Boly et al., (2011)^b, De Melo et al., (2009)^c, Tomczyk, (2011)^d, Dewick, (2009)^{e,f,g} and Agnihotri et al., (2008)^h.

5.2 Materials and Methods

5.2.1 Antimicrobial Assay (MIC and MBC)

Following the initial antimicrobial screen, a full MIC assay was conducted for *P. reptans* at 10 serial concentrations using the microbroth dilution method (2-0.004 mg/mL). Materials and methods were based on methodology in 3.2.3 (Rios and Reico, 2005; Cos et al., 2006; NCCLS, 2009). Dried crude (48 mg) was reconstituted in 1 mL (240 μ L DMSO and 760 μ L nutrient broth) and sonicated for 30 min prior to diluting in 11 mL sterile nutrient broth (4 mg/mL with DMSO at 2%). The antimicrobial activity was calculated using the formula give in 3.2.5 with MIC₅₀ and MIC₉₀ being 50% and 90% inhibition of bacterial growth and >99% inhibition determined as a full MIC value. Minimum bactericidal concentration (MBC) was confirmed by inserting a sterile loop into the well of lowest concentration with no visible growth, streaked onto a sterile nutrient agar plate, incubated at 37 °C for 18 h and checked for any bacterial growth.

5.2.2 Optimised HPLC Method

The original HPLC method described in 4.2.2 was optimised to achieve resolution of the early eluting polar peaks. The optimised HPLC method was based on approach by Sánchez-Medina et al. (2007). Crude extract (2 mg) was dissolved in H₂O/methanol (25/75% v/v) and centrifuged for 5 min (13,000 rpm). The supernatant was transferred to a HPLC vial and diluted with the starting mobile phase to give a final concentration of 1 mg/mL. The starting mobile phase comprised aqueous 0.01% formic acid in distilled water (A) and methanol (B) with initial conditions set at 2% B with a linear gradient to 60% B at 30 min, 90% B at 31 min and retained for 4 min. The gradient was returned to 2% B at 36 min and the column equilibrated in the start conditions for 7 min giving a total gradient cycle of 43 min. The sample injection was 20 μ L (n=3). Coefficient of variation for peak t_R and area were determined to ascertain reproducibility of HPLC data.

5.2.3 Optimised HPLC-PCA

Data from the optimised HPLC method was analysed using PCA methods detailed in 4.2.3 (Gao et al, 2010; Roos et al., 2004) to determine t_R of the compounds significantly contributing to the antimicrobial activity in the loading plot. The HPLC chromatogram data for each file was divided into 6450 discrete regions by acquiring three data points every second between 0.00 and 43.00 min, with the last seven minutes eliminated to remove background noise during column equilibration, resulting in 5400 data points retained for further analysis.

5.2.4 Liquid Chromatography Mass Spectrometry (LC-MS)

The optimised HPLC approach was used to develop an in house method for LC-MS analysis in order to identify molecular mass for metabolites of interest. A reference standard was prepared using quinic acid (1 mg) dissolved in 1 mL of acetonitrile with 100 μ L diluted tenfold, centrifuged at 13,000 rpm for 5 min and 200 μ L supernatant transferred to LC-MS vial. Plant extract (1 mg) was dissolved in 250 μ L H₂O and 750 μ L acetonitrile, sonicated for 20 min and centrifuged at 13,000 rpm for 5 min. Supernatant (200 μ L) was transferred to a vial and stored at 4 °C ready for analysis.

The Shimadzu LC-MS system comprised pump (LC-20AB), degasser (DGU-20A), autosampler (SIL-20A), UV-VIS detector (SPD-20A) and mass spectrometer (LCMS-210EV) controlled by LMSolutions software (version 3.2) and used to analyse the data. The UV detector was set to acquire data in a range of 200-360 nm with 210 nm selected for monitoring. Chromatographic separation was performed on an Eclipse XDB-C18 analytical column (150 x 2.1 μ m id, 5 μ m particle size) with the oven temperature set at 27 °C. The starting mobile phase comprised aqueous 0.01% formic acid (A), and acetonitrile (B) with initial conditions set at 5% B with a linear gradient to 30% at 40 min; 90% at 45 min (3 min) and gradient returned to 30% at 50 min and column equilibrated to initial conditions at 55 min. The total gradient cycle was 60 min with a flow rate of 0.25 mL/min. The *P. reptans* root decoction and 75% EtOH root extracts were injected (5 μ L) in triplicate.

The mass spectrometer was set to acquire spectral data from m/z 100 to 1000 in both negative and positive ion mode with alternating sampling frequency at 2 Hz. The heater block temperature was set at 250 °C and CDL at 200 °C; nebulising gas flow (1.5 L/min), detector voltage (1.50 kV) and the IG vacuum at 1.3e-003 Pa.

5.2.5 High Resolution Electrospray Ionisation Mass Spectrometry (HR-ESI-MS)

Sample preparation is detailed in 5.2.4. Accurate mass measurements and MSⁿ analyses were performed by Geoffrey Kite at the Jodrell Laboratory, Royal Botanic Gardens Kew, using a Thermo Scientific 'LTQ-Orbitrap XL' linear ion trap-orbitrap hybrid mass spectrometer with sample introduction via a Thermo Scientific 'Accela' LC system. The instruments were controlled by XCalibur software (version 2.0.7). Chromatographic separation was performed on a Phenomenex Luna C18 (2) column (150 mm × 3 mm i.d., 3 µm) using a 1 mL/min mobile phase gradient of 90:0:10 to 0:90:10 (water/methanol/acetonitrile + 1% formic acid) over 20 min, followed by 5 min column wash in end conditions. The column was equilibrated in the start conditions for 3 min prior to sample injection of 5 µL. The column eluate was monitored by a photodiode array detector over the range of 200-600 nm before introduction into the mass spectrometer.

The interface with the mass spectrometer was via a Thermo Scientific 'Ion-Max' electrospray source, tuned according to the manufacturer's standard recommendations and operated in polarity switching mode. The orbitrap analyser of the hybrid mass spectrometer monitored the ion beam in positive mode at high resolution (30,000), while the ion trap surveyed the ion beam in the range m/z 125-2000 in both positive and negative modes at low resolution. The ion trap survey scans in both polarities were each followed by data dependent MS² and MS³ scans. The most abundant non-excluded ion in the MS¹ survey scan was selected for MS² analysis and excluded from re-analysis for 6 seconds, while the two most abundant ions in each MS² spectrum was

subjected to MS³ analysis. For all MSⁿ spectra the ion isolation window was +/- 2 m/z units and the normalised collision energy 35%. Both the orbitrap and ion trap were mass calibrated according to the manufacturer's standard procedure (Kite et al., 2013).

5.2.6 Identification of unknowns

The HPLC-PCA _{210 nm} loading plots indicated t_R of compounds having a significant contribution to the antimicrobial activity. Initial LC-MS analysis provided putative molecular weights and a compound database was compiled from the literature. Accurate mass and mass spectra were obtained for compounds of interest and compared to in-house spectral reference library (Jodrell Laboratory). Matches were searched for in one or more databases including the Dictionary of Natural Products (<http://dnp.chemnetbase.com>), NIST Chemistry WebBook, (<http://webbook.nist.gov>) and Pub Chem database (<http://pubchem.ncbi.nlm.nih.gov>). A phytochemical literature search was conducted for putative compounds regarding *m/z* values, reported structures and MIC values for antimicrobial activity against gram-positive and gram-negative pathogens commonly found in wounds.

5.3 Results and Discussion

5.3.1 Antimicrobial Assay

The MICs for *P. reptans* root extracts and controls against gram-positive and gram-negative bacteria are given in Table 5.2. The 75% EtOH root extract was the only sample to exhibit a MIC₅₀ to full MIC range (31.25 to 1000 µg/mL) against *S. aureus* and a MIC₅₀ (500 to 1000 µg/mL) against *E. coli* and *P. aeruginosa* respectively. Against *E. coli*, the root decoction (MIC₅₀ 3.9 µg/mL) was as effective as the reference antibiotic chloramphenicol at the lowest concentration.

Table 5.2 The MIC₅₀ values (µg/mL) of *P. reptans* root extracts for growth inhibition against Gram-positive and Gram-negative pathogens commonly found in wounds.

<i>P. reptans</i> extracts	<i>S. aureus</i>	<i>B. subtilis</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
Decoction	62.5	125	-	3.9
75% EtOH*	31..25-1000	500	1000	500
Root wine	250	-	-	1000
Red wine control	250	-	-	
Chloramphenicol*	3.90-31.25	3.90-125	7.81-500	3.90-62.50
* Range of inhibition from MIC ₅₀ to total MIC (>99% inhibition)				

In this study the root extracts of *P. reptans* exhibited greater antimicrobial activity than the leaves and have demonstrated greater MIC values against *S. aureus*, *P. aeruginosa* and *E. coli* than those reported for other *Potentilla* species (Tomczyk et al., 2008).

5.3.2 Optimised HPLC Method

The HPLC-UV_{210 nm} chromatogram for the optimised method (Fig. 5.2) demonstrates the separation of early eluting polar peaks (t_R 1-5 min) evidenced in the initial HPLC screening and with increased resolution, this gradient approach was used to develop an in house LC-MS method.

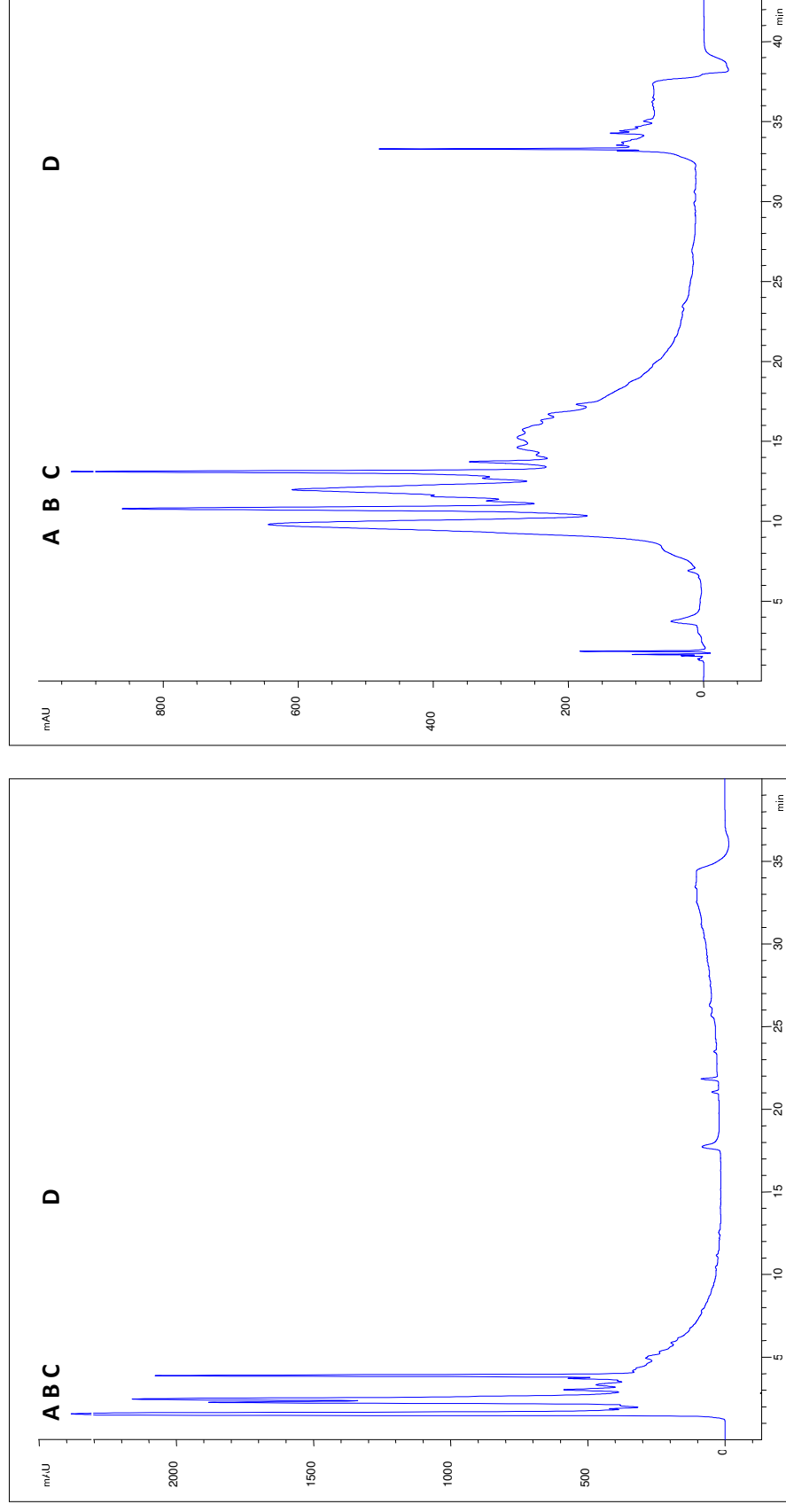


Fig. 5.2 HPLC-UV_{210 nm} chromatogram of *P. reptans* EtOH root extract for the initial HPLC method (left) with clustering of polar compounds eluting in the first 5 min and the optimised method (right) showing separation of peaks A - D and used to determine gradient for LC-MS analysis.

5.3.3 HPLC-PCA Mapping of Optimised Data

The HPLC-PCA $_{210\text{ nm}}$ score plots using the optimised data showed separation of the *P. reptans* extracts in a new position (Appendix 4.2). The main variance in the optimised score plots is due to the 1st principal component (PC1, 67%) with PC2 accounting for a further 13%.

The replications of each extract are more disparate and the 75% EtOH root extracts distinctly separate from the other samples. Supervision of the antimicrobial activity against *S. aureus* overlaid onto the score plot showed three distinct clusters: those at 200 µg/mL were poor to intermediate inhibitors; the 25% EtOH root samples at 40 µg/mL were more active and the 75% EtOH root extracts at 8 µg/mL exhibited the highest activity (Watkins et al., 2012). The loading plot for the optimised data depicts the relationship between the different samples and the loading line plot (Fig 5.3) was used to determine which of the HPLC peaks in the 75% EtOH root, were significantly different compared to all *Potentilla* extracts (24 replicates).

