ABSTRACT

WAGNER, CHARLES STEPHEN. Evidence Based Validation of Botanical Interventions from Scotland and Medieval Wales. (Under the direction of Dr. Slavko Komarnytsky and Dr. Jillian De Gezelle.)

The British Isles are a group of over 6,000 islands in the North Atlantic Ocean. From the first millennium BC onward, various tribes and invaders created a diverse cultural landscape of Celtic, Roman, and Germanic traditions, transforming the isles from barbarian outpost to world superpower. Within this hybrid landscape emerged a rich healing tradition, with a written record spanning approximately 2,000 years – from the Roman era through the Middle Ages, up until the early 20th century. This dissertation addresses the application of traditional Welsh and Scottish botanical interventions to modern health, and using current laboratory analyses, systematically evaluates specific botanicals and formulas for their potential to improve various human health outcomes.

Chapter 1 overviews the history of the Celtic linguistic community within the context of plant use and comparatively examines plant species and target health conditions found in the medieval Welsh herbal *Meddygon Myddfai* against herbal texts from Greco-Roman, Anglo-Saxon, and continental European sources. Although the majority of the recipes were based on the Mediterranean herbal tradition, they preserved unique herbal preparation signatures and six plants could be attributed to the Celtic (Welsh) herbal tradition. This review provides, for the first time, initial evidence for traces of Celtic framework in the wider Western herbal tradition and warrants further investigations of bioactivity and clinical applications of the described plant leads.

Chapter 2 examines the antibacterial potential of 83 of 138 plant species indicated in the medieval Welsh herbal *Meddygon Myddfai* to treat conditions related to microbial infections. In

an ethnobotanical survey of the Isle of Arran, a novel field assay for *in situ* targeted screening of antimicrobials was employed. 67 of the 83 plants identified in *Meddygon Myddfai* and an additional 14 of 18 plants from local knowledge showed detectable levels of antimicrobial activity. In a follow-up proof-of-concept study, bioassay-guided fractionation was performed to identify bioactive constituents from two high scoring hits that inhibited *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) bacterial growth. Sabinene, a natural bicyclic monoterpene from juniper "berries" (*Juniperus communis* L.) and alliin, a natural sulfoxide from garlic cloves (*Allium sativum* L.), were isolated and confirmed as primary antibacterial leads.

Chapter 3 evaluates the anti-colitic potential of a polyherbal recipe from the medieval Welsh herbal *Meddygon Myddfai* in the dextran sodium sulfate (DSS) mouse model of colitis. The *Myddfai* recipe, consisting of *Artemisia vulgaris* L., *Plantago major* L., and *Lamium album* L. boiled in goat's whey, dramatically alleviated body weight loss, disease activity index, and colon length shortening as compared to the DSS control. Beneficial microbiome modulation was also observed. Differential effects were observed from each individual ingredient suggesting that recipe was "designed" by the physicians that recorded it.

In Chapter 4, 242 oat (*Avena sativa* L.) flour samples were analyzed for free, bound, and *in vitro* digested bitter compounds (avenanthramides A-C, coumaric, ferulic, and caffeic acids.) The samples consisted of 109 varieties from the AFRI CORE diversity panel, each grown at two different locations. Early modern anthropologists noted the consumption of oats, which was not common in continental Europe, to be associated with the robust health of 17th through 19th century Scottish peasants. The acute anti-hyperglycemic effect of selected whole grain oat flours in the polygenic C57BL/6J mouse model of diet-induced obesity was evaluated and correlated to

metabolite composition. Pretreatment with oat cultivars high in avenanthramides showed marked reduction of post prandial glucose rise in mice.

Collectively, these findings support historical botanical interventions as effective dietary agents to improve human health. When backed by evidence and proper clinical integration, historical botanical interventions have great potential for preventing chronic disease, managing symptoms, and enhancing patient quality of life.

Evidence Based Validation of Botanical Interventions from Scotland and Medieval Wales

by
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DEDICATION

To Matt, for teaching me to look at the Moon.

"πάντες ἄνθρωποι τοῦ εἰδέναι ὀρέγονται φύσει."
-Aristotle, *Metaphysica*

BIOGRAPHY

Charles Wagner was born in Louisville, Kentucky. The child of two educators, Charlie was always fascinated by living things and their histories. A love of ideas, books, maps, and music led him to study philosophy at St. John's College in Annapolis, Maryland before transferring to North Carolina State University, where he completed his bachelor's degree in Plant Biology. His Orcadian heritage has inspired much of his work and travel, along with a desperate desire to live a simple life close to nature.

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Of course, in this same breath I must also include my sister, Elizabeth. There is no one on Earth that understands me the way you do and has shared my most inner life. You taught me to write, and I consider you my closest friend – through thick and thin.

Then there is my brother Matthew. He has been the guiding principle in my life and my family's. He lived a very difficult and complex life, yet he was always fearless, strong willed, and humorous. I am so grateful, and forever indebted to him. He saved me from the frivolity that so many live in and he taught me that life is so much more than what meets the eye. Rest in peace, dear brother.

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You have all been instrumental in my success. I will end by thanking all the people that came before me on those dark Isles. Our world is changing faster than it ever has, yet more than ever I feel solidly rooted. I persist because of this identity. On the cliffs of Westray "I felt the touch of the kings and the breath of the wind… with the ghosts of the men who can never sing again..." And for that, I am forever grateful.

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CHAPTER 1: CELTIC PROVENANCE IN TRADITIONAL HERBAL MEDICINE OF MEDIEVAL WALES AND CLASSICAL ANTIQUITY

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Abstract

The Celtic linguistic community dominated large spans of Central and Western Europe between 800 BC and 500 AD, but knowledge of their traditional medicine is very limited. Multiple progressive plant gains in Neolithic settlements along the Danube and up the Rhine valleys suggested that taxon diversity of gathered plants peaked at the Balkans and was subsequently reduced as crop and gathered plants packages were adopted and dispersed throughout Neolithic Europe. This process coincided with the Bronze Age migration of the R1b proto-Celtic tribes, and their herbal traditions were occasionally recorded in the classic Greco-Roman texts on herbal medicines. The provenance of Celtic (Gallic) healing methods and magical formulas as recorded by Pliny, Scribonius Largus, and Marcellus Empiricus can still be found in the first part of the medieval Welsh (Cymry) herbal manuscript *Meddygon Myddfai* (recipes 1-188). Although the majority of *Myddfai* I recipes were based on the Mediterranean herbal tradition of Dioscorides and Macer Floridus, they preserved the unique herbal preparation signatures distinct from continental and Anglo-Saxon counterparts in increased use of whey and ashes as vehicles for formulation of herbal remedies. Six plants could be hypothetically attributed to the Celtic (Welsh) herbal tradition including foxglove (*Digitalis purpurea* L.), corn bellflower (*Legousia speculum-veneris* L.), self-heal (*Prunella vulgaris* L.), sharp dock (*Rumex conglomeratus* Murray), water pimpernel (*Samolus valerandi* L.) and river startip (*Scapania undulata* L.) This review provides initial evidence for traces of Celtic framework in the Welsh herbal tradition and warrants further investigations of bioactivity and clinical applications of the described plant leads.

1. Introduction

The Celts were an Iron Age European cultural group that originated from a compact Proto-Indo-European linguistic community somewhere in the border region of Eastern Europe and Western Asia, and invaded into Central and Western Europe by land, as it seems on foot, circa 3000 BC. In their footsteps, proto-Celts likely followed the Balkan (Danube) route of the Neolithic Anatolian farmers (Hofmanová et al., 2016) and Yamna steppe pastoralists (Haak et al., 2015) to establish two secondary refuges in the Hallstatt region of Central Europe (Smrcka, 2009) and the Tartessos region of the Iberian peninsula (McEvoy et al., 2004). In doing so, they overtook the local Corded Ware groups (ca. 2400 BC) and admixed with the late Bell Beaker groups (ca. 2200 BC) in Central Europe by forming a succession of Unetice (2300-1600 BC), Tumulus (1600-1200 BC), and Urnfield (1300-750 BC) cultures likely ancestral to the Celtic speakers. Proto-Celts also spread their influence into the British Isles sometime after 800 BC, perhaps in a very similar manner to how the Bell Beaker culture replaced the late Mesolithic populations of Britain (Olalde et al., 2018) and Ireland (Cassidy et al., 2016). While some areas in Europe preserved the original non-Indo-European languages (Basque, Etruscan, Lusitanian, Rhaetian), populations that spanned the Hallstatt to Tartessos corridor adopted Celtic linguistic and cultural traits in connection with expanding trade networks. Today, the term Celtic generally refers to the living languages and respective cultures of Goidelic (Irish and Scottish Gaelic, and Manx Gaelic from the Isle of Man) and Brittonic (Breton, Cornish, and Welsh) groups spoken by the insular Celts of the British Isles and Brittany. The continental Celtic languages were gradually replaced by Vulgar Latin and Germanic languages sometime after 500 AD (Helix, 2010).

Knowledge regarding the history of traditional Celtic medicine is exceedingly uncertain. A small number of inscriptions and place names that survived in the Lepontic (500-300 BC), Gallic (200 BC-400 AD), and Celtiberian (200-100 BC) continental Celtic languages made no references to their medical tradition, while the earliest inscriptions in insular Celtic languages did not appear before 400 AD (Helix, 2010). As such, most of the original accounts of Celtic culture were recorded by foreign writers immediately before and during the period of the Roman Empire. The lack of direct textual or archaeological evidence of medical practice in the Celtic world largely obscured differentiation of local Celtic medical tradition from the later Mediterranean influences. In this review we argue that traces of Celtic herbal knowledge persisted in the wider European medical tradition by means of direct oral transmission, and albeit at low frequency, were incorporated into classic and medieval herbal compilations.

2. Methods

Middle Welsh, Anglo-Saxon, Roman, and Continental European sources were reviewed in their original languages with the aid of lexicons, dictionaries, as well as both antique and modern English translations. Using *Myddfai I* as one of the earliest known medical texts written in a Celtic language, the most pertinent Roman sources were reviewed in chronological order to observe species frequencies and gather facts relatable to *Myddfai I*. Conclusions are original to the authors and when possible corroborated by citations from other scholarly works. The criteria for the selection of antique works to compare plant species frequencies was based upon the lineage and historical popularity of each text, availability, and relevance to medieval Wales (chronology, location). Herbal preparation signatures were evaluated by calculating the frequencies of certain vehicles used to crush, dissolve, or extract plant material in order to

discern how different cultures were uniquely using similar plants. Methods, including translation notes, are discussed in more detail where relevant.

Pubmed, Scopus, Google Scholar, ScienceDirect, JSTOR, and NCBI were used to find and review relevant modern research articles. The plantlist.org was used for aligning scientific binomials. Archaeobotanical literature was reviewed with a focus on samples taken from the contexts (burials, pottery, pit fills, internal occupation deposits, hearths, ash layers, floors, burnt areas, and middens) likely to represent the accumulated debris from a range of intentional plant-related activities, including the processing of gathered plants and cultivated crops.

3. The Celts – an ethnolinguistic group

A possible date for the integration of tribal groups and enclaves into the Celtic lineage likely coincides with the merger of the ancestral Urnfield farmers with the Tumulus (kurgan) warlords. The survival of this social structure required continuous conquest and expansion into new territories. The evidence for this is seen in the Celtic migrations over much of Europe as far as the Ukrainian Carpathians, and seemingly universal acceptance of Celtic languages as *lingua franca*, the main communication language in the region (Scheeres et al., 2014). The control over salt mines of the Hallstatt region and the key trade routes to the Mediterranean coast, especially Massalia (Marseille), brought a surplus of trade and accumulation of wealth and power in the hands of the few Celtic elites – and ended as a direct result of the successful expansion of the Roman empire.

3.1. Genetic history of Celtic tribes

Modern European men are classified into 7 most frequent but distinct haplogroups based on the SNP (single nucleotide polymorphism) mutations found on the non-recombinant portion

of the Y chromosome (Jobling and Tyler-Smith, 2003). Haplogroup F moved out of Africa into the Arabian Peninsula and Near East circa 45000 BC, and produced a succession of haplogroups K > I, N, P > R in Western Central Asia (Soares et al., 2010). The two R1 subclades, R1a and R1b, established themselves in the Pontiac steppes of Ukraine, while the N2 and N3 subclades spread out to Northeastern Europe circa 25000 BC (Haak et al., 2015). Haplogroup F also produced haplogroup I that moved into the Balkans around 20000 BC. Together, these haplogroups survived the Last Glacial Maximum in the Iberian, Balkan, Ukrainian, or Siberian refuges and re-emerged as major Mesolithic hunter-gatherer groups in Europe when climates improved. The other populations arrived to Europe as Neolithic Anatolian farmers that belonged to the G2a, J2, and the African E3b lineages (Kivisild, 2017).