The red squares in Fig. 5.3 indicate the optimised HPLC peak in the *P. reptans* 75% EtOH extract with a t_R of ~33.38 min having a significant contribution to the antimicrobial activity and corresponds to the HPLC-UV peak **D** in Fig. 5.2. It is noticeable in this particular extract, PC1 is attributable to a high level of procyanidins (t_R 8-30 min) and therefore with only a minimum amount of material available, LC-MS was selected as the method for separation and identification of the active compounds.

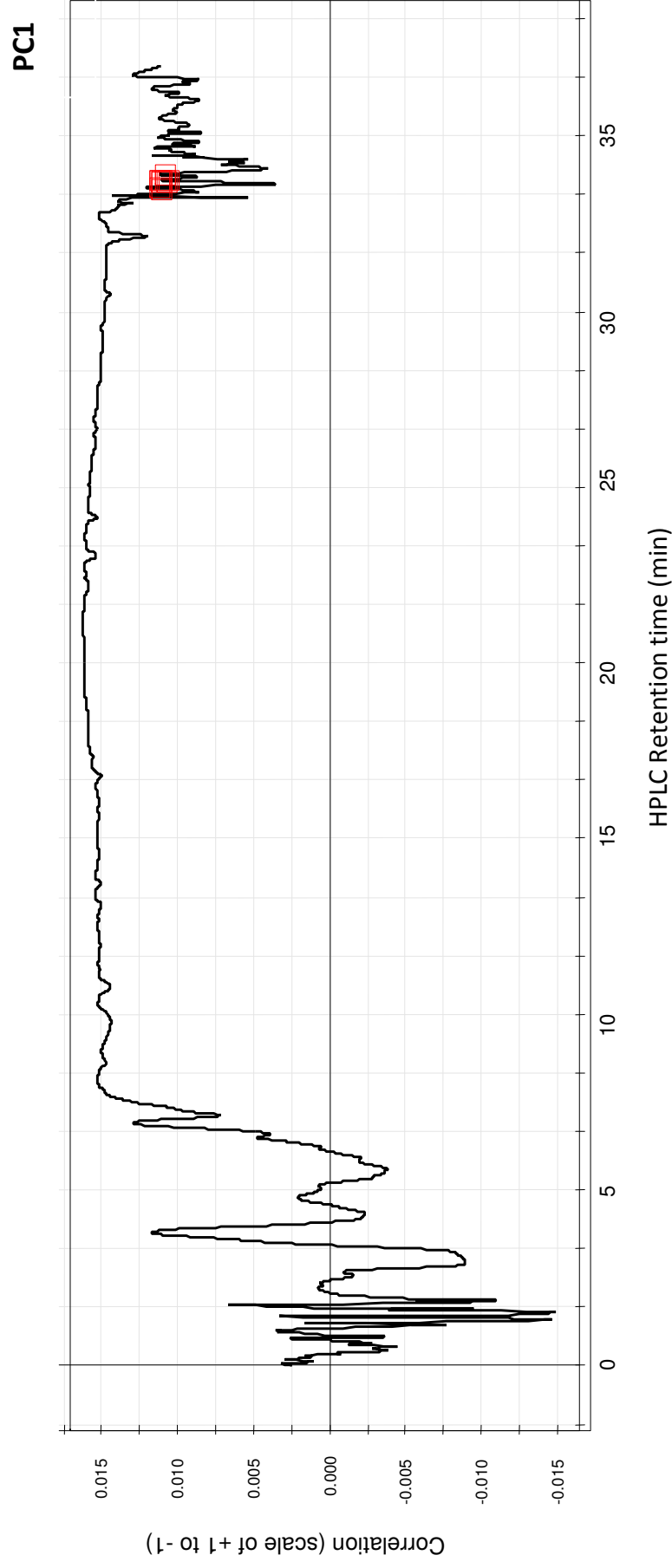


Fig. 5.3 Contribution line plot for PC1 from HPLC-PCA_{210 nm} loading plot for *P. reptans* (optimised HPLC data over 37 min). The red squares indicate the peak region (t_R 33.88 min) as having a significant contribution to the antimicrobial activity and correlates to peak **D** in the HPLC-UV_{210 nm} data (Fig. 5.2) and peak **F** in the HR-MS data (Fig. 5.4).

5.3.4 LC-MS and HR-ESI-MS Analysis

The *Potentilla reptans* decoction and 75% EtOH root extracts were analysed using the Shimadzu instrument in order to compile a list of potential candidates in positive and negative ion mode, molecular weight and chemical formula. HR-MS chromatograms and accurate mass data for both extracts were provided by Dr Geoffrey Kite, Jodrell Laboratory at Kew. Similar chromatogram profiles were obtained on the Shimadzu and Accela systems with the latter used to confirm accurate mass for the compounds of interest (Fig. 5.4).

The HR-ESI-MS chromatograms for both root extracts in negative ionisation mode (Appendix 5.1) showed a series prominent molecular peaks at m/z 387.1 (t_R 1.7 min), m/z 577.1 (t_R 5.22 min), m/z 289.2 (t_R 5.84 min) and m/z 695.3 (t_R 15.7 min). The mass spectra for both extracts in positive ionisation mode showed prominent molecular peaks at m/z 579.1 (t_R 5.2 min), m/z 291.1 (t_R 5.8 min) and m/z 489.4 (t_R 16.1 min) shown in Fig. 5.4. All of the major peaks observed in both positive and negative ion mode are shown in Table 5.3 with those of interest labelled **A-G**. A number of HPLC peaks not investigated further include compounds that matched reference standards and have been reported for antimicrobial activity (Table 4.1); for example, β -sitosterol (t_R 24.8 min) is ubiquitous in plants and present in *Potentilla freyniana* (Wu et al, 2009).

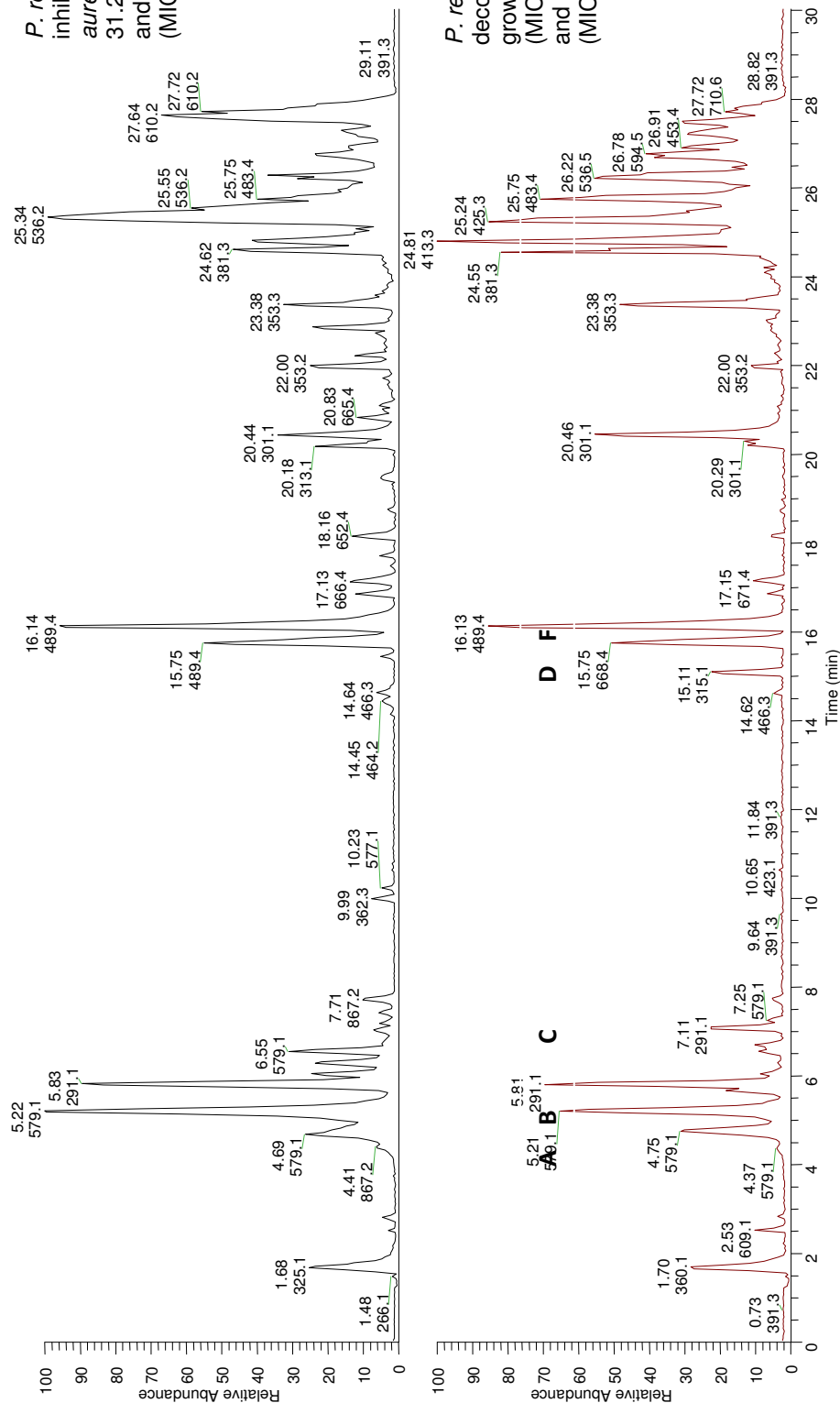


Fig. 5.4 HR-ESI-MS chromatograms in positive ionisation mode for *P. reptans* 75% EtOH root and root decoction.

Table 5.3 HR-ESI-MS peak retention times, chemical formula and putative compound for main chromatographic peaks (Fig. 5.3).

Peak Ref.	t _R (min)	HR-ESI-MS m/z [M+H] ⁺	MS/MS	Chemical Formula	Putative Compound
A	5.22	579.1495	559.1, 451.1, 426.1, 425.0, 408.1, 407.1, 289.1, 245.0	C ₃₀ H ₂₆ O ₁₂	Procyanidin B type
B	5.68	291.0856	271.1, 245.1, 231.1, 205.0, 203.1, 179.0, (MS ²)	C ₁₅ H ₁₄ O ₆	Epicatechin
C	7.71	867.2133	739.1, 713.1, 695.0, 587.1, 561.0, 543.1, 451.2, 425.0, 407.1	C ₄₅ H ₃₈ O ₁₈	Procyanidin C1
	1.68	325.1124	360.1496, 343.1233	C ₁₂ H ₂₀ O ₁₀	unknown
	1.7	360.1496	365.1051, 343.1232, 325.1124	C ₁₂ H ₂₅ O ₁₁ N	[M+NH ₄] ⁺ of sucrose
	2.53	609.1240	610.12, 611.12	C ₃₀ H ₂₄ O ₁₄	Quercetin glycoside
	15.11	315.1223	459.1, 441.3, 423.2, 357.1, 315.1 (MS ²), 297.1, 279.1, 207.0, 188.9, 163.0, 161.1, 135.0, 121.1 (MS ³)	C ₂₄ H ₂ O ₁₀	unknown
F	15.11	477.1752		C ₂₄ H ₂₈ O ₁₀	Agrimonomide-6-O-glucopyranoside
D	15.75	489.4	650.6, 489.1, 471.2, 453.2, 425.2, 407.3 (MS ²)	C ₃₆ H ₆₂ O ₁₀ N	[M+NH ₄] ⁺ Tormentric acid glucosyl ester or glycoside
	15.75	668.4371		C ₃₆ H ₆₁ O ₁₀ N	[M+NH ₄] ⁺
	17.13	666.4218	649.1, 487.1, 469.2, 451.3, 441.3, 423.4, 405.4 (MS ²)	C ₃₆ H ₅₉ O ₁₀ N	[M+NH ₄] ⁺
	24.81	413.3		C ₂₉ H ₅₀ O	β-sitosterol
	25.34	536.2	518.9, 502.9, 299.8, 298.7, 282.0, 281.0 (MS ²)	C ₃₉ H ₂₂ O ₂ N	[M+NH ₄] ⁺
HR-ESI-MS					
		m/z [M-H]⁻			
E	10.75	301.0	301.0, 284.0, 257.0, 229.0, 201.0, 185.0 (MS ²)	C ₁₄ H ₆ O ₈	Ellagic acid
G	13.49	394.9	No accurate mass	C ₁₆ H ₁₈ O ₉	Chlorogenic acid + K

5.3.4.1 Compounds A, B and C

The HR-MS chromatograms for *P. reptans* root decoction and 75% ETOH extracts in the positive ionisation mode (Table 5.3) showed two major $[M+H]^+$ peaks at m/z 579.1 (**A**) and m/z 291.1 (**B**). These protonated molecules may correspond to compounds with molecular weights of 578.1 and 290.1 and chemical formulae of $C_{30}H_{26}O_{12}$ and $C_{15}H_{14}O_6$ (578.142430 and 290.079040 amu respectively). There was an additional peak at m/z 867.2133 for the 75% EtOH root extract (**C**). This protonated molecule may correspond to a compound with a molecular weight of 866.2 and a possible chemical formula of $C_{45}H_{38}O_{18}$ (866.205820 amu). All three compounds matched putative structures found in the Jodrell Laboratory reference library (Fig. 5.5) as condensed tannins comprising an epicatechin (**A**), a procyanidin dimer type B (1, 2, 3, 4, 5, 6 or 8) (**B**), and a procyanidin trimer type C1 (**C**). Structures and molecular weights for these compounds were confirmed in the literature (Benavides et al., 2006; Nunes et al., 2008; Takahashi et al., 1999).

The antimicrobial activity of condensed and hydrolysable tannins is well reported with procyanidins having an intracellular action by inhibiting the α DNA polymerase (Saito et al., 2005) and catechins, acting by damage to the bacterial membrane (Cushnie and Lamb, 2005; Daglia, 2011). Procyanidins and epicatechins in *Theobroma cacao* L. exhibited IC_{50} values of 12 μ g/mL on lipid peroxidation in rat liver microsomes and in a radical-scavenging DPPH assay EC_{50} values of 6.2 μ g/mL (Hatano, 2002). Epicatechins have demonstrated analgesic properties; cyanidin found in tart cherries exhibited IC_{50} values of 60 μ M compared to 1050 μ M in aspirin (Harborne and Williams, 2000). A total of 27 condensed and hydrolysable tannins have been studied in the *Potentilla* species with *P. erecta* demonstrating moderate antibacterial and antifungal activities against *S. aureus*, *E. coli* and *Candida albicans* (Tomczyk and Latte, 2009).

5.3.4.2 Compounds D and E

The same chromatograms for both extracts showed a base peak at m/z 489.4 (**D**) with an associated ion species at m/z 668.4 (Table 5.3). The protonated $[M+NH_4]^+$ molecular ion at m/z 668.4371 may correspond to a molecular formula of $C_{36}H_{58}O_{10}$ (650.403000 amu) plus ammonia ion. The MS^2 spectrum showed a fragment ion pattern at m/z 489.1, 471.2 and 453.2 that matched a structure (in-house reference library) for either tormentic acid glucosyl ester or a tormentic acid glycoside + NH_4 (Fig. 5.6). A β -D-glucopyranosyl ester of tormentic acid has been isolated from a variety of plants including *Rubus glaucus* Benth as well as *Potentilla anserina* L. and *Potentilla erecta* L. (Mertz et al, 2007). Tormentic acid and its derivatives were recently reported to be apoptotic, acting by inhibition of α and β -DNA polymerases in a range of human tumour cell lines (Csuk et al., 2012; Tomczyk and Latte, 2009). Kite et al. (2007) reported isobaric glycosyl esters of tormentic acid and euscaphic acids present as major saponins of *Potentilla tormentilla* with compounds generating $[2M-H]^-$ and $[2M+CH_3COO]^-$ using negative ion electrospray. Saponins including ursolic acid and euscaphic acid have been reported in some *Potentilla* species (Bilia, et al., 1994; Wu et al., 2009; Tomczyk and Latte, 2009).

The full mass spectra of the 75% EtOH root extract in negative ion mode showed a deprotonated molecular ion at m/z 301.05 (**E**) in Table 5.3. The MS^2 spectrum displayed a major fragment ion at 257 and subsequent ions at 229, 201 and 185. This fragmentation pattern matched a potential structure in the in-house library for ellagic acid that corresponds with a molecular formula of $C_{14}H_6O_8$ (302.006270 amu) shown with chemical structure in Appendix 5.2. Fractions of EtOH root extract of *Rubus ulmifolius* Schott produced ellagic acid and derivatives that inhibited *S. aureus* biofilm formation at 50 μ g/mL and increased antibiotic susceptibility to 15 clinical isolates of the same species and in another study, four ellagic acid derivatives were found to inhibit growth of yeast by damaging the cellular DNA (Quave et al., 2012; Xu et al., 2003).

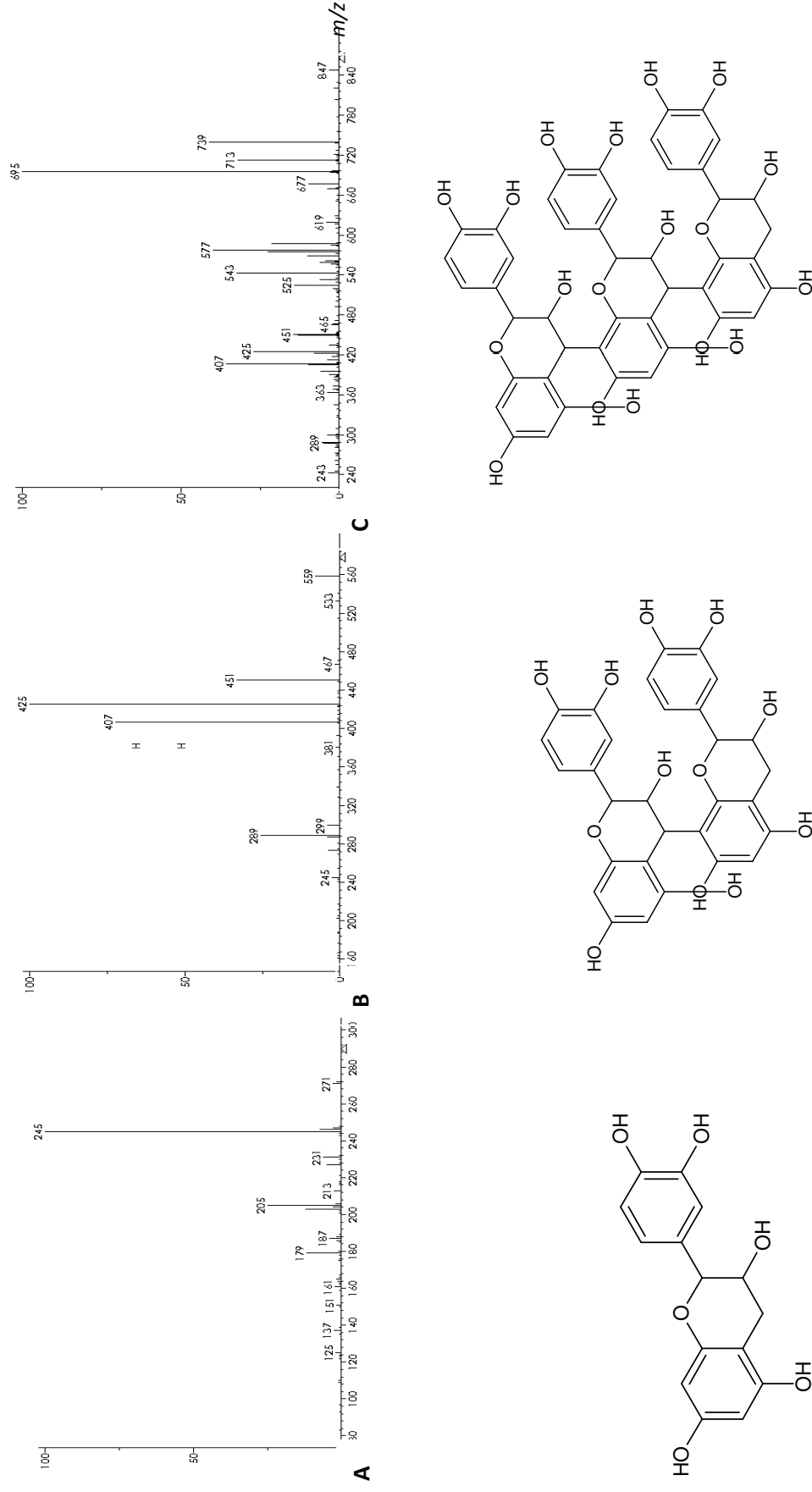


Fig. 5.5 Fragmentation patterns that matched Kew in-house HR-ESI-MS library structures. Putative compounds for *P. reptans* root decoction and 75% EtOH root extracts are Epicatechin (**A**), procyanidin type B1 (**B**) and procyanidin type C1 (**C**).

5.3.4.3 Compounds F and G

The HR-ESI-MS chromatogram for the root decoction in positive ion mode showed a base peak at m/z 315.1 with major peaks at 477.1 (**F**) and 494.2 that corresponded with $[M+H]^+$ and $[M+NH_4]^+$ respectively (Table 5.3). These were sodiated peaks giving a molecule with a potential molecular weight of 476.1 and a molecular formula of $C_{24}H_{27}O_{10}$ (476.168250 amu). A MS^3 scan showed fragmented ions at 297.1, 279.1, 207.0 and 188.9. By cross referencing the molecular weight in the Dictionary of Natural Products database a putative structure was located as agriminolide-6-*O*-glucopyranoside for the chemical formula $C_{24}H_{28}O_{10}$ (Fig. 5.7). The negative ion high resolution mass spectra show that this peak is not present in 75% EtOH root extract and may explain the activity for the root decoction (Appendix 5.1). A survey of 43 naturally occurring and synthetic coumarins demonstrated a wide range of activity against clinical isolates of Gram-positive and Gram-negative bacteria from nearly 50%, exhibiting no activity at 1000 $\mu\text{g/mL}$ compared to 8-iodo-5,7-dihydroxycoumarin, the drug of choice for MRSA infections (Smyth et al., 2009).

A prominent molecular peak at m/z 394.9 (**G**) in Table 5.3 corresponded with $[M-K]^-$ giving a molecule with a possible molecular weight of 354, consistent with chlorogenic acid plus potassium adduct (354.095085 amu). Chlorogenic acid is known to exhibit antibacterial activity by increasing the membrane permeability thereby disrupting cell wall synthesis (Wang et al., 2013).

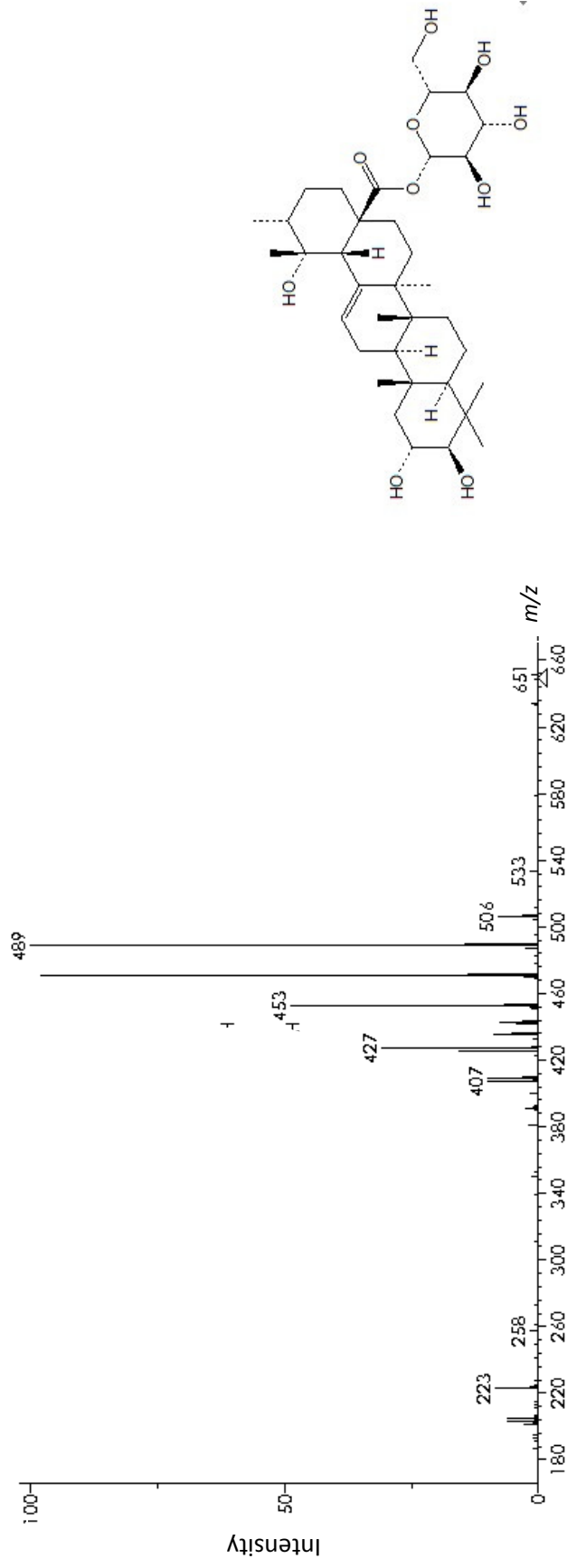


Fig. 5.6 Product ion fragment pattern for base peak at m/z 489.1 in positive ion mode present in *P. reptans* root decoction and 75% EtOH root extracts. This peak corresponds to a molecular formula ($C_{36}H_{58}O_{10}$) that may be a tormentonic acid glycosyl ester or tormentonic acid glycoside plus ammonia ion. Chemical structure reported for tormentonic acid (An et al., 2011).

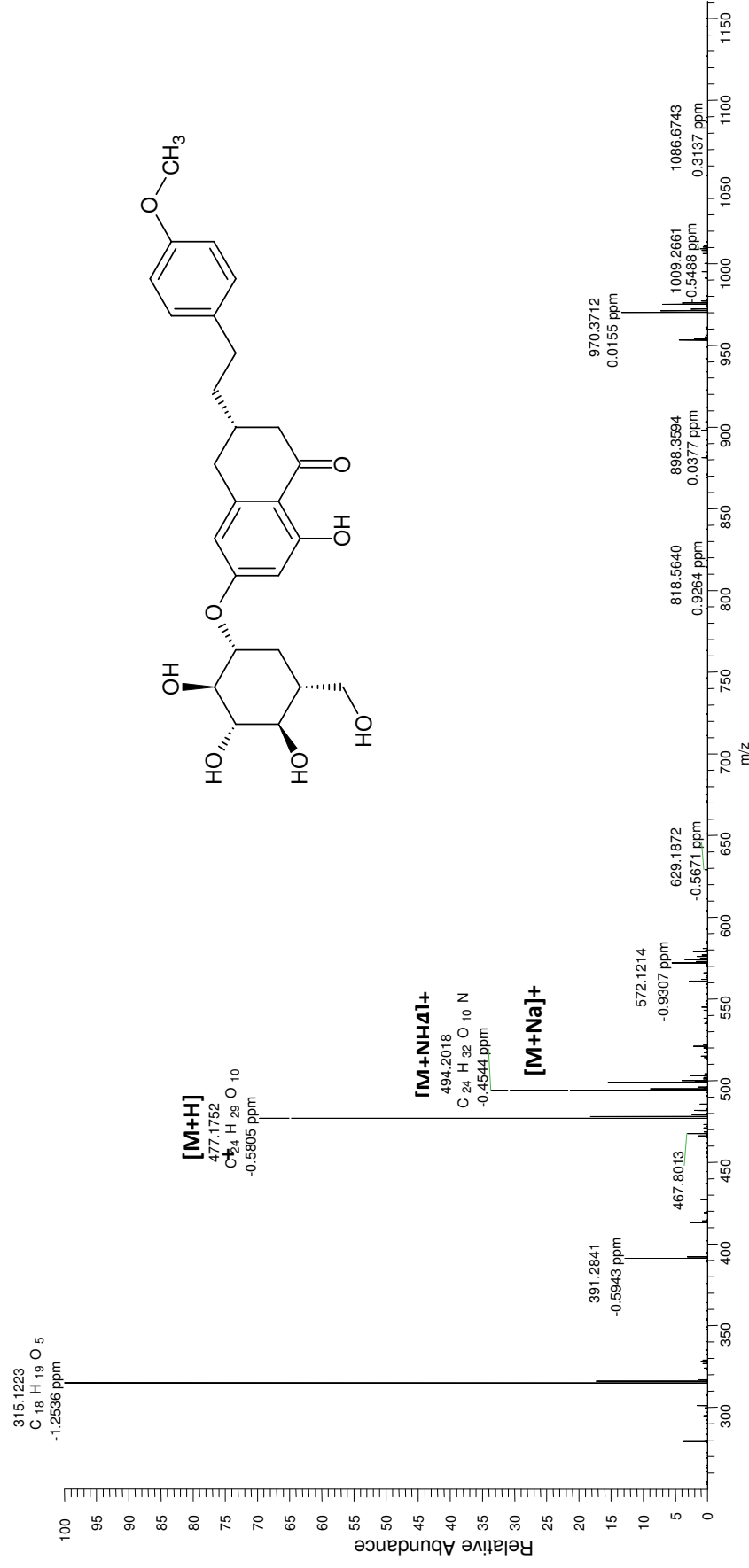


Fig. 5.7 Putative compound for *P. reptans* root decoction from base peak at m/z of 315.122 with chemical formula C₂₄H₂₈O₁₀ (476.1752 amu) is agrimonolide-6-*O*-glucopyranoside. The decoction exhibited an inhibitory growth of *S. aureus* (MIC₅₀ 62.50 µg/mL) and against *E. coli* (MIC₅₀ 3.9 µg/mL). Chemical structure reported for agrimonolide-6-*O*-glucopyranoside (Kato et al., 2010).