The late Neolithic-early Bronze archaeological horizon of Europe (2900-2400 BC) was dominated by the R1a Corded Ware people in the east and the late R1b Bell Beaker culture in the west. Both cultures met and partially overlapped at the Upper Danube region of modern Germany and Austria (Allentoft et al., 2015). As Yamna steppe pastoralists (predominantly R1b) arrived to the same area via the Balkan Danube route, they displayed an opposing approach to the existing cultures in the region, which experienced a rapid decline in human activities due to climatic changes 4000-3000 BC (Kolář et al., 2018). While they largely displaced and pushed the Corded Ware people further to the east, they rather admixed and spread their influence over the Bell Beaker people to the west. This would be possible if we assumed that both the late Bell Beaker and Yamna cultures were at least partially related as evident by the common R1b haplogroup, and preserved some ethnic, linguistic, or cultural identity that allowed for a certain degree of integration. If this assumption is correct, it allows the acceptance of the original theory of east to west migration of the R1b people (R1b-V88), followed by their retreat and subsequent

expansion from west to east (Iberian refuge), followed by the second east to west migration of Yamna R1b groups (Rb1-L51) to the area (Haak et al., 2015). Preliminary evidence for a direct genetic R1b link between Yamna and late East Beaker cultures was recently described from Alsace, France (Brunel, 2018).

The subsequent Unetice culture of the Upper Danube and Upper Rhine basins (**Figure 1**) shows a continuity of R1b1a1 haplotypes presumably contributed by the Beaker/Yamna admixture (Allentoft et al., 2015) and I2a subclades of Mesolithic hunter-gatherers from the Balkans (Mathieson et al., 2018). This suggests that the Unetice culture served as a local nucleation center for agriculture, horse-assisted trade, prospecting, and metallurgy, probably speaking languages ancestral to Germanic and proto-Italo-Celtic (R1b-L11). The lack of DNA samples and complex genetic analyses from Tumulus and Urnfield groups prohibits us from observing a direct continuum from the Unetice people to the more recent haplogroups strongly associated with Celtic populations of Irish and Scottish (R1b-M222) origins, Germanic populations from the Rhine valley (R1b-S21/U106), Italic populations of the Liguria coast (R1b-S28/U152), or Gascons, Basques, and Catalans (R1b-DF27). The Hallstatt culture (800-450 BC), centered around the Alps (R1b-Z36), and its western successor, the La Tene culture (450-100 BC), are considered the first classical Celtic cultures that contributed to Gaul and Briton lineages in France (R1b-L21) and Celtiberian lineages in Spain (R1b-DF27), but substantial genetic gaps yet need to be filled before these conclusions can be ascertained.

3.2. Early archaeobotanical evidence from excavated sites

Neolithic European settlements present in Europe since the migration of Anatolian farmers until early Bronze Age (circa 7000-1500 BC) hold the first archaeological records of

humans gathering wild plants. The Late Mesolithic Lepenski Vir site within the Iron Gates gorge on the Danube (Serbia) produced early traces of wild grass grains such as einkorn and emmer wheat (y'Edynak and Fleisch, 1983), likely introduced by the Anatolian migrants circa 6000 BC (Mathieson et al., 2018). The lowest levels of the near-continuous Upper Paleolithic-Neolithic settlement at Franchti Cave (Greece) contained the largely uncharred carbonized seeds of field gromwell (*Lithospermum arvense* L.), alkanet (*Alkanna orientalis* L.), and bugloss (*Anchusa* sp.) (20000-11000 BC). Wild lentils (*Lens* sp.), vetch (*Vicia* sp.), pistachio (*Pistacia* sp.), almonds (*Prunus dulcis* Mill.), oats (Avena sp.), barley (*Hordeum spontaneum* K. Koch), and pear (*Pyrus amygdaliformis* Vill.) were recovered from the next Mesolithic layer (11000-7000 BC). The cultivated emmer wheat (*Triticum dicoccon* Schrank) also appeared at this site circa 6000 BC, similar to that of the Lepenski Vir settlement, followed by the cultivated two-row hulled barley (*Hordeum distichon* L.) and lentil (*Lens culinaris* Medik.) (Hansen and Renfrew, 1978). The comparative analysis of archaeobotanical data from 40 aceramic Neolithic sites from the eastern Mediterranean identified a near-identical founder crop package among these locations that consisted of emmer (*Triticum dicoccon* Schrank), einkorn (*Triticum monococcum* L.), hulled barley (*Hordeum vulgare* L.), flax (*Linum usitatissimum* L.), and four pulses – lentil (*Lens culinaris* Medik.), pea (*Pisum sativum* L.), bitter vetch (*Vicia ervilia* L.), and chick pea (*Cicer arietinum* L.). The adoption of this crop package led to an immediate reduction in vegetational taxon diversity at the settlements (Colledge et al., 2004).

It seems that an early tradition of collecting and using wild plants followed the same adoption pattern. Plants gathered at Franchti Cave and identified to the genus level (*Adonis, Alkanna, Anchusa, Calendula, Capparis, Colchicum, Fumaria, Lithospermum, Malva, Medicago, Phalaris, Salvia*, and *Silene*), could be considered medicinal by modern standards

(Hansen and Renfrew, 1978). Moreover, transition to the early Neolithic settlements such as Theopetra (Greece), Revenia (Macedonia), or Sesklo (Greece) significantly increased variety of wild plant taxa at those sites, thus hinting at the diversity in early wild plant resources (Kotzamani and Livarda, 2018). When archaeobotanical data from 250 Neolithic sites was grouped into 22 European and eastern Mediterranean geographic regions (Coward et al., 2008), a clear east-west alignment in the direction of the Neolithic farming migration routes along the Danube and up the Rhine valley, but not the Mediterranean coast, was supported by multiple progressive plant gains (**Supplementary Table 1**). The diversity of wild plant findings showed a distinctive pattern of gains in Thalesian Greece, peaked in the Balkans (Bulgaria and Macedonia), and stabilized in the areas dominated by the Linear Pottery culture (LBK). Gathered plant gains included verbena (*Verbena officinalis* L.), agrimony (*Agrimonia eupatoria* L.), nightshade (*Atropa belladonna* L.), henbane (*Hyoscyamus niger* L.), ribwort plantain (*Plantago lanceolata* L.), poppy (*Papaver somniferum* L.), and wild strawberry (*Fragaria vesca* L.) Further migrations in the direction of Benelux, Scandinavia, and Southern Britain regions correlated with a divergent pattern of gathered species losses (Coward et al., 2008). The Mesolithic and Neolithic migrations into British Isles took place predominantly via Atlantic coastal routes (Farrell, 2015). As such, they were defined mostly by losses as compared to the traditional Neolithic Mediterranean plant assemblies (Coward et al., 2008). Notable gains here were puffball mushrooms (*Calvatia* sp.) from the Neolithic Skara Brae site (3180-2500 BC) in Orkney, Scotland (Watling and Seaward, 1976) and henbane (*Hyoscyamnus niger* L.) seeds in grooved ware pots at the Balfrag settlement (2900 BC) in Fife, Scotland (Barclay et al., 1993). Meadowsweet (*Filipendula ulmaria* L.) was found at the Bronze Age cairn at Fan Foel,

Carmarthenshire (Wales) as well as in incised beakers at the burial cists of Ashgrove, Fife and North Mains Strathallan (Scotland) (Henshall, 1963).

Although the putative health applications of these plants cannot be confirmed directly, their intended use can be implied by high frequency storage finds or detection in the gastrointestinal system of human remains. The examples included discovery of 8,000 perforated and intact seeds of purple gromwell (*Lithospermum purpurocaeruleum* L.) present within the same pot, or 22,400 seeds of common gromwell (*Lithospermum officinale* L.) in a goblet within a larger pot, dated to the Neolithic Cucuteni-Trypillia culture (Solcan et al., 2014). More than 54,000 seeds of pigweed (*Chenopodium album* L.) were found in a pot from the Neolithic settlement of Niederwil (Switzerland), as well as in the intestines of 7 European Iron Age bog bodies (Behre, 2008). This is also true for hazelnut (*Corylus* sp.) and crab apple (*Malus* sp.) found in many in Neolithic assemblages and used to supplement everyday diets (Kirleis et al., 2012).

No significant changes in gathered wild plant profiles were observed in the findings from the 15 late Neolithic settlements of the Funnel Beaker culture from northern Germany (Kirleis et al., 2012) or 6 late Neolithic excavation sites attributed to Baden and Jevisovice groups in eastern Austria (Kohler-Schneider and Caneppele, 2009). However, the next two archeological horizons in Europe that were dominated by pastoral populations in the east (Corded Ware) and the west (late Bell Beaker), relied on different agricultural strategies and focused on single isolated farmsteads that often did not leave a distinct signal of cultivated and gathered wild plants. In those rare instances when these settlements could be identified as in case of the Engen-Welschingen “Guuhaslen” site, a similar set of wild gathered plants has been noted that also included ruderals nettle (*Urtica dioica* L.), hemlock (*Conium maculatum* L.), white violet (*Viola*

alba Besser), elderberry (*Sambucus nigra* L. and *Sambucus ebulus* L.), and broadleaf plantain (*Plantago major* L.) (Lechterbeck et al., 2014). The history of plant assemblages from graves and megalithic tombs due to intentional activities toward their putative ritual and therapeutic use is lacking.

The east to west Paleogenetic trends of early Europe coupled with the emerging archaeobotanical pattern would suggest that the flora of the Thessalian Greece – Thracian Bulgaria and Macedonia – Central Europe axis along the Danube migratory route constituted the core pharmacy of proto-Indo-European medical traditions, predating the diversification of tribes into Italic, Celtic, Germanic, and Slavic. As history went on, this core tradition would be further diversified by the evolution of distinct ethnic groups, local oral knowledge, and plants gained and lost due to changes in environment. Ultimately these core plants, became fixed or standardized as "European medicine" by the written tradition of Greece and Rome as a result of Romanization, with small vestiges surviving in folklore and oral tradition.

3.3. Proto-Celtic cultures of pre-Roman conquest (800-275 BC)

The proto-Celtic tribes reached the area of the upper Danube and Rhine basins around 2500 BC. It is generally agreed that the first Celtic groups distinguished themselves from earlier Urnfield (proto-Celtic) and Villanova (proto-Etruscan) people ca. 800 BC through a succession of Hallstatt C-D and La Tene cultures centered around iron trade and salt mines of Hallstatt and Hallein (Dürrnberg) (**Figure 1**). This is the era of complex societies that ensured stability of bronze and later iron trade after the collapse of Southwest Asian and Mediterranean sources (Thurston, 2009). Despite numerous archaeological records of graves, there are only a small number of Hallstatt and La Tene period settlement sites known, of which only a few have been

excavated. Archaeobotanical remains from the Eberdingen-Hochdor site (600-400 BC) contained a few cultivated and wild plants, including carrot (*Daucus carota* L.), wild strawberry (*Fragaria vesca* L.), celery (*Apium graveolens* L.), camelina (*Camelina sativa* L.), parsley (*Petroselinum crispum* Mill.), hazel (*Corylus avellana* L.) and dyer's weed (*Reseda luteola* L.) (Stika, 1996). The first certain identification of *Cannabis* in Europe was also in the Hallstatt-period *Fürstengrab* of Hochdorf near Stuttgart, dated to ca. 500 BC (Merlin, 2003). While the use of opium, mandrake, and henbane were well documented in the Greco-Roman world prior to contact with the Celts, the ritual use of *Cannabis* was not a widespread practice within Greek and Roman societies, and could be likely attributed to direct early Indo-European influences of the Linear Pottery culture situated between Prut and Dniester rivers in Ukraine (Larina, 1999).

True botanical imports, including plants that were difficult to cultivate north of the Alps such as black pepper (*Piper nigrum* L.), nutmeg (*Myristica fragrans* Houtt.), sesame (*Sesamum indicum* L.) or cumin (*Cuminum cyminum* L.) were found predominantly within Roman military camps in the Rhine frontier zone (Livarda, 2011). A similar observation was made from the archaeobotanical assemblages of Roman Britain, where imports (fig, mulberry, grape, olive), herbs (coriander, celery, dill, fennel, summer savory, and marjoram), and oils (black mustard, hemp) were much more common in major military sites and towns, especially in the southeast. Fruit (apple, pear, cherry, plum, damson, and walnut) and vegetable (carrot, cabbage, turnip, parsnip, and leaf beet) findings were better represented at Roman era rural sites, suggesting that cultivation was taken up by common people, and that some crops (e.g. apple) were present in their native wild form in the Late Iron Age. Dates, almonds, pine nuts, lentils, mulberry, and grapes were also found in the ceremonial context as votive offerings exclusively in Roman London temples, burials, and shrines (Veen et al., 2008). Since no imported Greek and Roman

vessels and plants were found in Celtic rectilinear enclosures (known as *Viereckschanzen*) spread across Czech Republic to France and dated to 200-100 BC (Murray, 1995), this data indicated that Celtic culinary (and likely herbal) practices had little Roman influence until the end of the 1st century BC. Moreover, the first archaeobotanical records of imported non-native plants do not occur until after the Roman conquest of Britain (43 AD.) These included seeds of olive (*Olea europaea* L.), celery (*Apium graveolens* L.), coriander (*Coriandrum sativum* L.), and dill (*Anethum graveolens* L.) from the high status Silchester site, dated to 50 AD (Lodwick, 2014).