5.4 Conclusion

Mapping the activity for each extract to the PCA loading plot followed the methodology of Gao et al., (2010), whilst unknown peaks contributing to the greatest variance were identified as targets responsible for the antimicrobial activity (Watkins et al., 2012). The multivariate analysis was also used to direct optimisation of the HPLC and develop a LC-MS method for the separation of target compounds to determine chemical formulae and molecular mass. The dilute plant extracts contained a high concentration of tannins, suggesting nuclear magnetic resonance spectroscopy (NMR) would not be appropriate prior to fractionating the compounds of interest and thus require collecting more plant material. An alternative method would have been to use HPTLC-densitometry; a method recently developed by Tomczyk et al. (2012), to separate and quantify four quercetin derivatives in selected *Potentilla* species: miquelianin, isoquercitrin, hyperoside and rutin, all known antimicrobial agents and previously reported. However, with no access to this instrument, LC-MS was chosen to separate target peaks and indentify putative compounds using accurate mass cross referenced with chemical databases and spectral libraries.

Combination plant formulations are used in China as adjuvants in orthodox treatments for lung cancer (Gao et al., 2010) and evidence is gathering for using botanicals to assist in regaining susceptibility of antibiotics to resistant pathogens (Savoia, 2012). The immunomodulating properties of *P. erecta*, was shown to be an effective adjuvant in the treatment of HIV/TB infected patients as part of a combination formula in an open label, Phase II clinical trial (Nikolaeva et al., 2008). According to Tomczyk and Latte (2009), *Potentilla sericea* L. is used as adjuvant as part of the orthodox medicinal approach in treating cutaneous tuberculosis, suggesting that some of the *Potentilla* genus may prove to be useful adjuvants in combating infection, biofilm formation and reducing the amount of time for wounds to heal.

By contrast, the *P. reptans* root extracts in our antimicrobial study were more active than those reported for other *Potentilla* species (Tomczyk et al., 2007). The HPLC peaks identified by the PCA analysis of the 75% EtOH root extracts may contribute to the antimicrobial activity for example, tormentic acid, isolated from *Rosa rugosa*, has been shown to have anti-inflammatory activity and inhibit the production of nitric oxide in RAW26.4 cells (An et al., 2011) and has also been reported for *P. erecta* (Table 5.1).

Two peaks that may account for the activity in *P. reptans* root decoction suggested by the PCA analysis were the base peaks at m/z 609.1 and m/z 476.1 and not present in the 75%EtOH root extract. The root decoction showed greatest inhibition of Gram-negative *E. coli* (MIC₅₀ 3.9 µg/mL) which may be explained by the presence of the procyanidins (Daglia, 2011) or potentially, the putative compound identified as agrimonolide-6-*O*-glucopyranoside. This compound is a 3,4 dihydroisocoumarin glycoside recently isolated from *Agrimonia pilosa* (Kato et al., 2010) and whilst coumarins have been reported for a number of the *Potentilla* species including *P. erecta* (Sohretoglu and Kirmizibekmez, 2011) this specific compound has not been reported for any of the *Potentilla* species.

In the Anglo-Saxon formulations, *P. reptans* is specified as a decoction of the whole plant and applied topically for burns and ulcerous sores, as a drink for snakebite and to staunch bleeding (Table 2.7). These ancient indications are similar to traditional uses of *Potentilla* species around the world. Clinical studies have found extracts of *P. erecta* rhizome to be effective in the treatment of ulcerative colitis (Huber et al., 2007) and in a randomised control trial, diarrhoea due to a *Rotavirus* infection (Subbotina et al., 2003). In this study, the aqueous and EtOH root extracts of *P. reptans* have shown to be appropriate for treating bacterial infection of gram-positive and gram-negative pathogens commonly associated with wounds.

6.0 Conclusions

There have been a number of calls to research ancient medical literature (Buenz et al., 2004; Holland, 1996; Riddle, 1974) but to date little has resulted in scientific evaluation of the Anglo-Saxon herbal texts. Cameron (2006) argued that at least two thirds of the formulations were pragmatic and medicinally useful although he did not conduct any laboratory research. Brennessel et al. (2005) reported poor antimicrobial activity for eleven Anglo-Saxon preparations and as yet, the full experimental methods and data have not been published. To date, this work described herein is the robust methodology used to determine whether plants used in Anglo-Saxon formulations would have been fit for purpose (Watkins et al., 2002).

The scope of work here is the first comprehensive study investigating the historical and botanical context of three major Anglo-Saxon herbal texts in conjunction with analysing the bioactivity and phytochemistry of the plants. The research focus was native British plants used in treating bacterial infection and wounds; an area that continues to be important to global health with the increase of antibiotic resistance and the management of chronic conditions as seen in diabetic leg ulcers. A systematic review of the 10th century Anglo-Saxon herbal literature by Watkins et al., (2011) was the first contribution to scientific knowledge for this project. The paper showed a medical system was present in early medieval England ranging from the gold standard *Leechbook* compiled for use by the royal physicians to short instructional texts for the lay practitioner and simple monastic texts. Some of the plants would have been relevant for treating the condition cited and worthy of further investigation to recover herbal knowledge lost to Western herbal medicine.

Researching the 10th century herbal literature raised many issues surrounding language, translation and context; the conditions and diseases portrayed in the texts, identification of plants used and the manner in which the formulations were prepared (Banham, 2002; Cameron, 2006; Van Arsdall, 2002). A number of historians have attempted to show the texts were of value

with empathy on the part of the practitioner, pragmatic herbal preparations and an overall intention that the patient should get well (Crawford, 2010; Meaney, 2002; Voigts, 1979). More than 130 plants referenced in the Anglo-Saxon medical texts are listed in modern herbal pharmacopoeias (D'Aronco and Cameron, 1998) with many of these plants still used in WHM (Barker, 2001; Tobyn et al., 2011). This study contributes to the overall development of WHM by informing the herbal profession of forgotten knowledge regarding medicinal native species and the ancient practice of using topical and systemic herbal formulae to treat wounds. To a Western herbal practitioner, the Anglo-Saxon formulations could be considered stylistically similar to those found in contemporary herbals with minimal preparatory instruction (Culpeper, 1654; Grieve; 1931; Hoffman, 2002). Whilst looking at the layout of Bald's *Leechbook* and *Lacnunga* formulations, the author observed a number of repeated patterns in the illuminated letters that await evaluation as to whether they are an integral part of the formulation.

A key element of this project was the plant collection – firstly the logistics' of obtaining permission from the Wildlife Trusts and private landowners to harvest whole specimens from uninhabited locations and secondly, the ethnobotanical approach of being sympathetic to the 10th century collection methods. Quantities of plants harvested for each species were kept to a minimum, meaning only analytical techniques that required micrograms as opposed to grams of material were used. The screening and analytical processes utilised <30 g of dried material of each species (Watkins et al., 2012); a model that could be adopted by others to screen bioactivity of plants referenced in historical texts.

Plant diversity changes with evolving agricultural methods and some plants considered ubiquitous in the 10th century are no longer abundant as seen with *B. officinalis*. In the last 25 years, the plant has declined by nearly 50% in southern England. Culpeper (1654) considered the dandelion to be so common that it did not warrant a description which shows how herbal knowledge is easily lost to future generations reading his herbal. Investigating the ethnobotanical

use of medicinal plants and demonstrating bioactivity can help create awareness of the importance in protecting species in decline. The medicinal use of substitute plants used according to the local environment is seen with *A. minus*, *P. media* and *P. reptans* and perhaps, one that medical herbalists should reconsider to reduce the carbon footprint of herbal products when replenishing their dispensary.

Plant mixtures contain thousands of compounds and whilst individual metabolites can be identified as having antimicrobial activity in a pure form, the true activity may be the result of a synergistic effect of multiple compounds having one or more targets (Jalencas and Mestres, 2013; Williamson, 2001). Complex plant mixtures are being used as adjuvants in orthodox treatment of lung cancer in China (Gao et al., 2010) and there is evidence of plant compounds being used to synergistically enhance antibiotic activity, whilst at the same time reducing the amount of antibiotic given (Hancock, 2005). Gibbons (2008) provides a convincing argument for plants as a source of antimicrobial agents in that they produce metabolites as part of their chemical defence; there is ethnobotanical evidence of plants being used topically and systemically to treat antibacterial infection and there is chemical diversity in plants and natural products.

In this study, all six plants exhibited IC_{50} values in one or more extracts against Gram-positive microorganisms showing that all of the plants would have provided some beneficial effect compared to a using plant exhibiting no activity. The *Betonica officinalis* leaf extract was the most potent infusion against *S. aureus* suggesting the plant may have wider applications than those applied in current Western herbal practice. The *P. reptans* root decoction was the most potent extract against *E. coli* in the antimicrobial screening. Further *in vitro* studies showed this dilute extract to have activity against *E. coli* (MIC_{50} 3.9 $\mu\text{g/mL}$) comparable to chloramphenicol, a broad spectrum antibiotic control, at the lowest concentration. This suggests that the isolated compound could be more potent, thus being of pharmaceutical interest (Gibbons, 2008). Relatively

few plants inhibit growth of Gram-negative bacteria and potentially, once known, the mechanism of the active compound in *P. reptans* may be of scientific interest. *Potentilla reptans* may also possess other pharmacologically active compounds beneficial to wound healing for example, anti-inflammatory and tissue repair, reported for other *Potentilla* species (Tomczyk and Latte, 2009).

A literature review of the phytochemistry for *Potentilla* species showed only eight compounds (mainly flavonoids) reported for *P. reptans* leaves and to date, nothing for the roots (Tomczyk and Latte, 2009). With little reported phytochemistry, the challenge here was that the active compounds were unknown. By metabolomic mapping of the pharmacological activity onto the chemical features, the PCA was used to discern different HPLC peaks between the extracts of each species that may account for the antimicrobial activity. This approach is relatively new to determine known markers within a complex plant mixture (Bailey, 2004; Gao et al., 2010) and arguably, a novel approach for unknown complex plant mixtures. For the first time, seven compounds were putatively identified in *P. reptans* roots: caffeic acid, ellagic acid, ferulic acid, chlorogenic acid, β -sitosterol, tormentic acid and agrimonolide-6-*O*-glucopyranoside. The first five are ubiquitous in plants, known to have strong antimicrobial activity and some already reported for *P. reptans* leaves (Fig. 5.1). However, the latter two are of interest in that tormentic acid has been reported for *Potentilla* species other than *P. reptans* and agrimonolide-6-*O*-glucopyranoside, has not been reported for any *Potentilla* species. Derivatives of agrimonolide have been reported for *Agrimonia pilosa* which is the same family (Rosaceae) as *Potentilla*. If this compound is confirmed by NMR spectroscopy, it will be the first report for *P. reptans*.

This study has laid the foundation for multidisciplinary research to consider the early medieval literary heritage as a credible basis for systematic investigation of pharmacologically active compounds and therapeutic indications from native British plants, some of which are currently lost to Western herbal medicine (Watkins et al., 2012).

6.1 Specific Future Work

- Explore the hypothesis that there is a formulaic relationship between the zoomorphic letters and the herbal formulations in the 10th century Anglo-Saxon texts.
- Test the pure compounds available for those reported in *P. reptans* decoction and 75% EtOH root extracts to determine MIC/MBC activity.
- Investigate the wound healing properties of *P. reptans* in anti-inflammatory and tissue repair *in vitro* assays.

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Research Project: Ethno-medical approaches to discovering novel antimicrobials from England's cultural Anglo-Saxon heritage

Name: *Agrimonia eupatoria* L.

Family: Rosaceae

Vernacular Name(s): Agrimony, OE 'Garclife'

Date of collection: 28 August 2010 @12:37pm

Locality: TL06526 **Altitude:** 49M

Habitat/ecological notes: Native herbaceous perennial common throughout British Isles except most of northern Scotland (Stace, 2010, p259). First recorded in Hertfordshire in 1814; last found in 76% of tetrads in old undernourished pastures, roadside verges and woodlands. Species population steadily decreasing as fields are enriched (James, 2009, p.209).

Supplementary data: Pinnate leaves, yellow flowers on long terminal racemes; stems ± erect, to 1m, diffusely branched, hairy with long and short non-glandular hairs, main leaflets 3-6 pairs, rather bluntly serrate; fruiting hypanthium obconical, deeply grooved ± to apex, with outermost bristles. Fragrant when crushed (Stace, 2010).

Uses: Anglo-Saxon medicinal formulations included treating sore eyes, abdomen and spleen; ulcerous sores, battle wounds, snakebite, warts and surgery (Van Arsdall, 2001, p.164). Fresh or dry, aerial parts and root used as an infusion, decoction and poultice: 'Against bite of snake, take this same wort, by weight of two drachms, and two draughts of wine. Give this to drink; wonderfully it removes the poison' (Cockayne, 1864, p.131)

Determined by: Dr Brenda Harold

Date: 24 September 2010

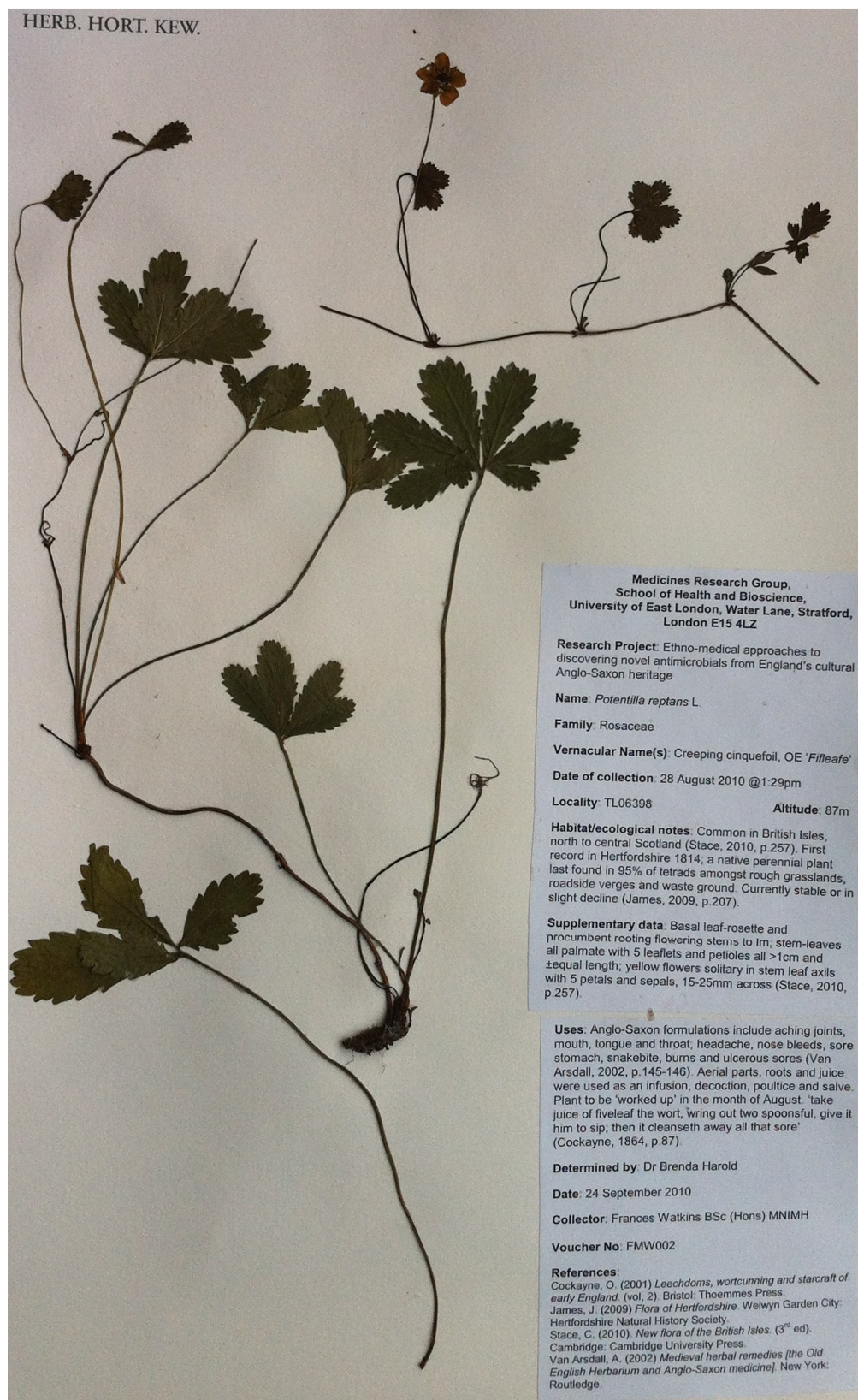
Collector: Frances Watkins MNIMH

Voucher No: FMW001

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Appendix 1.1 *A. eupatoria* herbarium label designed to demonstrate connection of plant to Anglo-Saxon medical literature and the reason for providing herbarium specimens of native British plants (Watkins et al., 2012).



Appendix 1.2 Herbaria sheet for *P. reptans* (voucher number FMW002)
lodged at the herbarium, Royal Botanic Gardens, Kew for future reference.

Plant Material	25% EtOH	75% EtOH	100% Red Wine	Infusion or Decoction
Leaves				
<i>A. eupatoria</i>	AGL25	AGL75	AGLW	AGLINF
<i>A. minus</i>	LBL25	LBL75	LBLW	LBINF
<i>B. officinalis</i>	BTL25	BTL75	BTLW	BTINF
<i>C. erythraea</i>	CTL25	CTL75	CTLW	CTINF
<i>P. media</i>	PTL25	PTL75	PTLW	PTINF
<i>P. reptans</i>	CIL25	CIL75	CILW	CIINF
Roots				
<i>A. eupatoria</i>	AGR25	AGR75	AGRW	AGDEC
<i>A. minus</i>	LBR25	LBR75	LBRW	LBDEC
<i>B. officinalis</i>	BTR25	BTR75	BTRW	BTDEC
<i>C. erythraea</i>	CTR25	CTR75	CTRW	CTDEC
<i>P. media</i>	PTR25	PTR75	PTRW	PTDEC
<i>P. reptans</i>	CIR25	CIR75	CIRW	CIDEC
Red Wine 2010	-	-	WO	-
Red Wine 2011	-	-	WOW	-

Appendix 2.1 Experimental codes assigned to leaf and root plant extracts using EtOH, red wine and H₂O.

HPA Colindale
61 Colindale Avenue, London NW9 5HT

Switchboard: 020 8200 4400
Website: <http://www.hpa.org.uk/SRMTTests>
Email: Infections@HPA.org.uk



FAO: FRANCES WATKINS
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ROMFORD ROAD
LONDON

E15 4LZ

Sender's ref. No. FW/UEL57/230420
HPA ref. No. H1 2172 0225
Date received 24.04.2012

Billing reference
Outbreak/Investig. No
Ilog number
Project code

Hospital No.
Sex Date of birth Age Y.
NHS number Patient postcode
Name SOURCE UNKNOWN
Date of collection 19.04.2012 17:00

Culture isolated from: Information not provided

Laboratory report

Final report from the Laboratory of Gastrointestinal Pathogens

Gastrointestinal Infections Reference Unit (GIRU)

Identification	Serotype	Phage type
<i>Escherichia coli</i>	Rough	

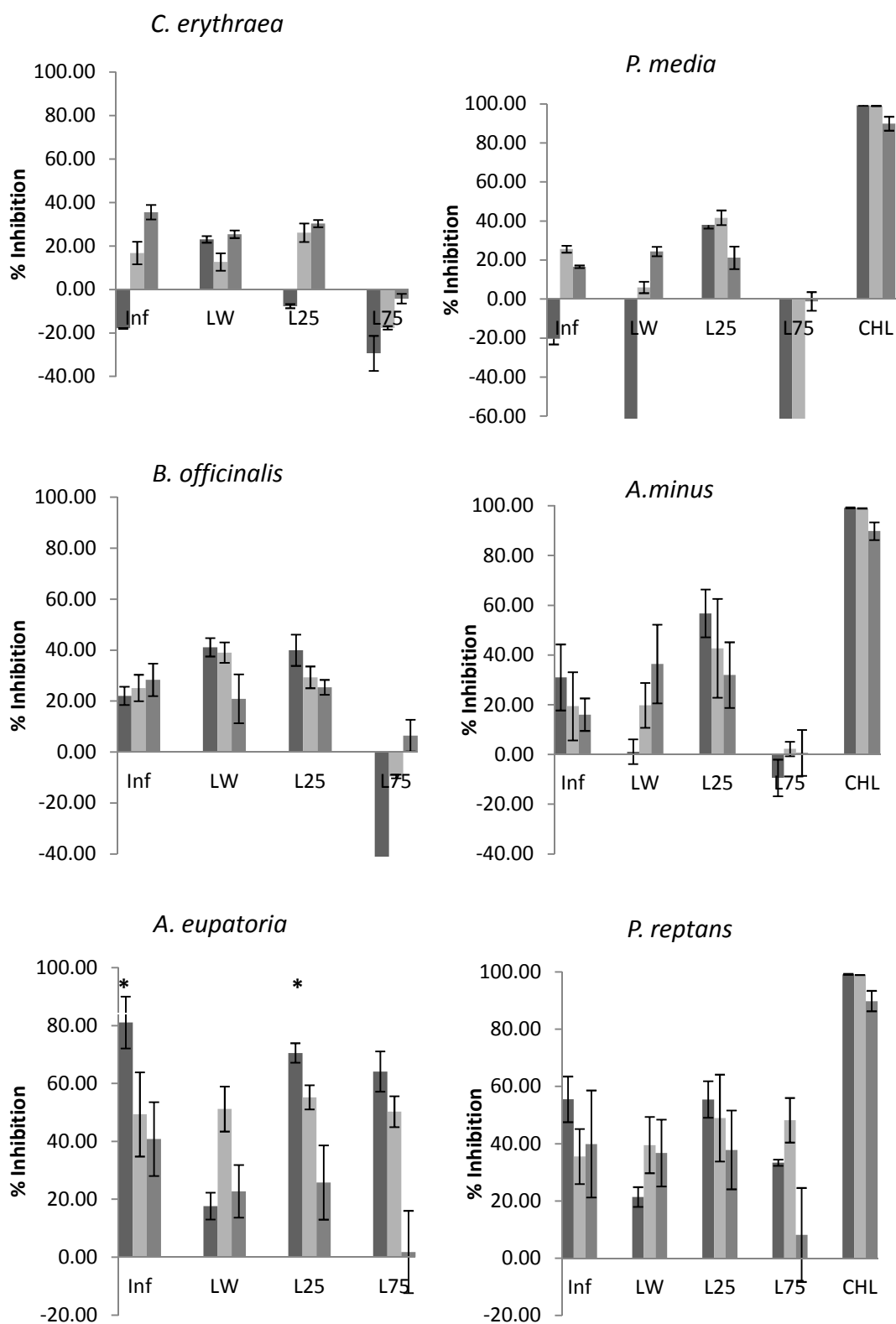
Authorised by Claire Jenkins (Laboratory of Gastrointestinal Pathogens) Date reported: 01.05.2012 11:28
Page 1/1 Date printed: 01.05.2012 11:28



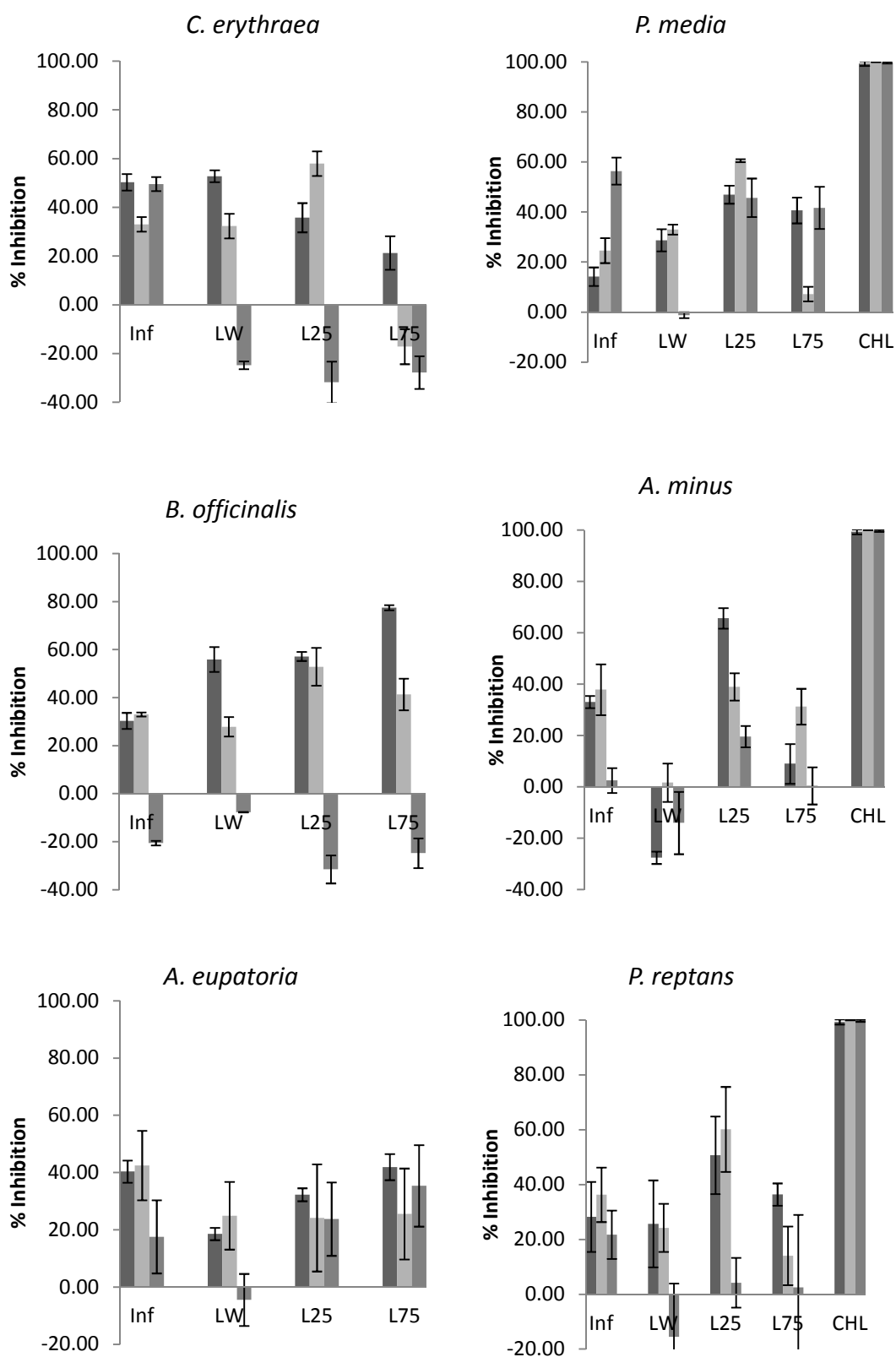
Appendix 3.1 Confirmation letter from Health Protection Agency for *E. coli* strain (UEL 57). Email correspondence from Marie Chattaway (20 December 2012) confirmed serotyping as H49.