4. Written records from the era of Roman conflict (275 BC – 476 AD)

Gallic expansion into Italy ca. 500 BC penetrated the Po valley and culminated in the battle of Allia and sack of Rome (390 BC). Boii and other Cisalpine Gaulish tribes often allied with Etruscan and Carthaginian armies against Rome, and their social organization and military tactics were extensively recorded by Roman writers. The military conflict intensified after the defeat of Carthage in 202 BC and resulted in annexation of *Gallia Cisalpina* in 192 BC, *Gallia Transalpina* in 121 BC, Gaul in 52 BC, and Britannia in 43 AD (**Figure 1**). It is speculated that capture of Rome and the Great Celtic expansion that followed were due to population pressure, political instability, and to establish secondary Gallic states of Tylis in Thrace (279-212 BC) and Galatia in central Anatolia (279-64 BC). Here, the knowledge of traditional Celtic medicine was recorded for the first time in several Graeco-Roman herbal manuscripts focused on the medicinal flora of the Eastern Mediterranean region.

4.1 Dioscorides – *De Materia Medica* (ca. 40-90 AD)

Pedanius Dioscorides, a Greek physician possibly employed in the Roman army and a native of the Roman province of Cilicia situated south of Galatia in Asia Minor, wrote a 5-volume treatise on the preparations, strengths and dosage of 600 herbs utilized in some 1,000 medicines. Adding an additional 100 plants over the *Historia Plantarum* of Theophrastus, he ignored classification using botanical characteristics in favor of direct medicinal properties and uses of herbs. *De materia medica* directly influenced the writings of the Greek physicians Galen (ca. 129-210 AD, *De simplicibus*), Theodorus Priscianus (ca. 380 AD, *De virtutibus pigmentorum*), Oribasius (320-403 AD, *Collectiones medicae*), and many more authors. Although the original Greek version of Dioscorides was continuously inscribed for the next several centuries, the text was also rearranged in the alphabetical order (i.e. *Vienna Juliana Anicia Codex* ca 515 AD) and translated into Latin on multiple occasions. The early Latin translation was designated as *Dioscorides longobardus*, while the later translations were based upon the alphabetical version of the manuscript and often combined with information extracted from the Pseudo-Apuleius herbal (*Dioscorides vulgaris*) (Collins, 2000). Subsequent copies, interpretations, and translations of these manuscripts laid the foundation of the European herbal tradition of 8^{th}-14^{th} centuries (Singer, 1927) (**Figure 2)**.

Dioscorides owes his universal acceptance to the development of an empirical tradition of herbal remedies throughout the Mediterranean region (Nissen, 1958). In the analysis of plant locations mentioned in his work, 67 refer to Asia Minor, 43 to Arabic world, 38 to Africa, 30 to Greece and Balkans, 12 to Italy, 8 to Spain, and 5 to the more northern territories (Denham and Whitelegg, 2014), suggesting that Dioscorides was only scarcely familiar with the plants of the Danube and Rhine basins. A section of Dioscorides, however, contains a collection of plant

synonymous names (*Synonyma plantarum*) in multiple languages including Dacian and Gallic. Generally believed to be a later addition (Popa, 2010), the list nonetheless highlights the medicinal herbs of importance to the local regions of the empire as it generally excludes all exotic and cultivated plants, as well as animal and mineral treatments. The focus of the synonym list was likely to assist with identifying and sourcing local wild medicinal herbs in the regions outside of Greek and Latin tradition. A direct comparison between the plant lists that include geographically distinct Dacian and Gallic names may indicate a subset of native plants of high relevance to each area (**Table 1**). Several additional plants were also directly noted by Dioscorides as a part of the Iberian Celtic tradition, including eruca (*Erucastrum gallicum* Wild.) used as an aphrodisiac, and fennel gum (*Foeniculum vulgare* Mill.) as a more effective eye medicine than the plant juice. Finally, Celtic nard (*Valeriana celtica* L.) endemic to the Celtic Alps, was described as a tonic to the spleen, stomach, liver, and kidneys, that could be taken in wine with wormwood (*Artemisia absinthium* L.) as a "narcotic," likely meaning a painkiller. Dioscorides reported very few superstitious practices, including the use of anchusa (*Echium* sp.) charms against bites.

4.2 Pliny's Account of Gallic Druids (23-79 AD)

Pliny the Elder, a Roman author born in Como, Lombardy, described the Celtic healers, or druids as "magicians" and "priests" and talked of their fondness for plants (*Naturalis Historia,* Book XIV). Special attention was given to mistletoe (*Viscum album* L.), especially when grown upon the English oak (*Quercus robur* L.), as medicine and religious sacrament of the Celts. Pliny described other Celtic plants including *glastum* or dyer's woad (*Isatis tinctoria* L.) as a source of blue dye, the use of beech (*Fagus sylvatica* L.) ashes for reddish hair, hellebore (*Helleborus*

niger L.) as arrow poison, and *selago* similar to savin (*Huperzia selago* L.) for eye infections. Even though much of Pliny's material came from Theophrastus or from shared sources, he described several new herbs including *britannica*, a plant that grew on the islands off the Frisian coast and was used as a cure for scurvy, quinsy, and snake bites. This name could be possibly attributed to the extremophile English scurvygrass (*Cochlearia anglica* L.) rich in vitamin C (Nawaz et al., 2017) (**Table 1**).

The herbal practices of Hellenized Anatolia prioritized a rational approach to making and prescribing remedies, therefore deliberately avoiding magical formulas. Latin herbal writers, however, continuously mention magical cures in the form of spells, charms, and incantations, even while being hostile to these practices, as in the case of Pliny. Among 27 magical remedies listed in his work, only 4 could be attributed to Greek and Latin origin. One of them utilized reseda (*Reseda alba* L.) to treat inflammation and originated in the vicinity of a Roman colony Ariminum (Gaillard-Seux, 2014), in the area of Northern Italy held by Celtic tribes since the 6th century BC. The majority of other formulas, as attested by Pliny, were supplied by unknown *magi*. On the other hand, *Codex Ardmachanus*, a 9th century Irish manuscript written mainly in Latin, specifically applied this term to those who in the Irish tradition were called *Druadh* (Skene, 1886). It seems that Pliny's sources have been referring to Celtic *magi* (druids), and their magical formulas were incorporated into the Latin works by direct geographical proximity and societal overlap of Roman and Celtic cultures after 250 BC.

4.3. Marcus Cato – *De re rustica* (ca. 234-149 BC)

A separate branch of herbal knowledge rather independent from Theophrastus, Pliny, and Dioscorides works was initiated by Marcus Cato of Tusculum, known for his conservatism and

opposition to Hellenization. He recorded the folk knowledge related to the agricultural and herbal tradition of the Italic and Celtic tribes, including the use of offerings, charms, and incantations for healing practices. Cato advised on the use of cabbage (*Brassica oleracea* L.) to heal multiple inflammatory and gastrointestinal disorders, the use of urine collected after cabbage ingestion, and underlined the higher therapeutic potency of wild cabbage compared to its cultivated relatives.

4.4 Celsus – *De medicina* (ca. 25 BC-50 AD)

Cornelius Celsus was most likely associated with *Gallia Narbonensis*, which became a Roman province in 121 BC. This relationship was suggested based on his remarks about a very specific vine (*marcum*) which, according to Pliny, was native to Narbonese Gaul (Langslow, 2000), as well as close familiarity with Gallic hunting poisons and their practices to cauterize the blood vessels. The writings of Celsus were contemporary to Pliny and Dioscorides, and took place shortly before Claudius, the "Gallic" emperor, addressed the senate to allow Gallic aristocrats to enter the Roman senatorial class in 48 AD. This idea was abandoned after the Gallic revolts of 68-70 AD, and even Tacitus, a Gaul himself, believed that the oppression of northern Gauls was a necessary evil (Woolf, 2000). At the same time, the last major stronghold of Celtic druids at Anglesey, Wales was destroyed and brought to the Roman empire in 60-78 AD. Although Celsus provided an extensive description of using wild and pot-cultivated herbs for medicinal purposes, none of them is explicitly stated by him to be attributed to a Celtic tradition.

4.5 Scribonius Largus – *Compositiones* (ca. 1-60)

Scribonius Largus was believed to accompany Claudius on the British campaign of 43 AD and assembled his own version of herbal prescriptions similar to Cato and Celsus. His birthplace remained uncertain with contradicting sources pointing to Sicily or *Gallia Narbonensis*, and he clearly shared knowledge or some common sources with Celsus as evident from their descriptions of Theriaca and Mithridatic remedies (Baldwin, 1992). Following his predecessor's trends, Scribonius also preserved the superstitious and highly magical nature of many prescriptions, however unlike Celsus, he placed a strong emphasis on exact dosing of individual components, in addition to the general description of the herbal types and mixtures. Scribonius describes a variety of eye diseases which were among the most common afflictions in Gaul, and the use of the dissected ointment sticks (*collyria*) in preparation of an eye salve. This form of treatment (dry packaging in large batches) was rather unique to Gaul and the British Isles and could be possibly explained by limited and often difficult access to Eastern Mediterranean herbs required for these preparations (Baker, 2011).

4.6 Marcellus Empiricus – *De medicamentis liber* (ca. 395-410)

The Celtic magical formulas found in the writings of Pliny, Cato, Celsus, and Scribonius peaked in the *De medicamentis liber* of Marcellus Empiricus. For example, Marcellus advises certain plants to be collected with the waning of the moon, the use of iron forbidden in digging or cutting the plant, requiring certain plants to be collected with the left hand. This is highly similar to Pliny's description of *Samolus valerandi* or water pimpernel. The marsh dwelling plant was said to be gathered by a fasted druid with his left hand (Stannard, 1973).

In addition to relying on previous writers, Marcellus clearly stated that the bulk of his recipes came from the local population (*sed etiam ab agrestions et plebeis*). He listed 12 Celtic plant names, ten of which were accompanied by a Greek or Latin synonym. These plants included *baditis* (water lily, *Nymphaea alba* L.), *biricumus* (mugwort, *Artemisia vulgaris* L.), *calliomarcus* (colt's foot, *Tussilago farfara* L.)*, gigarus* (snake lily, *Dracunculus vulgaris* Schott)*, gilarus* (thyme, *Thymus serpyllum* L.), *odocos* (elder, *Sambucus ebulus* L.), *ratis* (ferns, *Pteridophyta),* and *visumarus* (clover, *Trifolium* sp.). The two plants without synonyms were tentatively identified as *vernetus* or *viridis* (alder, *Alnus* sp.) and *blutthagio* (buttercup, *Ranunculus* sp.) (Stannard, 1973) (**Table 1**). Turning to polypharmacy, most of his recipes contained 10-20 plant constituents, and magical formulas, charms, and incantations formed an intrinsic part of his therapeutic strategies.

Marcellus work marked the turning point in our direct knowledge of Celtic herbal tradition, as the druids left no original or surviving writings, and the contemporary Roman culture experienced a drastic social and political decline following the Antonine (165-180 AD), Cyprian (251-270 AD), and Justinian (541-542 AD) plagues that killed as much as 30-40% of the population in the affected areas, and devastated the Roman army. From here, we can only discuss the surviving Celtic herbal framework within the fringes of the British Isles (Wales, Scotland, Ireland and the Isle of Man).

5. *Meddygon Myddfai* in the Red Book of Hergest (shortly after 1382)

The demographic pressure from Germanic tribes along the Rhine and Danube frontiers, combined with Anglo-Saxon and Norman expansion into Brittany confined Celtic tribes to the western regions of the British Isles and Ireland. The advance of Christianity and associated

Crusades consolidated Western Europe as a unified Christian force, and it seems its medical tradition followed suit. The herbal texts of Medieval Europe were dogmatically transcribed and translated from existing works, mostly different versions of Dioscorides and simplified Pseudo-Apuleius. This also included a series of translations from Arabic sources (al-Razi, Ibn-Sina, Haly Abbas) by Constantine Africanus and other writers. Medical schools of Salerno and Padua also maintained the Graeco-Roman herbal tradition of the existing herbal manuscripts and their interpretations such as Odo Magdunensis (*Macer Floridus*), Matthaeus Platearius (*Circa instans*), John of Milano (*Regimen sanitatis*), Nicolai Salernitanus (*Antidotarium parvum*) and Rufinus (*Liber de virtutibus herbarum*) (**Figure 2**).