Plant Code	INF	DEC	25%	75%	Wine
Leaves					
AGL	329	-	104	154	295
BTL	277	-	367	224	290
CIL	285	-	143	171	283
CTL	242	-	428	335	512
LBL	530	-	172	162	357
PTL	306	-	256	224	187
Roots					
AGR	-	267	209	236	682
BTR	-	544	325	403	544
CIR	-	1232	199	232	769
CTR	-	61	-	42	510
LBR	-	313	118	152	682
PTR	-	262	168	197	434
WO1	-	-	-	-	668
WO2	-	-	-	-	542

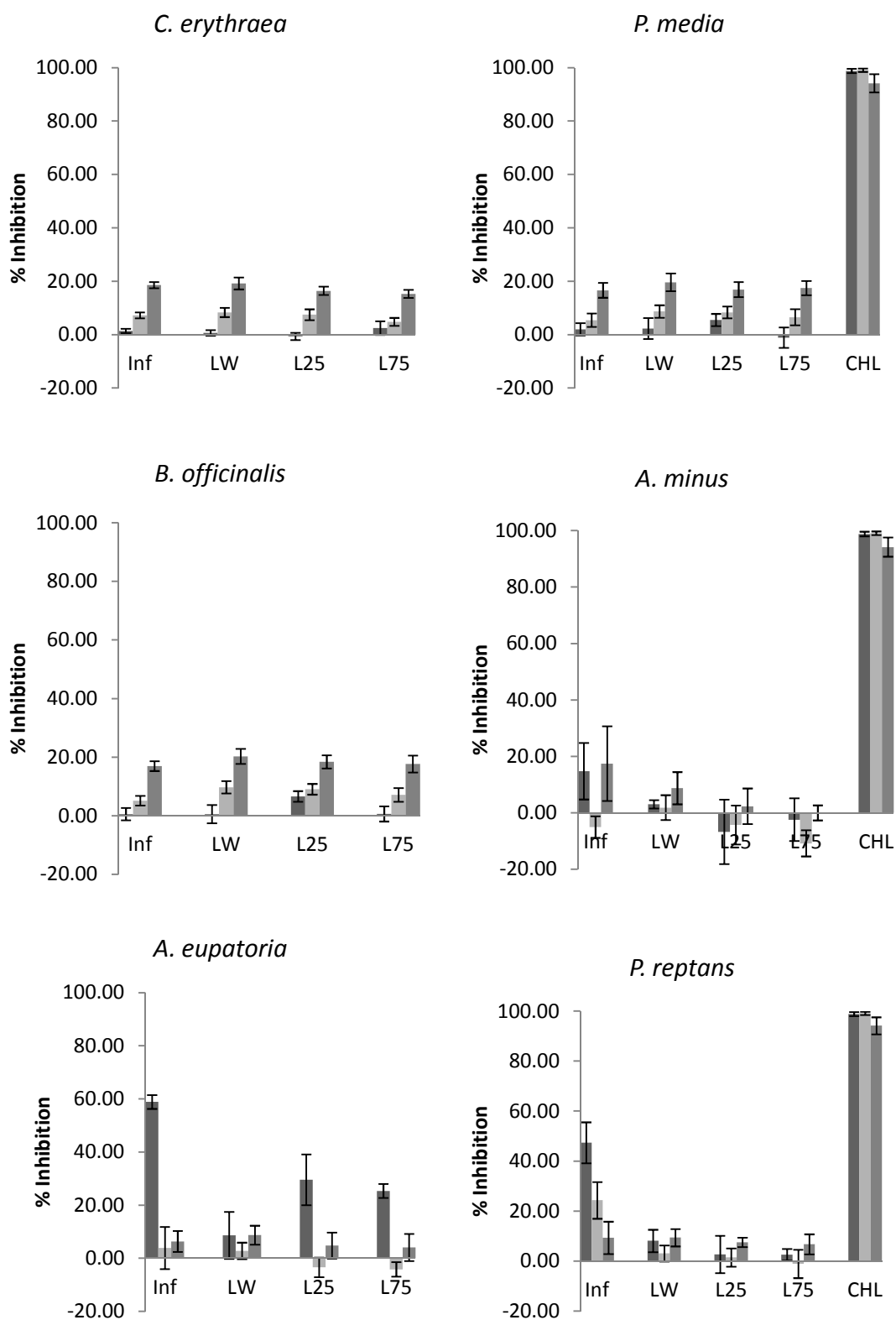
Appendix 3.2 Dried yield (mg) from 2 g of *A. eupatoria*, *B. officinalis*, *P. reptans*, *C. erythraea*, *P. media* and *A. minus* leaf and root extracts and red wine controls stored at 4 °C.



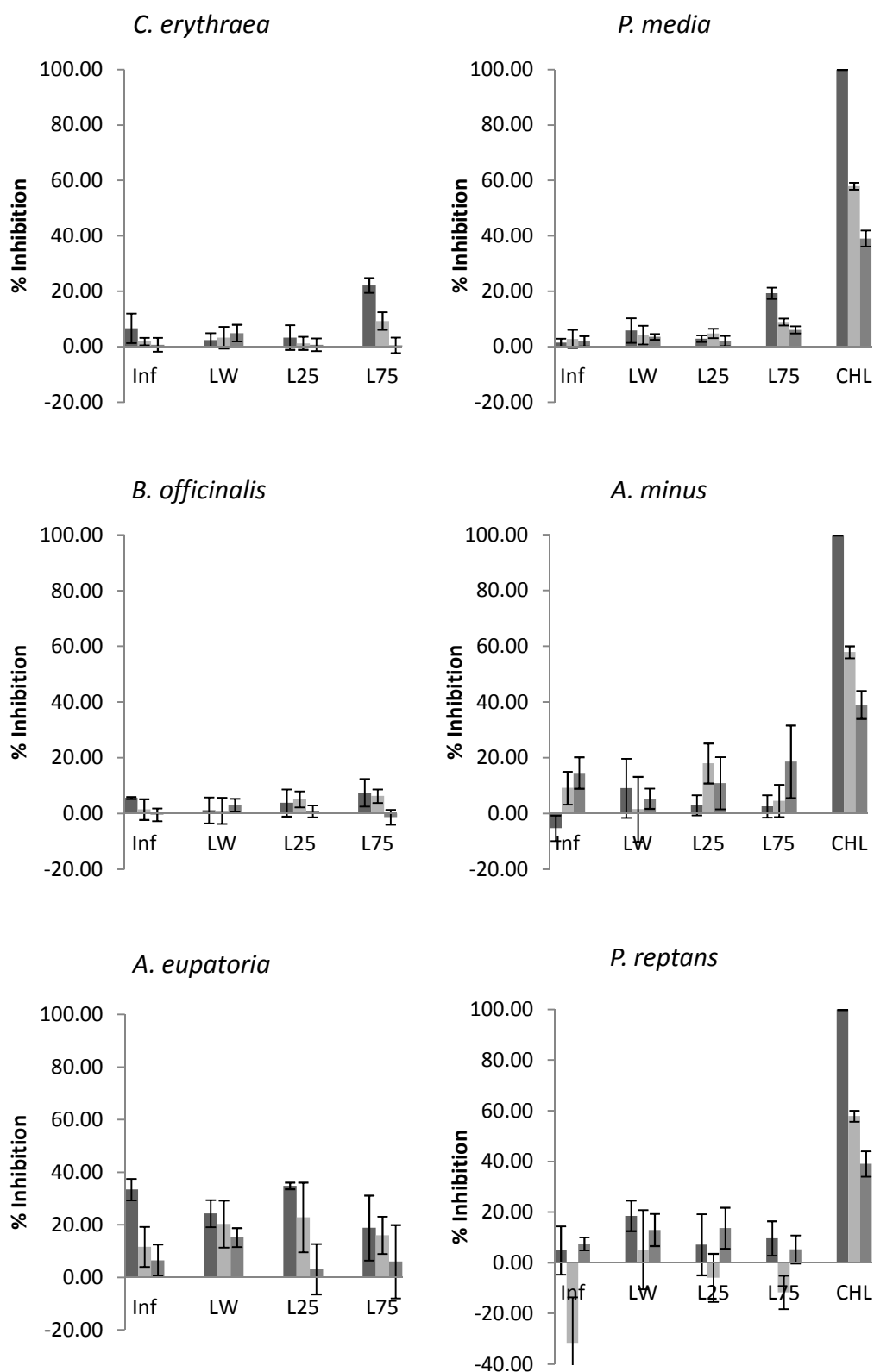
Appendix 3.3 *S. aureus* antimicrobial screening results for leaf extracts (from left to right) at 200, 40 and 8 µg/mL. **Key:** **Inf** Leaves in boiling H₂O; **LW** Red Wine; **L25** 25% EtOH; **L75** 75% EtOH and **CHL** Chloramphenicol (positive control). * denotes significant inhibition (p < 0.05).



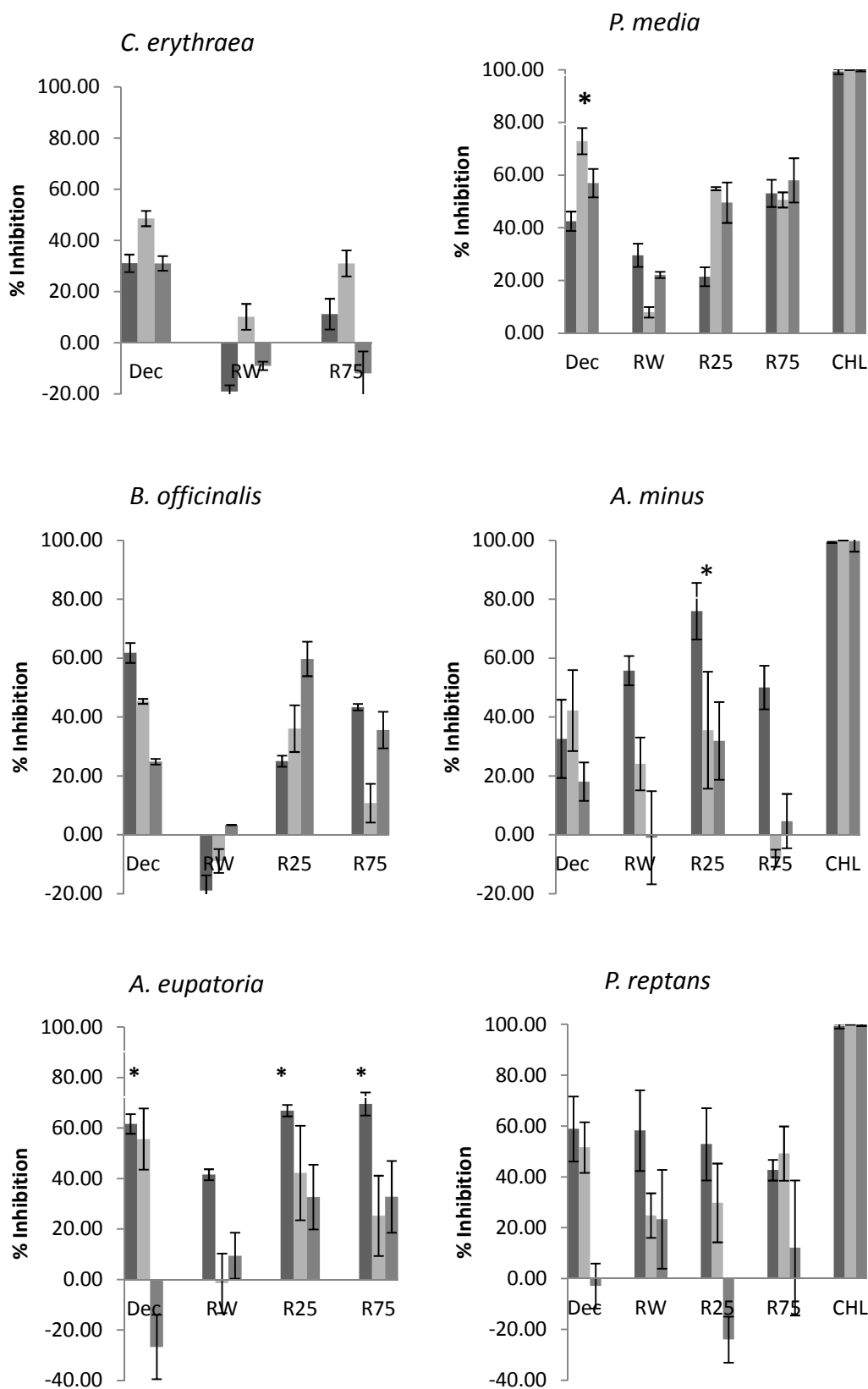
Appendix 3.4 *B. subtilis* antimicrobial screening results for leaf extracts (from left to right) at 200, 40 and 8 µg/mL. **Key:** **Inf** Leaves in boiling H₂O; **LW** Red wine; **L25** 25% EtOH; **L75** 75% EtOH and **CHL** Chloramphenicol (positive control).



Appendix 3.5 *E. coli* antimicrobial screening results for leaf extracts (from left to right) at 200, 40 and 8 µg/mL. **Key:** **Inf** Leaves in boiling H₂O; **LW** Red wine; **L25** 25% EtOH; **L75** 75% EtOH and **CHL** Chloramphenicol (positive control).

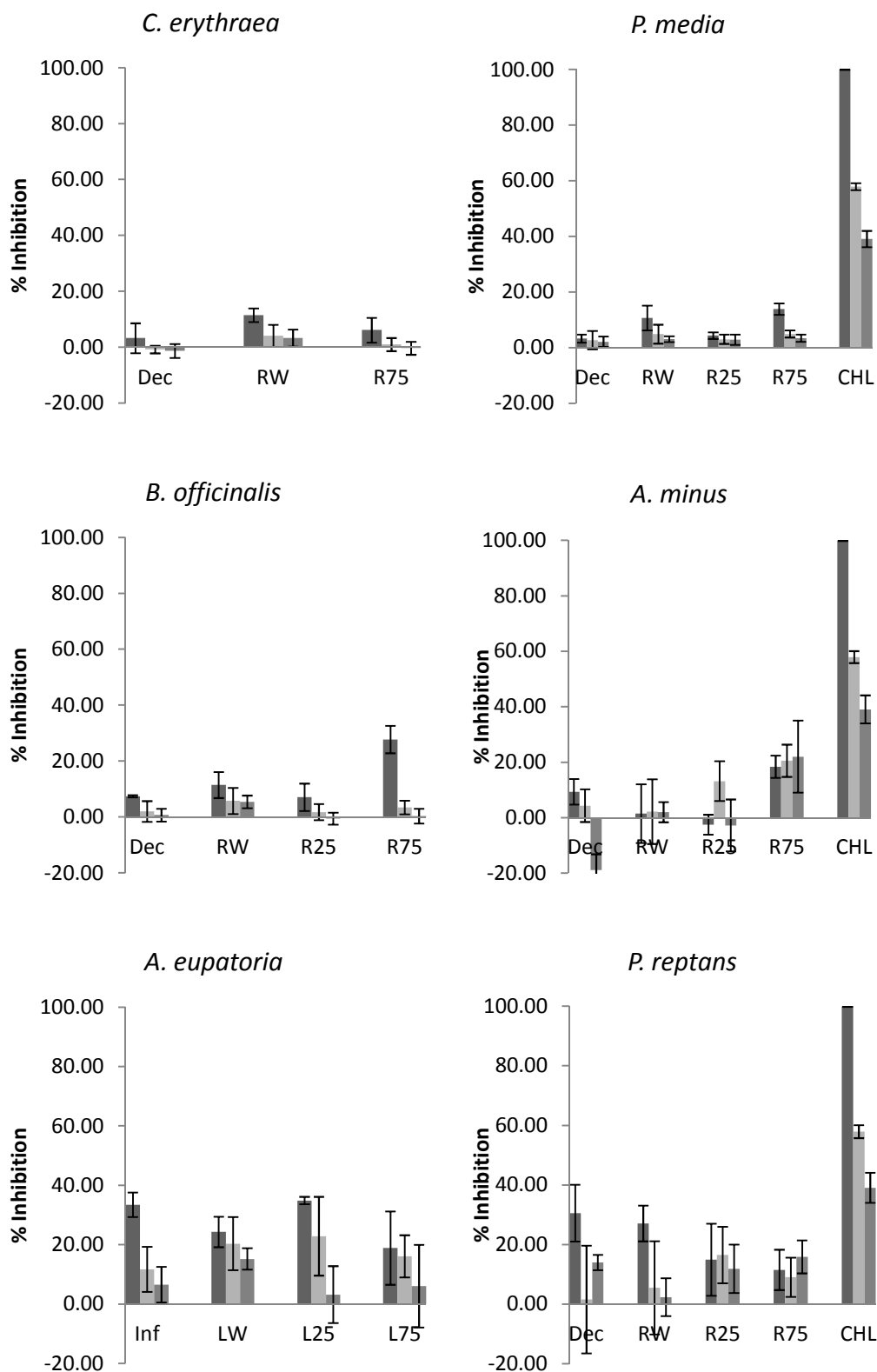


Appendix 3.6 *P. aeruginosa* antimicrobial screening results for leaf extracts (from left to right) at 200, 40 and 8 µg/mL. **Key:** **Inf** Leaves in boiling H₂O; **LW** Red wine; **L25** 25% EtOH; **L75** 75% EtOH and **CHL** Chloramphenicol (positive control).

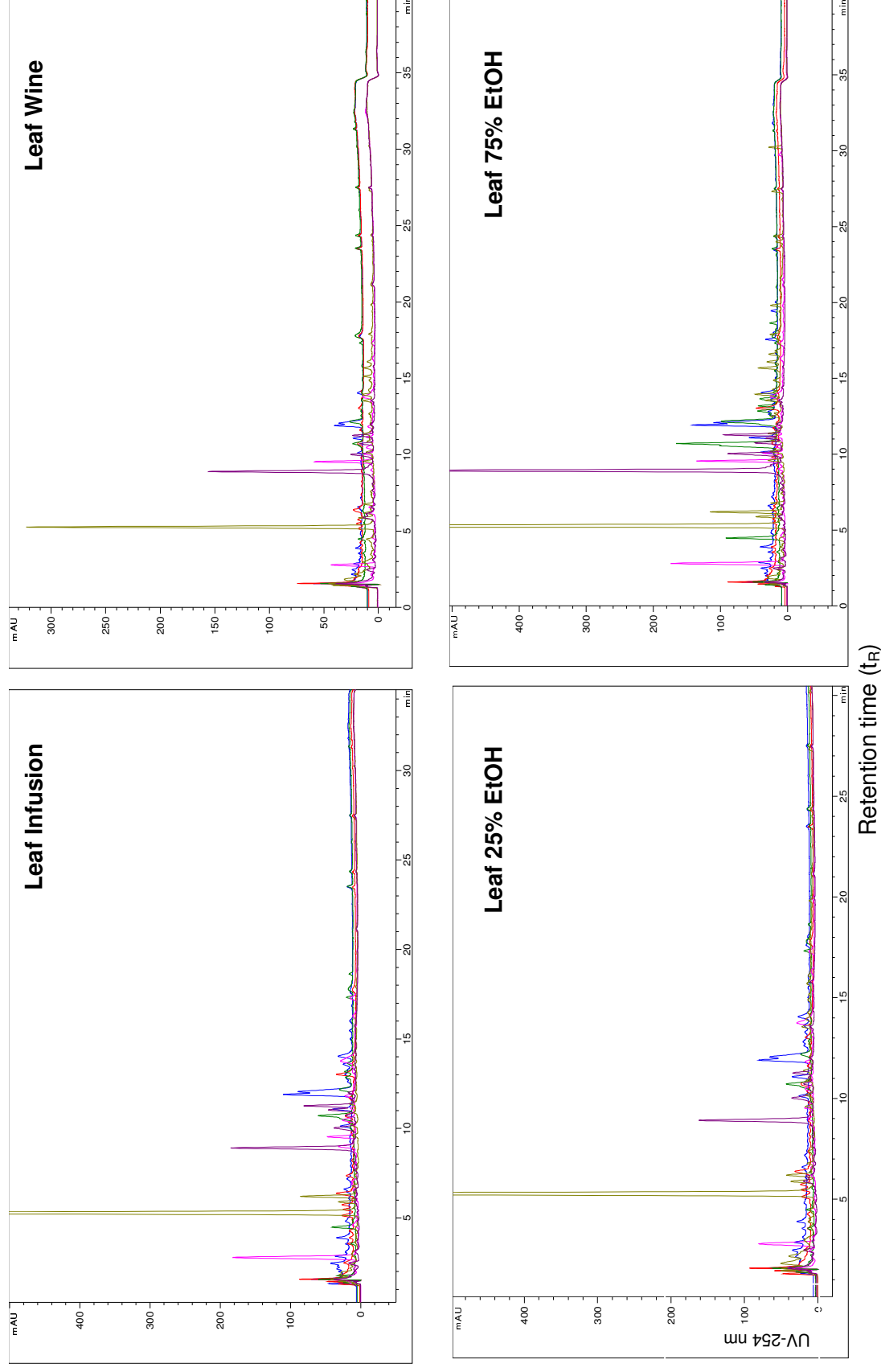


Appendix 3.7 *B. subtilis* antimicrobial screening results for root extracts (from left to right) at 200, 40 and 8 $\mu\text{g/mL}$. **Key:** **Dec** Roots in boiling H_2O ; **RW** Red wine; **R25** 25% EtOH; **R75** 75% EtOH and **CHL** Chloramphenicol (positive control).

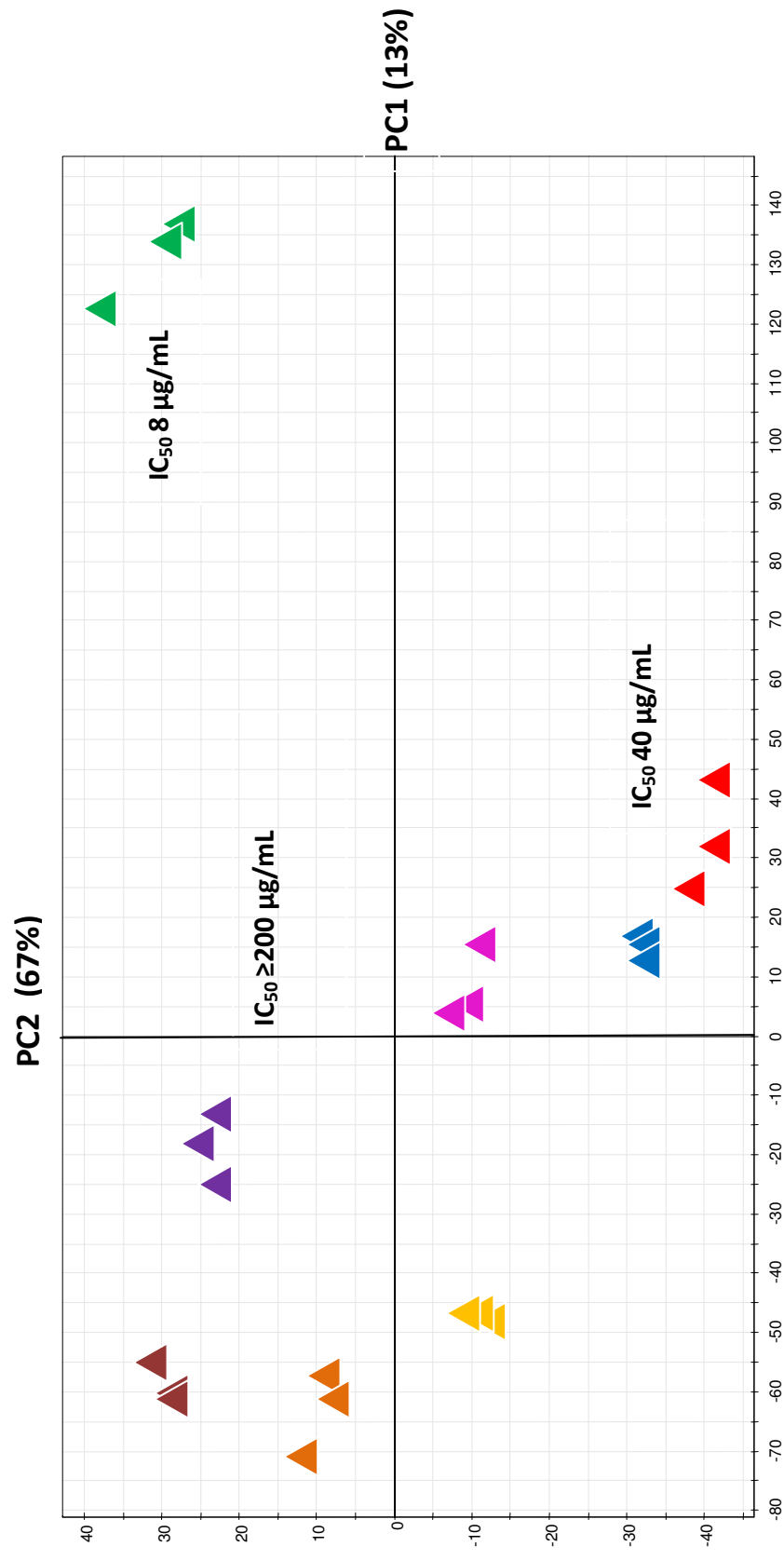
* denotes significant inhibition ($p < 0.05$).