Away from the Mediterranean, the classical herbals and their translations were of ever decreasing relevance, which prompted a revision and fixation of a distinct and presumably independent vernacular herbal knowledge. It was the turn of the outskirts of Medieval Europe to develop their own herbals by synthesizing the classic sources and local tradition, as evident from reappearance of magic formulas, incantations, and vernacular plant names in these works. Three manuscripts that fall under this category include the 9th century Anglo-Saxon Bald's Leechbook (*Medicinale anglicum*), the 12th century Germanic *Physica* (*Liber simplicis medicinae*) by Hildegard of Bingen (1098-1179), and the 14th century Welsh *Meddygon Myddfai* as a part of the Red Book of Hergest (Bodleian MS 111, shortly after 1382). The latter was said to incorporate the healing tradition of Rhiwallon Feddyg, the physician to Rhys Gryg from the Cymry Celtic kingdom of Deheubarth (ca. 1234). The manuscript is divided in two parts: *Myddfai I* (recipes 1-188 considered original to 1382) and *Myddfai II* (recipes 189-815 allegedly drawn by Iolo Morganwg from the continental herbal tradition (Luft, 2018). Both *Bald's Leechbook* and *Meddygon Myddfai* seem to contain larger amounts of magical formulas and superstitious

treatments than the classical herbal manuscripts of Pliny, Dioscorides, and Galen, and more resemble the approach of Celsus, Scribonius Largus, and Marcellus Empiricus in incorporating magical elements and elaborate herbal preparations with multiple constituents. It is rather likely that these manuscripts shared common sources that to a large extent relied upon Marcellus Empiricus and Alexander of Tralles (*Alexandri yatros practica*) and appealed to personal experiences in discovering and testing the herbal remedies.

5.1 Organization of *Meddygon Myddfai*

In contrast to Dioscorides, whose treatments were organized by substances (earlier Greek and old Latin manuscripts), or revised into an alphabetical order (later Greek and Latin translations), *Myddfai I* did not follow an obvious organizational structure and seemed to represent a loosely collected list of recipes that was developed over time. This also differed from the *Bald's Leechbook*, which preserved the classical head-to-toe order. The botanical descriptions in *Myddfai I* were often significantly reduced or eliminated altogether, suggesting that the manuscript was developed as a quick reference guide of directions (steps) to be performed by a person versed in the herbal tradition and medicine, rather than a medicinal text to be read and interpreted by a medieval scholar. Straightforward passages such as "*for an illness.., take a number of plants.., crush them in a vehicle medium.., set aside.., have a patient use…*" differed drastically with other herbal collections, and did not intend to educate readers in the art of identification and collection of medicinal plants. Another two features that set *Myddfai I* aside from the contemporary and classical herbals were i) the use of numerous and diverse media for the preparation of herbal remedies, and ii) the emphasis on magic formulas, charms, and incantations. Both groupings garnered very little attention in past scholarly works, however they

are more likely to preserve the original references and vernacular knowledge of Welsh belief systems and practices as it applies to Celtic medicine as a whole.

Herbal preparation signatures can be easily evaluated in the herbal manuscripts by calculating the frequencies of certain vehicles (media) used to crush, dissolve, or extract plant botanical material during formulation of the remedies. As evident from Dioscorides, classical Greco-Roman herbal tradition relied heavily on using wine, vinegar, and oil in addition to water as media for preparation of plant remedies. This signature clearly survived in Pseudo-Apuleius (4th century) and its Old English translation (9th century) despite multiple scribes, edits, rearrangements, and translation of the recipes. The signature is still evident in the work of Marcellus Empiricus (however, his formulations show limited use of oil and increased use of ashes), and multiple manuscripts of Italian and German tradition such as works attributed to Petrocelli (9th century) or Hildegard (12th century). It is interesting that *Bald's Leechbook II* manuscript also falls under this category, while *Leechbooks I, III*, and the *Lacnunga* maintained sets of remedies that used a different, presumably Anglo-Saxon, herbal preparation signature based on an increased use of milk, butter, and beer (**Figure 3**). The *Myddfai I* signature generally follows a Mediterranean tradition with several exceptions such as a decreased use of wine, and an increased use of whey (not frequent in other manuscripts) and ashes (similar to Marcellus Empiricus). *Myddfai II*, in concordance with stipulation of being a recent 18th century compilation from multiple herbal sources, shows a mixed herbal preparation signature with multiple formulation media present at nearly equal frequencies, including an increased use of milk and beer typical to the Anglo-Saxon tradition and largely limited in *Myddfai I*. It would be very interesting to extend the analysis of the herbal preparation signatures to other herbal manuscripts from the European (Salerno, Montpellier, Padua) and Arabic tradition, as well as

different versions of the same manuscript scribed in the geographically distinct areas and time periods.

5.2 Plants of *Meddygon Myddfai I*

In an attempt to further distinguish the Welsh herbal tradition, we performed a systematic analysis of plant species and the target health conditions described in the *Myddfai I* manuscript (Oxford Jesus College MS 111) against herbal texts of various medical traditions (**Figure 2**). The correct identification of modern plant names in the text was challenging due to the cryptic nature of Middle Welsh, as well as possible mistakes during scribing and incorrect previous translations as noted in **Table 2**. Longer recipes such as 11, 12, and 19 contained the most errors in translation and corrupt phytonyms. For instance, common burnet (*Sanguisorba officinalis* L.) is not actually present in the Welsh text but is found in the English translations of recipe 19. Uncertain plant names of Welsh origin were further checked against Davies' *Welsh Botanology* (1813), Richard's *Antiquae linguae Britannicae thesaurus* (1815), and Pughe's *Dictionary of the Welsh Language* (1832). In some cases, the common name was too general to resolve, such a *redyn* for "fern" or *redegaȏc* for "liverwort". Plant names not found to match Dioscorides were also corroborated against other sources, including Dunbar's *A New Greek and English Lexicon* (1844) and the Natural History of Pliny.

Direct counts of plant species listed in these herbal manuscripts indicated that Macer Floridus *De viribus herbarum* shared the most extensive overlap with *Myddfai I* (out of 77 plant species found in Macer Floridus, 68 (88%) were also mentioned in *Myddfai I*) (**Figure 4**). Approximately 114 plant species overlapped between Dioscorides' *De Materia medica* and *Myddfai I* as well, and the former contributed a set of 51 plant species not found in Macer

Floridus to *Myddfai* I recipes. One more plant species (primrose, *Primula vulgaris* Huds.) was mentioned in *Regimen sanitatis*, thus bringing the total number of *Myddfai I* plant species of classic Mediterranean herbal tradition to 120. Of those, only 5-6 species had to be imported in dried form from the warmer regions, suggesting that the manuscript was intentionally developed to exclude the most exotic species due to cost and/or lack of availability. The remaining 9 plant species could be hypothetically allocated to the herbal medical tradition of the British Isles and Ireland. Three plant species were derived from the *Bald's Leechbook* or the common shared sources between the two manuscripts, including silver birch (*Betula pendula* Roth), woodruff (*Galium odoratum* L.), and fir clubmoss (*Huperzia selago* L.). The remaining 6 plants (**Table 3**) (and their various names,) which were not mentioned in any text but *Myddfai I,* are discussed briefly below.

5.2.1 Foxglove, *Digitalis purpurea* L. (Plantaginaceae) – *ffiol y ffrud*

The plant is native to west and west-central Europe, including the British Isles. Foxglove is likely the closest claim to success for the Celtic and subsequent Welsh herbal tradition, as the plant was historically used to treat dropsy (congenital heart disease) and the decoction of its leaves was introduced into medical practice by the Scottish doctor William Withering in 1775. Chemical analysis revealed that foxglove contains a number of cardiac glycosides, including digoxin and digitoxin, that inhibit the activity of the Na/K-ATPase in the myocardium (Kjeldsen and Bundgaard, 2003). In the Digitalis Investigation Group clinical study (n=6800), digoxin did not reduce overall mortality, but it reduced the rate of hospitalization both overall and for worsening heart failure (Digitalis Investigation Group, 1997). No CAM herbal supplements derived from foxglove are currently on the market due to safety and overdose concerns.

5.2.2 Corn bellflower, *Legousia speculum-veneris* Fisch. (Campanulacaeae) – *dyrcheigyuac*

Formerly known as *Campanula hybrida*, the plant is found in chalky cornfields of the British Isles and other parts of Europe. Due to its status as a weed, corn bellflower has been subjected to ecological and molecular phylogenetic studies. However there is no current scientific evidence of its medicinal properties and its bioactive principles remain to be elucidated. *Myddfai I* cited it as a remedy for quinsy or tonsillar abscess.

5.2.3 Self-heal, *Prunella vulgaris* L. (Lamiaceae) – *wennela6c, ueidya6c*

Although self-heal is common throughout Britain, Europe, Asia, and North America, its traditional use was generally restricted to Asia, where it forms a part of traditional Chinese medicine targeting liver function, thyroid swellings, and inflammation. Flowering spikes of this plant contain up to 6% rosmarinic acid, one of the highest sources of this ingredient among plants (Lamaison et al., 1991). Otherwise, the phytochemical complexity of the plant is rather unknown. As a part of the polyherbal formulation, selfheal was explored as a pain reliever in combination with celecoxib in 181 Korean patients with rheumatoid arthritis (Song et al., 2007).

5.2.4 Sharp dock, *Rumex conglomeratus* Murray (Polygonaceae) – *turth*

Sharp dock is another plant of a wider Eurasian distribution that was utilized for its medicinal properties mostly outside of the Western Europe. In Turkey, the plant locally known as *labada*, was used for treatment of purgative disorders and dysentery. The use of docks in Europe is predominantly restricted to the British Isles and the Carpathian basin (Vasas et al., 2015). The roots of docks are a rich source of anthraquinones and naphthalenes. *Myddfai I* recommends its use in treatment of pneumonia, which can be related to the antimicrobial

properties of dock plants (Orbán-Gyapai et al., 2017). There is some certainty that the Welsh text is referring to *R. conglomeratus* in specific, due to the fact that species within the genus *Rumex* had unique Welsh names. Dock (*Rumex*) in general is often referred to as *tafolen.* Species follow such, *R. sanguineus* (*tafolen goch*), *R. crispus* (*tafolen grych*), *R.acutus* (*tafolen mair*), etc. The Welsh name *turth* appears to only have been attributed to *R.conglomeratus.*

5.2.5 Water pimpernel, *Samolus valerandi* L. (Primulaceae) – *glaerllys*

The plant is found in a variety of wet habitats all over Europe and other parts of the world. Due to a unique cosmopolitan distribution, water pimpernel has been subjected to extensive molecular phylogenetic studies (Jones et al., 2012). However, there is no current scientific evidence of its medicinal properties; and its bioactive principles remain to be elucidated. The plant was also mentioned by Pliny in connection to druid practices, providing additional evidence for attribution of the water pimpernel to the Celtic and subsequent Welsh herbal tradition.

5.2.6 River startip, *Scapania undulata* L. (Scapaniaceae) – *gynglenyd, cynglenydd*

Moss-mimicking leafy liverworts are the second largest group of plants, and their traditional use for addressing abdominal and liver disorders stemmed from a magical Doctrine of Signatures that believed in beneficial effects of plants to heal certain organs and body parts by resembling their shapes (Pearce, 2008). Traditionally, they were used crushed or intact to alleviate bruises, burns, and wounds, and their biochemistry exhibits a wide range of biologically active compounds (Asakawa, 2008). Liverworts, including *Scapania undulata*, contain highly specialized oil bodies enriched with sesqui- and diterpenoids, many of which have not been

found in higher plants (Adio et al., 2004). *Scapania undulata* is the most common liverwort in South Wales and the name *cynglenydd* in the Welsh text is different than that of the generic *hepatica* (*redega*ɔ*c*), suggesting a more detailed knowledge of this particular species. However, it is possible that this refers to the entire genus *Scapania* This translation was corroborated by Davies, 1813.

5.3 Spiritual healing practices

While the medicine of Greece and Hellenized Anatolia made great strides to separate formulations, vehicles, and single herb preparations (simples) into a pharmacologically-relevant treatments, *Myddfai I* herbal preserved the tradition of Roman and Celtic writers to incorporate magical formulas, scoring charms, evil spirits, and incantations into their healing practices. The reason could be argued that a later and often incomplete conversion to Christianity at the Celtic fringes of the British Isles preserved syncretism with pagan rituals including recognition of the fourfold principle, the luck of white cattle, eels, and roosters, lucky and unlucky days, as well as the legend of the Lady of the Lake – in which the authors described the way by which they received healing powers – from what was essentially a water spirit.

Many herbal preparations also preserved the significance of numbers 3 and 9 in selection of ingredients, number of manipulations, and frequency of dosing – often a medicine made of 3 components was taken thrice daily for 9 days. The number 3 (and thus 9=3x3) was significant in Celtic religion prior to Christianity and in syncretic Celtic Christianity (Arthurian legends), symbolizing the Trinity. However, the Trinity itself predates Christianity in Indo-European religions including Roman, Celtic, Germanic, and Hindu polytheism and may be supported by the trifunctional hypothesis of Dumezil. Triple deities such as the Matronae (Gallic), the

Morrigan (Irish), and Brigid (Irish), are prominent examples of the significance of three in Celtic culture along with symbols such as the triskele. In *Tain Bo Cuailnge,* a 12th century Irish legend, the Morrigan appears in battle as three animals (an eel, a wolf, and a white cow), sustains three wounds, and is cured by three drinks of milk. Welsh mythology similarly abounds with the appearance of the number three: the deity Llyr has three children, and there are often three demons or plagues found in myths. The theme of threefold death is found in the Welsh *Myrddin Wyllt,* and this concept may be supported by the remains of the *ca.* 1st century AD Lindow bog body which sustained a hanging, head wound, and cut throat and was even found with mistletoe pollen grains in his stomach (Hutton, 2011). Interestingly, Marcellus Empiricus references Esus in part of a remedy for treating throat infections, a Gallic god worshipped also in Roman Britain. Esus is part of the triple god entity containing Teutates and Taranis, documented by the Roman poet Lucan in the 1st century AD, as well as depicted on the Pillar of the Boatman (along with a sacred bull, Tarvos Trigaranus).

A distinctive Celtic ethnographic framework of traditional healing beliefs and practices also survived in part with local population and a network of hereditary scholarly physicians of the Western Isles of Scotland (i.e. Macleans in Skye, O'Conachers in Argyll, Beatons in Islay and Mull) (Anonymous, 1906). A peculiar glimpse of these practices could be found in Martin Martin's *A description of the Western Islands of Scotland* (ca 1695). Neil Beaton of Skye was said to treat "*Lilium Medicinæ, and some other practical pieces that he has heard of, with contempt. The success attending his cures was so extraordinary that people thought his performances to have proceeded rather from a compact with the devil, than from the virtue of simples. To obviate this, he pretends to have had some education from his father, though he died when he hurnself was but a boy.*" A more contemporary account of a direct oral transmission of

herbal tradition of the Western Isles can be also found in the Maclagan Manuscripts (1892-1903) collected throughout the Highlands and the Inner Hebrides (Turner, 2014). More of this knowledge was also captured in the recent written records of Mary Beith (*Healing Threads*, 1995), Tess Darwin (The Scots Herbal, 1996), and Bridgewater & Milligan (Flora Celtica, 2004).

Contrary to magic formulas, charms, and incantations which may exert beneficial results on human body by affecting the neurobiological mechanisms of individual expectations and psychosocial placebo effects (Benedetti et al., 2005), herbal remedies fall within a category of evidence-based scientific research that can be applied to rigorously test the efficacy and safety of plant preparations, validate their traditional use, and provide novel biochemical leads to the existing drug development pipelines (Palatini and Komarnytsky, 2018). The herbal potential of medieval plant collections to fight microbial infections was successfully explored both for *Bald's Leechbook* (Harrison et al., 2015) and *Myddfai I* (Wagner et al., 2017), with the latter work also identifying additional plants effective against microbes from the modern tradition of the Scottish School of Herbal Medicine, including the leaves of the endemic Arran whitebeam (*Sorbus arranensis* Hedl.) These findings, however, only further strengthen the critical need for correct translations, botanical identification of vernacular and old language plant names, understanding changes in formulation, biochemical composition, and efficacy of medicinal plants that varies tremendously based on plant species, environmental location, and tissues used in the preparation.

6. Conclusions

The Celtic fringe flora of the British Isles subsequent to the last glaciation shares many common species with Scandinavian plants (those that likely survived glaciation in the southern or mountaintop refuges), and the incomers from the Germanic and Mediterranean flora that

spread over the land bridges that existed at the time (Willis, 1995). As such, it is very difficult to differentiate between local versus continental herbal tradition that both share a very similar sets of plants. Westwards migrations of the Indo-European R1b tribes along the Danube and Rhine basins, superimposed on gathered plants automorphies found in the early settlements, clearly suggesting that the herbal knowledge of what later became Hellenized Anatolia was likely transferred to Central Europe and beyond, and this transfer coincided with the expansion of Celtic tribes. Appearance of some other plants of medicinal value was linked to later Roman introductions as in case with the greater celandine (*Chelidonium majus* L.) (Zielińska et al., 2018).

Traces of Celtic framework of traditional herbal remedies can still be found in classical and medieval herbal collections largely dominated by Mediterranean plants, as was shown for the foxglove (*Digitalis purpurea* L.) and several other plants. While the extent to which the later Welsh 14th century text (*Myddfai I*) manifest an earlier oral tradition is uncertain due to overlaps with the classical writers, later scribes, additions, and even forgeries of the manuscript (*Myddfai II*), as well as dubious translations of plant identities and human diseases. Recipes from *Myddfai I*, however, carry distinct herbal preparation signatures (use frequencies of certain formulations and vehicles), which are vastly different from earlier Graeco-Roman and contemporary Anglo-Saxon and Germanic traditions. The old practices also persisted in the Celtic fringes of the British Isles (Wales, Scotland, Ireland, and the Isle of Man) till very recently, but were rarely incorporated into the written tradition until the late 18-19th centuries. Consequently, many herbal remedies remained an integral part of prevention and treatment of human diseases until modern times, some were rediscovered as botanical drugs and incorporated into pharmacopoeias, while

the other neglected recipes can be further explored for novel interactions and pharmacological applications.

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8. Tables and Figures

Table 1. List of medicinal plants and their vernacular names from Celtic or Gallic herbal tradition, as compared to the Dacian plants of Dioscorides.

Botanical name	English name	Celtic (Gallic) name			Dacian name
		Dioscorides	Pliny	Marcellus	Dioscorides
Achillea millefolium L.	yarrow	beliucandas			diodela
Acorus calamus L.	sweet flag	piper apum			
Adianthum sp.	maidenhair fern				phithophthethela
Ajuga iva L.	bugle				dokela
Alnus sp.	alder			vernetus	
Amaranthus blitum L.	purple amaranth				bles
Anagallis sp.	pimpernel				kerker
Anchusa italica Retz.	anchusa				budalla
Anethum graveolens L.	dill				polpum
Arctium sp.	burdock				riborasta
Aristolochia rotunda L.	round birthwort	theximon			
Artemisia absinthium L.	wormwood				zeste
Artemisia scoparia Waldst	redstem wormwood				zired
Artemisia vulgaris L.	mugwort	ponem		biricumus	
Arum maculatum L.	arum				kourionnecum
Asarum europaeum L.	asarabacca	baccar			
Aster amellus L.	italian aster				rathibida
Atropa belladonna L.	nightshade				koikolida
Bryonia alba L.	white bryony				dicotella
Centaurium erythraea Rafn	centaury				stirsozila
Chelidonium majus L.	greater celandine	thona			koustane
Cochlearia anglica L.	engl. scurvygrass		britannica		
Conium maculatum L.	poison hemlock				zena
Lagenaria siceraria Molina	gourd				kinouboila
Cynodon sp.	dog's tooth grass				parithia
Cynoglossum sp.	hound's tongue				azila
Daphne laureola L.	spurge laurel	ousubim			
Daphne mezereum L.	daphne	ousubim			
Dioscorea communis L.	black bryony				priadela
Dipsacus sylvestris L.	wild teasel				skiare
Dracunculus vulgaris Schott	dragon lily			gigarus	
Eryngium campestre L.	eryngo				sikupnoex
Erucastrum gallicum Willd.	eruca	unknown			
Fagus sylvatica L.	beech		unknown		
Ficaria verna Huds.	lesser celandine				ebustrone
Filix	fern			ratis	
Foeniculum vulgare Mill.	fennel	sistrameor			
Geranium sylvaticum L.	cranesbill				aurumetti
Gnaphalium sp.	cudweed	gdasonen			
Hedera helix L.	climbing ivy	subites			arborria
Helleborus niger L.	black hellebore	laginum	unknown		prodiarna
Hyoscyamus niger L.	henbane	bilinuntiam			dielina
Hypericum hircunum L.	stinking tutsan				salia
Iris foetidissima L.	gladwin iris				aprus
Isatis tinctorial L.	dyer's woad		glastum		
Juniperus communis L.	juniper	jupicellusum			
Limonium sp.	sea lavender	iumbarum			

Table 1 continued.

Lithospermum tenuiflorum Lf.	gromwell				gonoleta
Lycopodium clavatum L.	ground pine				kodela
Huperzia selago L.	clubmoss		selago		
Matricaria recutita L.	chamomile	lusta			amalusta
Melissa officanlis L.	lemon balm	merisimorion			
Mentha pulegium L.	pennyroyal	albolon			
Mentha sylvestris L.	horsemint				teudila
Nepeta sp.	catmint				tanidila
Nymphaea alba L.	water lily			baditis	
Onobrychis caput-galii Born	sainfoin				aniarsexe
Panicum dactylon L.	switchgrass				kotiata
Papaver argemone L.	prickly poppy	corna			
Persicaria bistorta L.	bistort				adila
Plantago major L.	plantain	tarbidolopion			skinpoax
Polypodium vulgare L.	polypody				karopithla
Portulaca oleracea L.	pulsane				lax
Potamogeton compressus L.	pondweed				koadama
Potentilla reptans L.	creeping cinquefoil	pempedula			probedula
Quercus robur L.	oak		unknown		
Ranunculus sp.	buttercup			blutthagio	
Rosmarinus officinalis L.	rosemary				dracontos
Rubus canescens DC.	woolly blackberry				manteia
Salvia horminum L.	annual clary				hormea
Sambucus ebulus L.	dwarf elder	ducone		odocos	olma
Sambucus nigra L.	elderberry	scobie			seba
Samolus valerandi L.	water pimpernel		samolus		
Solanum nigrum L.	black nightshade	scubulum			
Thymus sp.	thyme			gilarus	mizeia
Trifolium sp.	clover			visumarus	
Tussilago farfara L.	coltsfoot			calliomarcus	asa
Urtica dioica L.	nettle				dyn
Valeriana celtica L.	celtic nard	unknown			
Verbascum sp.	mullein				diesapter
Veronica officinalis L.	speedwell	sapana			
Viscum album L.	mistletoe		unknown		
Withania somnifera L.	groundcherry				kykolis

Table 2. The list of *Myddfai I* plants and their health indications (recipes 1-188).

Latin name	English name	Middle Welsh name (*MM*)	Recipe(s)	Indication
Achillea millefolium L.	milfoil	uilffȯth	12, 16, 19, 121, 127, 132, 133, 136	fever, kidney stones, vomiting blood, worms, epistaxis, vomiting
Agrimonia eupatoria L.	agrimony	trydon, tryȯ	11, 12, 19, 20	pneumonia, fever, kidney stones, fertility
Agrostemma githago L.	corn cockle	tenteulys[1] uendigeit	11	pneumonia
Allium ampeloprasum L.	leek	gennyn, kenin	100, 135	dog bite, vomiting of blood, fertility, snake bite, ulcers, whooping cough, pneumonia, deafness, headache, bone healing, boils, increase strength, flatulency, worms
Allium sativum L, (var. *ophioscorodon* (Link) Döll)	garlic (and viper's garlic)	craf, garllec	24, 56, 96, 97, 138	wounds, prevent fatigue, swelling, pain, epistaxis, proud flesh
Allium ursinum L.	ramsons	craf y geiuyr	53	abdominal complaints
Anagallis arvensis L.	pimpernel	diwythyl	15, 17, 20, 21, 53	fever, abdominal complaints, fertility, menorrhagia
Apium graveolens L.	smallage (celery)	ysmalaes, apiȯm	116, 124, 128	smallpox, calming, ague
Aquilegia vulgaris L.	columbine	columbina	19,	kidney stones
Arctium minus Hill	small burdock	kyngaȯ man	12, 21	fever, menorrhagia
Aristolochia rotunda L.	round birthwort	hennllydan	11, 12, 42, 43, 45, 51	pneumonia, fever, toothache, inflammation, snake bite
Artemisia abrotanum L.	southernwood	brytȯn	102, 124	insanity, palsy
Artemisia absinthium L.	wormwood	wermot	12, 13, 65, 120, 128	fever, destroy fleas, general wellness, snake bite, ague
Artemisia dracunulus L.	taragon	dragrans	126	worms
Artemisia vulgaris L.	mugwort	gannwreid	12, 15, 25, 38, 51, 56, 66, 110, 111, 112, 128, 144	fever, worms, carbuncle, prevent fatigue, destroy flies, snake bite, difficult childbirth (ritual), ague
Asarum europaeum L.	asarabacca	alannon	11	pneumonia
Asplenium scolopendrium L.	hart's tongue	dauot yr hydd	60, 78	anaphrodisiac, general wellness
Avena sativa L.	oats	keirch	13, 53	poultice, dietary
Bellis perennis L.	daisy	hygat y dyd	11, 12, 64, 102, 161	pneumonia, fever, worts, insanity, tumor
Betula pendula Roth	birch	uedlȯyn	19, 50	kidney stones, impotency,
Borago officinalis L.	borage	glessyn	15, 17, 19	fever, abdominal complaints, kidney stones
Brassica oleracea L.	cabbage (red and green)	caȯl (coch)	12, 99	fever, nettle rash
Brassica rapa L.	turnips	eruin	108, 117	worms, surfeit?
Calluna vulgaris L.	heath	gruc	11, 12, 116	pneumonia, smallpox
Cannabis sativa L.	hemp	kywarch	12	fever
Capsella bursa-pastoris L.	shepherd's purse	phȯrs y bugeil	166	heartache
Carum carvi L.	caraway	garaȯyt, karaȯn, Iarderv	11, 166	pneumonia, heartache
Centaurea nigra L.	knapweed	benngalet	12, 38, 45	fever, carbuncle, snake bite
Centaurium erythraea L.	centaury	yscaȯl crist, centaȯrya[1]	12, 114, 115	fever, kidney pains, extreme thirst

Table 2 continued.