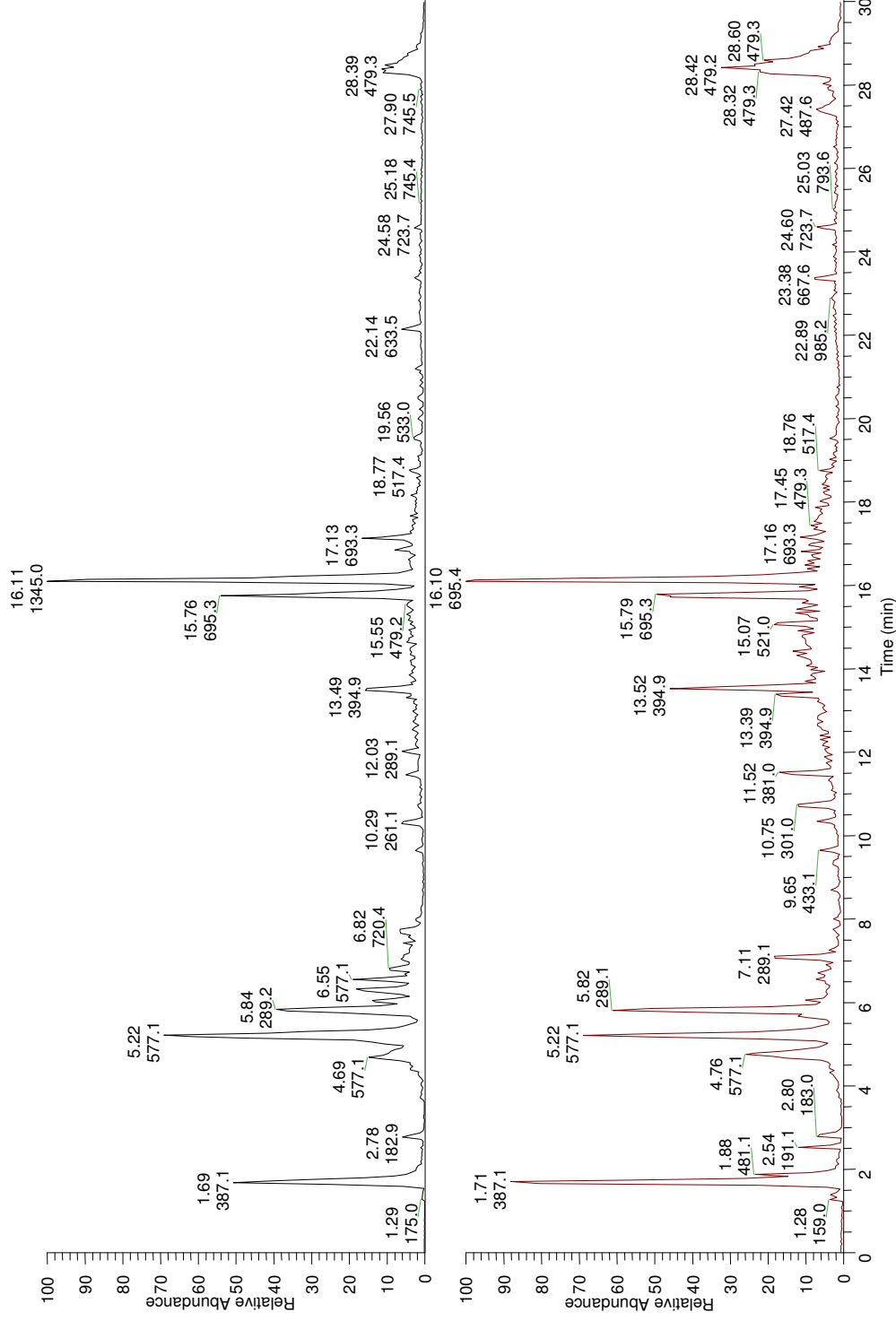
Appendix 3.8 *P. aeruginosa* antimicrobial screening results for root extracts (from left to right) at 200, 40 and 8 µg/mL. **Key:** **Dec** Roots in boiling H₂O; **RW** Red wine; **R25** 25% EtOH; **R75** 75% EtOH and **CHL** Chloramphenicol (positive control).



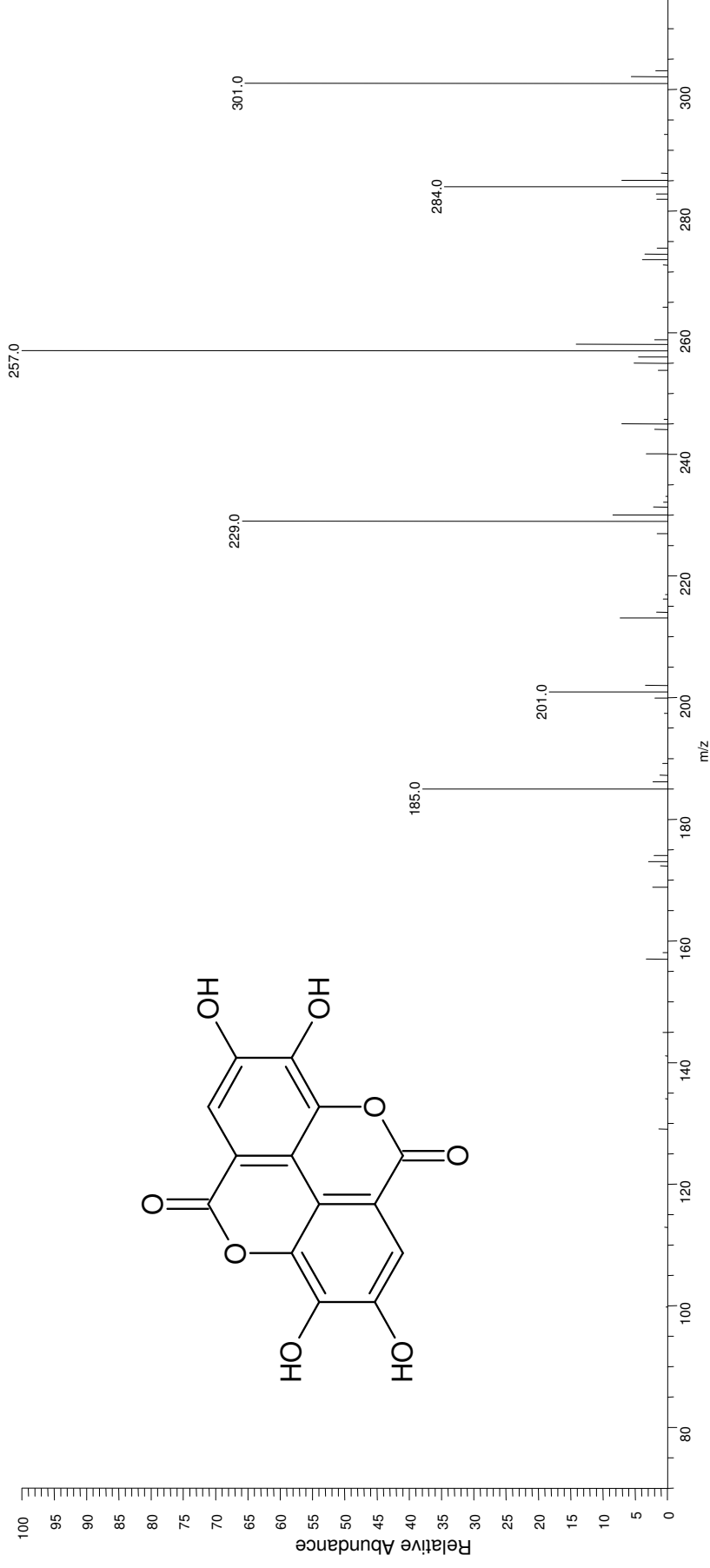
Appendix 4.1 HPLC-UV_{254 nm} chromatograms for leaf extracts (2 mg/mL). **Key:** Blue (*A. eupatoria*), Red (*P. reptans*), Green (*A. minus*), Pink (*B. officinalis*), Gold (*C. erythraea*) and Purple (*P. media*).



Appendix 4.2 Optimised HPLC-PCA_{210 nm} score plot for *P. reptans* extracts overlaid with antimicrobial activity against Gram-positive *S. aureus*. **Key:** Infusion (yellow) 25% EtOH leaf (brown) 75% EtOH leaf (purple) Leaf red wine (dark brown) Decoction (blue) 25% EtOH root (red) 75% EtOH root (green) and Root red wine (pink).



Appendix 5.1 HR-ESI-MS chromatograms in negative ionisation mode for *P. reptans* 75% EtOH root (top) and root decoction (bottom).



Appendix 5.2 Product ion scan for a molecular formula of $C_{14}H_6O_8$ that may be ellagic acid (18) with a LC-MS retention time of 10.75 min present in both *P. reptans* root decoction and 75% EtOH root extracts. Chemical structure (Tomczyk, 2011).



Anglo-Saxon pharmacopoeia revisited: a potential treasure in drug discovery

Reviews - POST SCREEN

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Three of the four major Anglo-Saxon collections reporting medicinal formulations in England from the 10th century, the *Old English Herbarium*, Bald's *Leechbook* and the *Lacnunga*, could contain leads and insights into new medicinal uses. Previous pharmacological studies of medicinal plants mentioned in Anglo-Saxon medical texts suggested that some were effective and led to the identification and isolation of natural compounds. For example, matricin from yarrow *Achillea millefolium* L., is a propionic acid analogue that yields chamazulene carboxylic acid with cyclooxygenase-2 activity similar to that of thuprofen. As we discuss here, multidisciplinary projects could further explore historical texts to discover additional plant metabolites with potential pharmacological applications.

Introduction

Chinese and Indian cultures consider their ancient texts of medicinal plants to be valuable resources in the search for novel compounds with potential pharmacological applications. In England, Anglo-Saxon medical texts have been under-researched until recently, as new translations now provide greater access to Anglo-Saxon pharmacopoeia for scientific investigation of this Western herbal tradition [1]. Anglo-Saxon medicinal plants have been reported as appearing in later herbals, although these formulations were greatly influenced by European, Arabic and Mediterranean pharmacopoeias of the time [2].

The Anglo-Saxon period spanned over 500 years, from 499 to 1066 AD. The *Anglo-Saxon Chronicle* was commissioned during the reign of King Alfred (871–899 AD) to record major events and natural phenomena from the time of Christ [3]. There is supporting evidence for the 'little optimum' or Medieval Warm Period, thought to be similar to current-day British weather patterns, with 38 established English vineyards listed in the Domesday survey of 1086 [4,5].

For more than half a millennium, battles, disease, droughts and famines in Anglo-Saxon England created great demands upon human health and the physicians attending the wounded and

the sick. Leech or 'laece' is an Old English word for healer or doctor. The Anglo-Saxon leech would have had many written sources at his disposal, including Latin and Greek medical books [6–8]. Prior knowledge of plants and herbal practices would have been required to make effective use of the herbal texts, to treat disease confidently and charge a *laecefah* or 'doctor's fee' for services and medication [8].

Some of the Anglo-Saxon settlements were near swamps and marshes, resulting in people suffering from asthma, eye and ear infections, pleurisy and rheumatism. Spring fever or 'lencten ail', possibly malaria, was recorded during the 8th century by the venerable Bede, historian and theologian, and remained evident throughout the Anglo-Saxon period. Disease encompassed plague and leprosy; battle wounds, amputations and burns; rheumatism, viral infections or 'flying venoms' and a variety of parasites, especially 'burrowing worms' [7,9]. The most common remedies found in the Anglo-Saxon medical texts are for skin disease, cough or lung disease, eye problems, headaches and fevers [10].

Treatment was empirical, pragmatic and, according to Cameron [7], two thirds of the remedies would have been effective for the prescribed condition. Internal and external applications were made from the whole plant or specified parts prepared as infusions, decoctions in wine or ale and pounded in

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www.drugdiscoverytoday.com 1069

Appendix 6.1 Review of plants used in 10th century Anglo-Saxon formulations published in Drug Discovery Today.



Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Antimicrobial assays of three native British plants used in Anglo-Saxon medicine for wound healing formulations in 10th century England

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ABSTRACT

Ethnopharmacological relevance: Three important Anglo-Saxon medical texts from the 10th century contain herbal formulations for over 250 plant species, many of which have yet to be evaluated for their phytochemical and/or pharmacological properties. In this study, three native British plants were selected to determine antimicrobial activity relevant to treating bacterial infections and wounds.

Materials and methods: Several preparations of *Agrimonia eupatoria* L., *Arctium minus* (Hill) Bernh. and *Patentilla reptans* L. were screened for antimicrobial activity against selected Gram-positive and Gram-negative bacteria of relevance in wounds using a 96 well plate microdilution method (200, 40 and 8 µg/ml). Minimum inhibitory concentration (MIC) values were determined for the most potent extracts from 2 to 0.004 mg/ml and HPLC chromatograms examined by multivariate analysis. Principle components analysis (PCA) was used to identify chemical differences between antimicrobial activity of the crude extracts.

Results: The HPLC–PCA score plots attributed HPLC peaks to the antimicrobial activity with all three plants inhibiting growth of Gram-positive *Staphylococcus aureus* by > 50% in four or more extracts. The first two principal components (PC) represented 87% of the dataset variance. The *P. reptans* 75% ethanol root extract exhibited the greatest range of activity with MIC₅₀ at 31.25 µg/ml to a total MIC that was also the minimum bactericidal concentration (MBC) at 1 mg/ml. Additionally, the root of *P. reptans*, inhibited growth of Gram-negative bacteria with the 75% ethanol extract having a MIC₅₀ at 1 mg/ml against *Pseudomonas aeruginosa* and the decoction a MIC₅₀ at 3.9 µg/ml against *Escherichia coli*.

Conclusions: The results indicate a moderate antimicrobial activity against common wound pathogens for *P. reptans* suggesting it may well have been effective for treating wound and bacterial infections. Anglo-Saxon literary heritage may provide a credible basis for researching new antimicrobial formulations. Our approach encompassing advanced analytical technologies and chemometric models paves the way for systematic investigation of Anglo-Saxon medical literature for further therapeutic indications to uncover knowledge of native British plants, some of which are currently lost to modern Western herbal medicine.

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1. Introduction

Three important Anglo-Saxon medical texts based on herbal formulations were compiled in England during the 10th century. The *Old English Herbarium*, a classical Latin text (4–5th century) containing many ‘simples’ or single formulations was reordered and translated into Old English. Bald’s *Leechbook* and the *Lacnunga*, by contrast, were written in the vernacular with no known Latin variants and include formulations from a variety of sources, including Latin and Greek; Old Irish and Aramaic (Voigts, 1979;

Pettit, 2001; Cameron, 2006). Bald’s *Leechbook* combines the best of classical and indigenous teachings into a coherent text suitable for attending the royal household whereas the *Lacnunga* is considered to be a personal collection of formulations used by a lay practitioner (Talbot, 1967; Meaney, 1984; Pettit, 2001; Pollington, 2008). Later European herbals from 16th and 17th centuries have been reviewed for rheumatism treatments (Adams et al., 2009) with reported activities that seem to support at least some of these historical formulations that have been handed down through the centuries.

Despite a rich history of medicinal plant use throughout the British Isles, much of the native flora listed in the Anglo-Saxon medicinal literature has yet to be evaluated for pharmacological and medicinal applications (Watkins et al., 2011). Following Cockayne’s translation of all the major Anglo-Saxon medical literature (1864–1865) many of the formulations were dismissed

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Appendix 6.2 Paper titled ‘Antimicrobial assays of three native British plants used in 10th century Anglo-Saxon medicine for wound healing formulations in 10th century England’, published in the Journal of Ethnopharmacology.



Student Declaration form

Student Name:	Frances Watkins
Student Number:	U0956768
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Degree for which thesis submitted:	PhD

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Signature of student

Date 31 May 2013

Appendix 6.3 Frances Watkins' Declaration 31 May 2013.