Chelidonium majus L.	celandine	llyssey y wennol	170	eye problems
Chenopodium album L.	goosefoot	roec, dudren	21, 166	menorrhagia, heartache
Cirsium vulgare Savi	common thistle	ysgall	122	constipation
Conium maculata L.	hemlock	kygget	11	pneumonia
Conopodium majus Gouan	earthnut	bywi	12, 15, 17	fever, abdominal complaints
Coriandrum sativum L.	coriander	koliandrǒm	128	ague
Corylus avellana L.	hazel	coll	186	cleaning teeth
Crataegus monogyna Jacq	white thorn	yspydat	36	worms
Crocus sativus L.	saffron	saffyr	57, 58, 146	remove drunkenness, induce happiness
Datura stramonium L.	thorn apple (dorycnion)	vennwen	42	toothache
Digitalis purpurea L.	foxglove	ffiol y ffrud	53, 74	abdominal complaints, tumors
Empetrum nigrum L.	crake berry	grygyon	11, 12	pneumonia, fever
Eryngium maritimum L.	eringo	mor gelyn	52	toothache
Eupatorium cannabinum L.	hemp agrimony	vedun chwerǒ	56, 78, 107	prevent drunkenness, general wellness, cough
Ficus carica L.	figs	ffigys	137	poison
Filipendula ulmaria L.	meadow sweet	erchǒreid, uedlys[1]	40	pneumonia, fever, kidney stones, haemorrhage
Filix	fern	redyn	98, 155	burns, hemorrhoids
Foeniculum vulgare Mill	fennel	ffenigyl	15, 79, 96, 100, 120, 123, 170	fever, general wellness, swelling, pain, dog bite, snake bite, digestion, eye problems
Fragaria vesca L.	strawberry	syui	10	eye problems
Fraxinus excelsior L.	ash	onn	31	deafness
Fumaria officinalis L.	fumitory	mǒc y dayar	13	fever
Galium odoratum L.	woodruff	udrot	11, 12, 79	pneumonia
Galium verum L.	yellow bed straw	kylyon, keulon	35, 162	spider bite, swelling
Geranium robertanium L.	herb Robert	troet rud	11	pneumonia
Geum urbanum L.	avens	uab coll	11, 12, 21, 41	pneumonia, menorrhagia, hoarseness
Glechoma hederacea L.	ground ivy	ganwreid rydegaǒc, eido y dayar, eidral[1]	15, 19, 72, 100, 129, 145	fever, kidney stones, eye problems, dog bite, ague
Hedera helix L.	ivy	eidorǒc	29, 30, 98	skin problems, toothache, burns
Helleborus foetidus L.	stinking hellebore	hylithyr	17	abdominal complaints
Hepatica	liverwort	redegaǒc	17, 19	abdominal complaints, kidney stones
Hordeum vulgare L.	barley	heid	33, 111, 163, 168	plaster, dietary, worms, boils,
Huperzia selago L.	fir clubmoss	tharǒ y mynyd	20	fertility
Hypericum androsaemum L.	tutsan	greulys (uendigeit) [1]	12 (English only), 15, 19, 95, 101	fever, kidney stones, inflammation
Hypericum perforatum L.	St. John's wort	erinllis	12, 19, 20, 38, 41	fever, kidney stones, fertility, carbuncle, hoarseness
Juglans regia L.	walnut	coll ffrenghic	36	worms
Knautia arvensis L.	field scabious	bennlas	12, 45	fever, snake bite
Lactuca sp.	lettuce	gǒylaeth, letus	80, 157	general wellness, fertility
Lamium purpureum L.	red nettle	dyna coch	12, 38, 51, 164	fever, carbuncle, strangury
Laurus nobilis L.	bay	dodeit	17, 19	abdominal complaints, kidney stones

Table 2 continued.

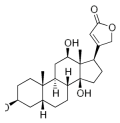
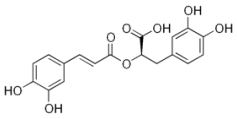
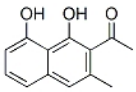
Legousia speculum-veneris Fisch	corn bell flower	drycheigyauc	22	quinsey
Lemna sp.	duckweed	linat	94, 103	abdominal complaints, constipation
Leonurus cardiaca L.	motherwort	vamyls	19	kidney stones
Lepidium latifolium L.	pepperwort	bybyrllys[1]	12	fever
Ligustrum vulgare L.	privet	rysswyd	12	fever
Lilium candidum L.	white lily	liliɷm gɷynn	98, 118	burns
Linum usitatissimum L.	linseed, flax	llin	7, 19	head wound, wounds
Lithospermum sp.	gromwel	grɷmyn	19, 151	kidney stones
Lonicera caprifolium L.	honeysuckle	gɷydwyd	30	toothache
Malus domestica Borkh.	apple	aualeu, aual	14, 15, 55	aprient, ritual
Malva sylvestris L.	mallows	hokys	15, 17, 59, 143	fever, haemorrhoids, abdominal complaints, general wellness
Matricaria sp.	chamomile	amrannwen	39, 125	wounds, reptiles in stomach
Melissa officinalis L.	lemon balm	gɷenyn	116	smallpox
Melittis melissophylum L.	bastard balm	wenenllys uan	12, 21	fever, menorrhagia
Muscus	moss	misyc, unsyc	15, 17	fever, abdominal complaints
Myrica gale L.	sweet gale	vrine	17	abdominal complaints
Origanum dictamnus L.	dittany	ditaen	126, 131, 160	worms, poison, pain
Oxalis acetosella L.	wood sorrel	suryon y coet	15, 34	fever, headache, joint pain,
Papaver somniferum L.	poppy	pabi	49	sleep
Persicaria amphibia L.	amphibious persicaria	granwreid benngoch	12, 15, 19, 20	fever, kidney stones, fertility
Petroselinum crispum Mill.	parlsey	persli	164	strangury
Pimpinella anisum L.	anise	ennyd	11	pneumonia
Piper nigrum L.	pepper (white and black)	pybyr	82, 100, 128, 138, 151, 157	general wellness, ague, proud flesh, kidney stones, fertility, bone healing
Plantago major L.	plantago major	erllyrat, plantaen	110, 127, 128, 161, 162, 163, 166	snake bite, worms, ague, tumor, swelling, boils, heartache
Potentilla reptans L.	creeping cinquefoil	ganwreid uelen	20	fertility
Primula vulgaris Huds.	primrose	briallu	68	loss of reason or speech
Prunella vulgaris L.	self-heal	wennelaɷc, ueidyaɷc	38, 42	carbuncle, toothache
Prunus persica L.	peach	persig	108	worms
Prunus spinosa L.	blackthorn	eiryn	167	dyspepsia
Quercus sp.	oak	derwhyden, keginderw	29, 166	skin problems, heartache
Ranunculus ficaria L.	lesser celandine	celidonia	96, 170	swelling, pain, eye problems
Raphanus sativus L.	radish	raphan, (r)hadigyl	120, 156	snake bite, dog bite
Rubia peregrina L.	little field madder	wreidrɷd lɷyt	19	kidney stones
Rubia tinctorum L.	madder	wreidrɷd	11,12	pneumonia, fever
Rumex conglomeratus Murray	sharp dock	turth, twrch	11	pneumonia
Rumex spp.	docken	tuaol	26, 42	abscess, toothache
Ruscus aculeatus L.	butcher's broom	iewydd, ieutaɷt[1]	12, 15	fever
Ruta graveolens L.	rue	rut	110, 111, 113, 120, 128, 137, 160	snake bite, worms, swelling, pain, ague, poisoning, anaphrodisiac
Salix sp.	willow	helic	165	warts

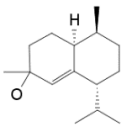
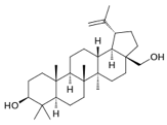
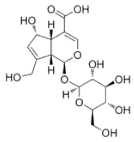
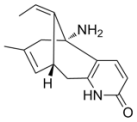
Table 2 continued.

Salvia officinalis L.	sage	saluia	102, 138	insanity, proud flesh
Salvia sclarea L.	clary clary	llygeit crist	59	anaphrodisiac
Sambucus ebulus L.	dwarf elder	gruelys war/uaɷr	12, 15	fever
Sambucus nigra L.	elder	ysgaɷ	15, 16, 36, 67	fever, haemorrhoids, worms, snake bite
Samolus valerandi L.	water pimpernel	glaerllys	19	kidney stones
Saxifraga granulata L.	saxifrage	saxifraga (tormaen)	151	kidney stones
Scandix pecten-veneris L.	shepherd's needle	greithic	11 ,24	pneumonia, wounds
Scapania undulata L.	river startip	gynglenyd	17, 106	abdominal complaints, liver disease
Secale cereale L.	rye	ryc	107, 138, 162	cough
Sedum telephium L.	orpine	ganwein	12, 20, 21	fever, fertility, menorrhagia
Sinapis alba L.	mustard	mɷstart	139	expel cold humors, snake bites, toothache, menorrhagia, digestion, colic, hair loss, tympanitis, dimness of sight, skin problems, palsy
Solanum dulcamara L.	bittersweet nightshade	elinyaɷc	36	worms
Solanum nigrum L.	black nightshade	morella	163	boils
Stachys officianlis L.	betony	dannaɷc, danhogen	5, 12, 19, 34, 42, 105, 121, 130	head wound, fever, kidney stones, headache, joint pain, toothache, epistaxis, vomiting blood, vomiting
Symphytum officinale L.	comfrey	chwefyrdan, consolida maior	26, 169	abscess, bone healing
Taraxacum officinale L.	dandelion	deint ylleɷ	13, 19, 23, 34	fever, kidney stones, exfoliation of the skull, headache, joint pain
Taxus baccata L.	yew	iaɷn	20	fertility
Tragopogon dubius Scop.	yellow goat's beard	gɷreid yr erweint	11, 12, 20	pneumonia
Triticum spp.	wheat	gɷenith	13, 34, 61	plaster, dietary
Urtica dioica L.	nettles	dynhaden	132	epistaxis
Valeriana officinalis L.	valerian	llysseu cadɷgaɷn	26	abscess
Veronica officinalis L.	speedwell	ieudaɷt	17, 38	abdominal complaints, carbuncle
Viola odorata L.	violet	violet, uiolet	5, 7, 55, 149	head wound, ritual

[1] Translation of plant names in recipes 11, 12, 15, 19 is corrupted.
- *Tenteulys uendigeit* (*Agrostemma githago* L.) could be a corruption of *greulys fendigaid* for tutsan (Hypericum androsaemum L.)
- *Yscaɷl crist* (*Centaurium erythraea L.)* could also indicate St. John's wort (*Hypericum perforatum* L.)
- *Erchwreid/orchwreid* (*Filipendula ulmaria* L.) (11,12,19) could be a corruption of *olchwraidd* (Sanicula europea L.) *Uedlys* (40) is correct
- *Ganwreid rydegaɷc* (*Glechoma hederacea* L.) (15) is actually creeping persicaria (*Persicaria* sp.); *eidral* (100) could indicate *Hedera helix; eido y dayar* is correct
- *Twrch* (*Hypericum androsaemum* L) (12) is likely sharp dock (*Rumex conglomeratus* Murray)
- *Bybyrllys* (*Lepidium latifolium* L.) is an incorrect translation (12) to peppermint
- Ieudaɷt (*Ruscus aculeatus* L.) is actually speedwell (*Veronica officinalis* L.)

Table 3. *Myddfai I* plants hypothetically original to the herbal tradition of British Isles

Tradition	Welsh	Welsh	Welsh	Welsh
Botanical print				
Latin name	*Digitalis purpurea* L.	*Legousia speculum-veneris* Fisch.	*Prunella vulgaris* L.	*Rumex conglomeratus* Murray
Common name	Foxglove	Corn bellflower	Self-heal	Sharp dock
Welsh name	*ffiol y ffud*	*drycheigyauc*	*wennelaǒc, ueidyaǒc*	*turth, twrch*
Indication(s)	Abscess, Heart	Quinsy	Carbuncle, Toothache	Pneumonia
Bioactive(s)	digoxin	unknown	rosmarinic acid	musizin
				

Welsh	Welsh	Anglo-Saxon	Anglo-Saxon	Anglo-Saxon
				
Samolus valerandi L.	*Scapania undulata* L.	*Betula pendula* Roth	*Galium odoratum* L.	*Huperzia selago* L.
Water pimpernel	River startip	Birch	Woodruff	Fir clubmoss
glaerllys	*gynglenyd*	*uedlǒyn*	*udrot*	*tharǒy mynyd*
Kidney stones	Abdominal, liver Scapanol	Urinary Betulin	Pneumonia Asperulosidic acid	Fertility Huperzine A
Unknown				

Supplementary Table 1. List of distinctive species gains or losses (autapomorphies) that defined the finds of wild gathered plants among the Neolithic settlements in Europe, with modifications after (Coward et al., 2008).

(1) Mediterranean coastal routes						
Southernmost Greece	**Sicily**	**Southern Italy**	**Portugal, Spain**	**Scotland**	**Ireland**	**Southern Britain**
Crete	Impressed ware, Stentinello	Impressed ware, Serra d'Alto, Diana, Lagnano da Piede	Impressed ware, Cardial, Chassey	Early	Early	Early
+Rumex sanguineus L.	*−Hordeum vulgare var. nudum* L.	*−Vicia ervilia* L.	***+Papaver somniferum* L.** *+Pisum sativum* L.	*−Lens culinaris* Medik. *−Triticum monococcum* L.	*−Hordeum vulgare var. nudum* L. *−Triticum moncoccum* L.	*+Arrhenatherum elatius* L. *−Asperula arvensis* L. *−Chenopodium hybridum* L. *−Echinochloa crus-galli* L. *−Galium spurium* L. *−Lens culinaris* Medik. *−Pisum sativum* L. *+Plantago major* L. *+Poa trivialis* L. *+Polygonum arenastrum* Boreau *+Prunella vulgaris* L. *−Rumex crispus* L. *+Urtica urens* L.
(2) Danube and Rhine valley routes						
Thessalian Greece	**Bulgaria Macedonia**	**Central Germany**	**Western Germany**	**Northern Italy**	**Austria, Western Carpathians**	**North France Benelux**
Sesklo	Karanovo, Starcevo	LBK	LBK	Square-mouthed pottery, Fagnigola, Impressed ware, Catignano	Eastern LBK, Koros	LBK, Swifterbant, Rubane, Group de Blicquy, Cerny

Supplementary Table 1 continued.

+*Agrostemma githago* L. +*Ajuga chamaepitys* L. +*Bromus secalinus* L. +*Cicer arietinum* L. +*Coriandrum sativum* L. +*Galium aparine* L. +*Galium spurium* L. +*Linum usitatissimum* L. +*Lolium temulentum* L. **+*Panicum miliaceum* L.** +*Portulaca oleracea* L. **+*Verbena officinalis* L.**	+*Adonis flammea* Jacq. **+*Agrimonia eupatoria* L.** +*Ajuga chamaepitys* L. +*Anagallis arvensis* L. +*Aphanes arvensis* L. **+*Atropa belladonna* L.** +*Bromus arvensis* L. +*Chenopodium murale* L. +*Chenopodium polyspermum* L. +*Cicer arietinum* L. +*Convolvulus arvensis* L. **+*Fragaria vesca* L.** +*Galium mollugo* L. +*Hibiscus trionum* L. **+*Hyoscyamus niger* L.** +*Lathyrus sativus* L. **+*Plantago lanceolate* L.** +*Poa annua* L. −*Polygonum lapathifolium* L. +*Polygonum minus* Huds. +*Portulaca oleracea* L. +*Rubus fruticosus* L. +*Rumex crispus* L. **+*Verbena officinalis* L.** +*Veronica hederifolia* L. +*Vicia ervilia* L. +*Vicia tetrasperma* L.	**+*Agrimonia eupatoria* L.** +*Asperula arvensis* L. +*Bromus arvensis* L. +*Chenopodium hybridum* L. +*Euphorbia helioscopia* L. **+*Hyoscyamus niger* L.** **+*Panicum miliaceum* L.** **+*Plantago lanceolate* L.** +*Plantago major* L. +*Polygonum aviculare* L. −*Polygonum persicaria* L. +*Rumex acetosella* L. +*Vicia ervilia* L. **+*Vicia faba* L.** +*Vicia tetrasperma* L.	**+*Agrimonia eupatoria* L.** +*Bromus arvensis* L. +*Euphorbia helioscopia* L. **+*Hyoscyamus niger* L.** **+*Panicum miliaceum* L.** **+*Plantago lanceolate* L.** +*Plantago major* L. +*Polygonum amphibium* L. −*Polygonum persicaria* L. +*Rumex acetosella* L. +*Vicia ervilia* L. **+*Vicia faba* L.** +*Vicia tetrasperma* L.	+*Agrostemma githago* L. **+*Fragaria vesca* L.** +*Linum usitatissimum* L. **+*Papaver somniferum* L.** +*Polygonum convolvulus* L. +*Polygonum persicaria* L. +*Portulaca oleracea* L. +*Rubus fruticosus* L.	+*Chenopodium album* L. +*Galium tricornutum* Dandy **+*Panicum miliaceum* L.** +*Polygonum convolvulus* L.	−*Anagallis arvensis* L. −*Arrhenatherum elatius* L. +*Chenopodium ficifolium* Sm. −*Papaver argemone* L. −*Phleum pretense* L. −*Poa trivialis* L. +*Polygonum amphibium* L. −*Polygonum arenastrum* Boreau −*Prunella vulgaris* L. −*Rubus fruticosus* L. −*Veronica hederifolia* L.

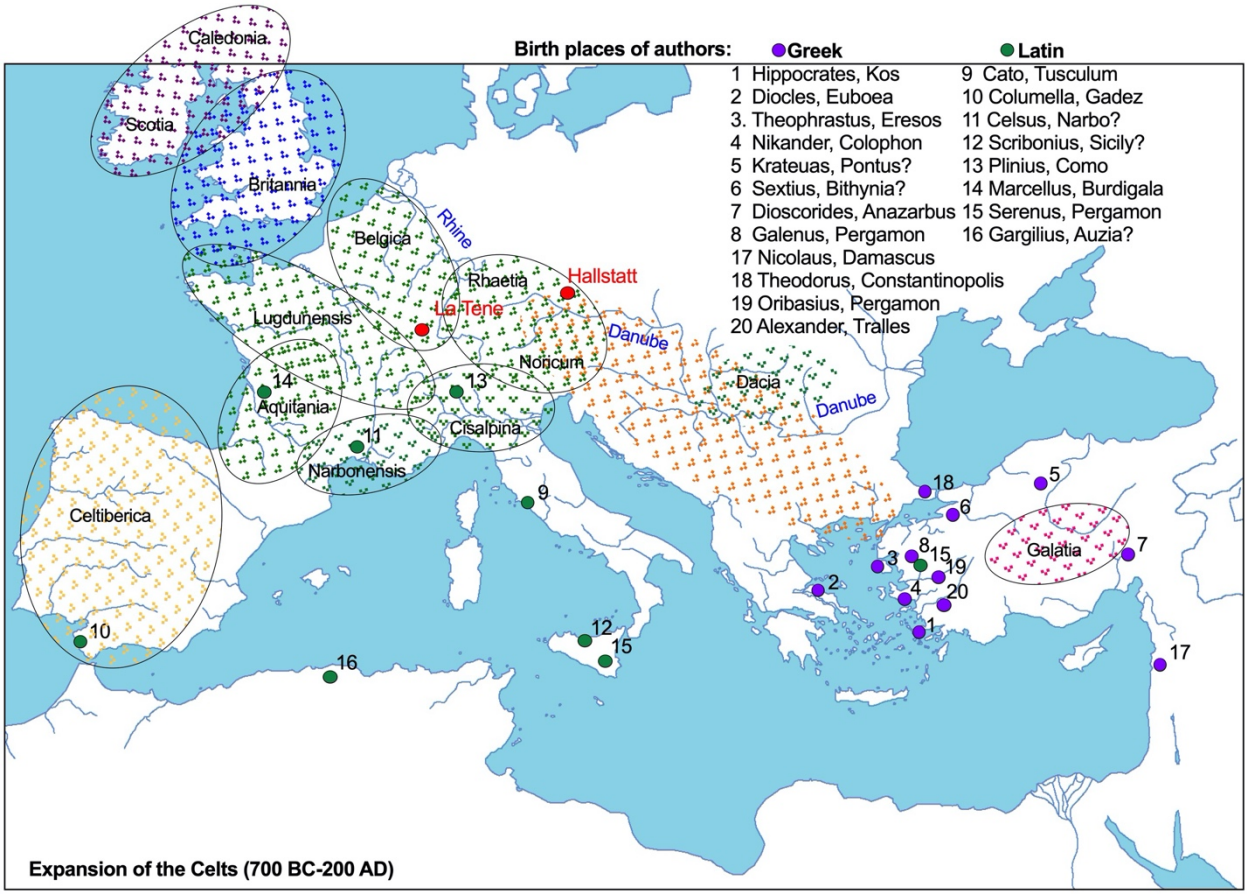


Figure 1. Birth places of classical medical writers in the Celtic world (700 BC-200 AD). Major spheres of Celtic influence are circled.

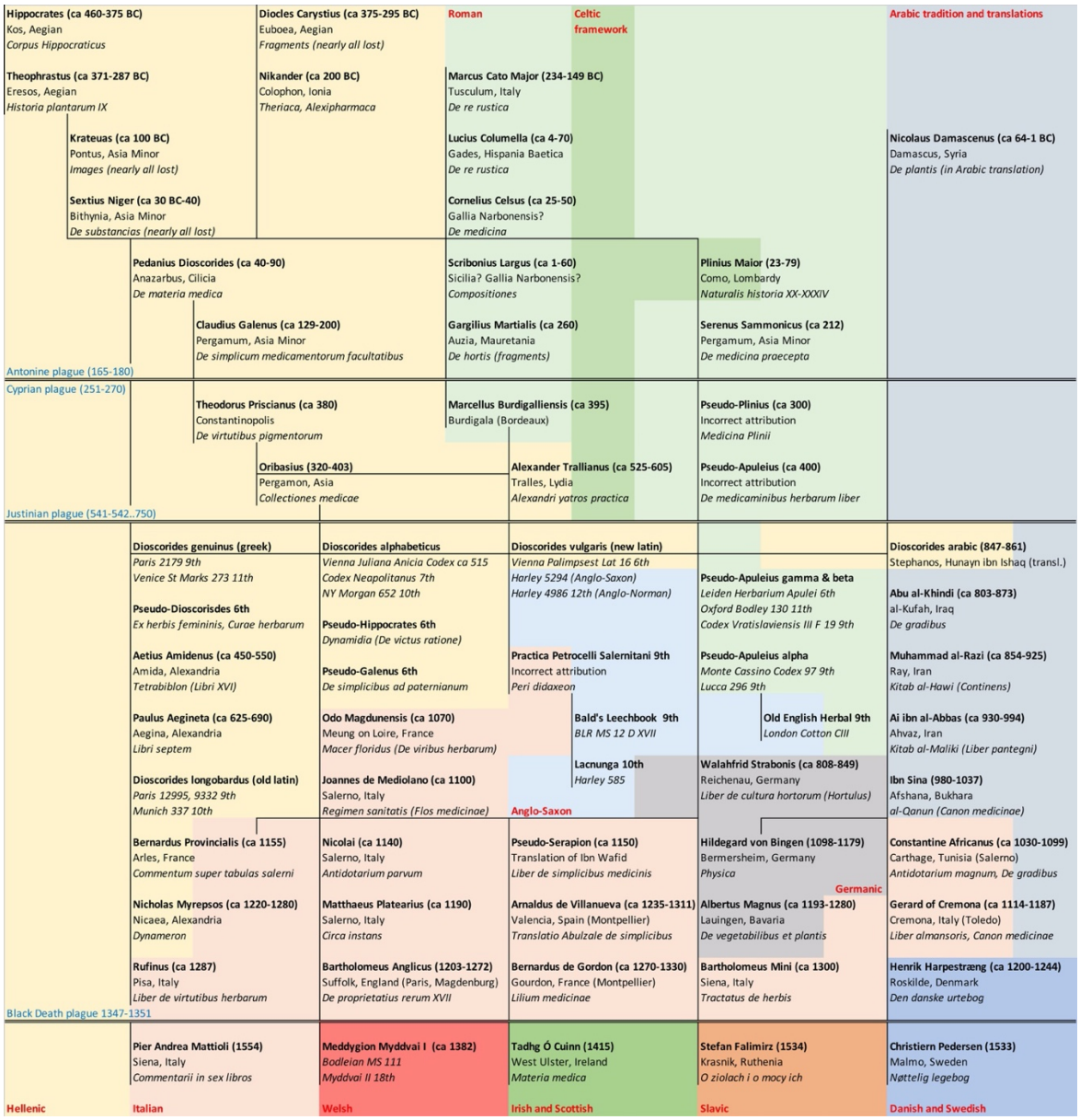


Figure 2. Lineages of major European medical traditions. Chronological outline of the Western European herbal tradition. Notable gaps in knowledge exist due to historic epidemics that drastically reduced the population of Europe. The Celtic framework exists first within the Roman authors and continues to be seen with additions from Dioscorides vulgaris and Pseudo-Apuleius, as well as the Anglo-Saxon, Germanic, and Salernian traditions -- ultimately manifesting in *Meddygon Myddfai I*.

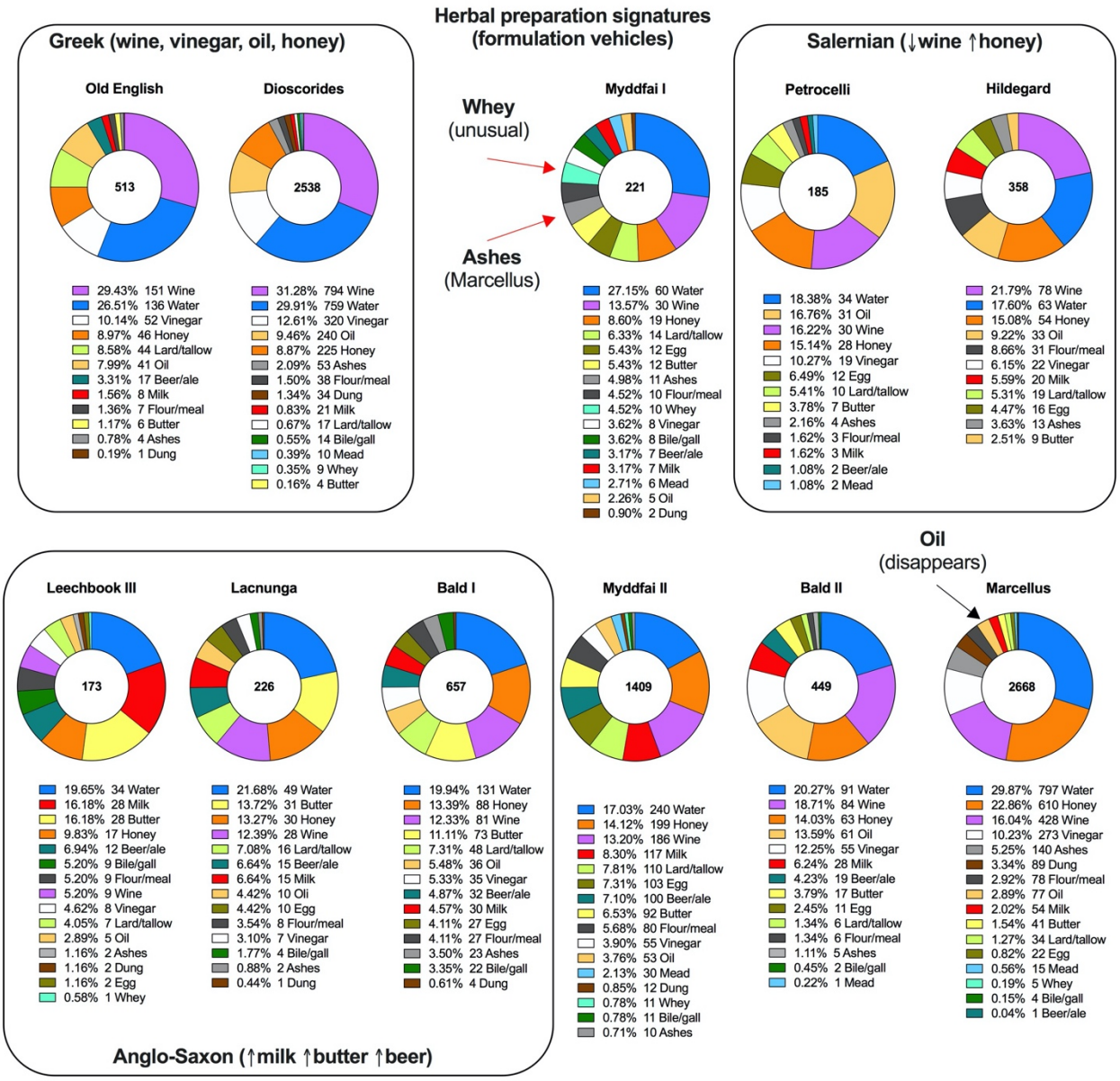


Figure 3. Herbal preparation signatures (formulation vehicles) of each medical text reviewed. The recipes in *Myddfai I* are unique in that they have a high frequency of usage of whey (primarily goat milk whey) as a remedy preparation vehicle.

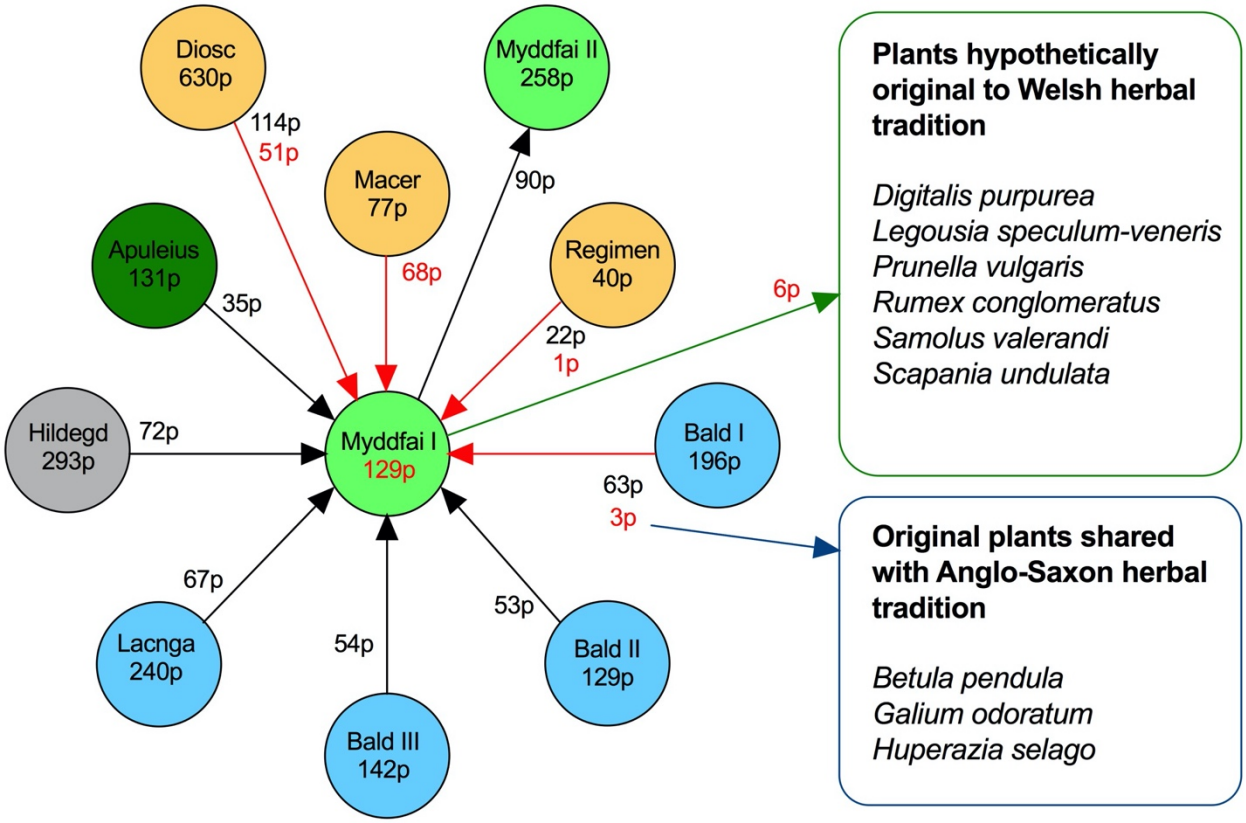


Figure 4. Overlap between *Myddfai I* and other sources. Arrows indicate influences while p indicates plant numbers.

CHAPTER 2: ANTIBACTERIAL ACTIVITY OF MEDICINAL PLANTS FROM *THE PHYSICIANS OF MYDDFAI,* A 14th CENTURY WELSH MEDICAL MANUSCRIPT

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Charles Wagner, Jillian De Gezelle, Maureen Robertson, Keith Robertson, Mickey Wilson, and Slavko Komarnytsky. (2017) *Antibacterial activity of medicinal plants from The Physicians of Myddvai, a 14th century Welsh medical manuscript*. Journal of Ethnopharmacology

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Abstract

Ethnopharmacological relevance: Antimicrobial drug resistance is a growing threat to global public health. Historical records and herbal texts relating to traditional Celtic medicine indicate an extensive pharmacopeia of plants for treating infections likely caused by microbes. However, a major barrier for successful integration of these remedies into mainstream practice is the current lack of accurate interpretation and scientific validation.

Materials and methods: We applied Mobile Discovery approach to the Isle of Arran, Scotland, for *in situ* targeted screening of 83 out of 138 plants identified in *Meddygon Myddfai* (a 14th century Welsh manuscript) to treat conditions related to microbial infections, and an additional 18 plants from modern ethnobotanical knowledge on the island. In a follow-up proof-of-concept study, bioassay-guided fractionation was performed to identify bioactive constituents from two high scoring hits that inhibited *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) bacterial growth. Results: 67 historical plants (80.7%) and 14 modern plants (77.8%) were found to have detectable levels of antimicrobial activity when tested using Mobile Discovery kits, with human saliva as a source of bacteria for screening. Sabinene, a natural bicyclic monoterpene from juniper "berries" (*Juniperus communis* L.) and alliin, a natural sulfoxide from garlic cloves (*Allium sativum* L.), were isolated and confirmed as primary antibacterial leads. Conclusion: Using historical medical sources such as those associated with traditional Celtic medicine to guide rigorous, evidence-based scientific investigation, provides additional leads for new and alternative bioactive molecules for combating bacterial and infectious diseases.

1. Introduction

Microbial infections date back to the dawn of humankind and are responsible for high mortality rates and a shorter life expectancy in medieval societies – especially among children, the malnourished, and wounded individuals. Diseases such as tuberculosis, typhus, diphtheria, typhoid, cholera, dysentery, and pneumonia took a large toll on the early medieval population of the British Isles, co-inhabited by ethnolinguistic Celts (Britton, Pict, and Gaelic tribes) and Anglo-Saxons (Germanic tribes) that arrived in the 5th century AD. Excavations of cemeteries from that time suggested a life expectancy into the mid to late thirties, but demonstrated a peak mortality rate in the teens and early twenties for those individuals who survived childhood (Fleming, 2010). From the 9th century AD, Celtic tribes became confined mostly to the west (Wales and Cornwall) and north (Scotland and the Western Isles). Despite their geographical divisions, the Celts of the British Isles shared in common the ancient healing traditions, handed down by word of mouth first by the druids and subsequently by leeches (from Gaelic *lighiche,* "physician") skilled in medical craft.

Similar to other ancient and medieval cultures, the Celts believed strongly that in nature there is somewhere and somehow a sovereign remedy for the management and treatment of diseases (Whittet, 1964). Historical records and herbal texts relating to traditional Celtic medicine indicated an extensive pharmacopeia of plants for treating infections likely caused by microbes (Beith, 1995; Dobson and Robertson, 2009; Henderson, 1994; Martin, 1703; Pughe, 1861). One of the most thorough and concrete historical texts concerning traditional Celtic plant medicines was recorded by the Physicians of Myddfai (*Meddygon Myddfai*, Carmarthenshire, Wales), held as a part of the Red Book of Hergest manuscript (Llyfr Coch Hergest, c. 1382). The text of the physicians fixed insular Celtic tradition of medicinal plants in a series of some 500

remedies that featured mostly native species and manifested the older, oral knowledge, and local apothecary from the 13th century AD. This knowledge was widely used by the hereditary scholarly physicians of the Western Isles (i.e. Macleans in Skye, O'Conachers in Argyll, Beatons in Islay and Mull). Many members of the medical families continued to practice traditional Celtic medicine in the Western Isles until modern days (Anonymous, 1906).

The Isle of Arran, the seventh largest Scottish island, is located in a mild oceanic climate zone (Figure 1). Arran's highest peaks may have been nunataks (rocky protrusions above the ice sheet) during the Pleistocene glaciations and provided protected places for plant life to survive. This feature may explain increased biodiversity and presence of tree species endemic to the area (McKirdy et al., 2007). With a long history of traditional use, the medicinal plants of Arran presented a unique opportunity for focused screening and validation of Celtic plant-based healing traditions.

Due to the rapid emergence of antibiotic-resistant bacteria (Tommasi et al., 2015) and the apparent lack of interest from the pharmaceutical industry in antibiotic research (Payne et al., 2007), we applied Mobile Discovery approach for *in situ* targeted screening of plants identified in the *Physicians of Myddfai* manuscript to treat conditions related to microbial infections. Two high scoring hits were further characterized for their ability to inhibit *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) bacterial growth. The antimicrobial constituents that they produce were also isolated and tested to establish preclinical MICs.

2. Materials and methods

2.1. Study area

The Isle of Arran belongs to the group of islands in the Firth of Clyde, separated from the Western Isles (also called the Inner and Outer Hebrides) by the Kintyre peninsula (Figure 1). Arran is divided into highland and lowland areas by the Highland Boundary Fault aligned southwest to northeast between Blackwaterfoot and Brodick villages (Barrow, 1912). Three collection sites were designed to capture geographical and biological diversity of the island, including (1) Northern site near Lochranza village, limited by Glen Catacol on the west, Gleann Easan Biorach on the east, and Beinn Bhreac on the south; (2) Western site between Blackwaterfoot and Tormore villages; and (3) Southern site between Shannochie and Kildonan villages. Collections occurred during May-July 2015, with the average daily temperature of 12.2 ^{o}C (6.6-17.1 ^{o}C) and the average of 10-12 precipitation days per month. Due to the nature of the Mobile Discovery approach (see below), only small samples (50-100 mg) of the fresh plants were collected and assayed daily to ensure a non-destructive method of testing. Plant identities were confirmed by the professional medical herbalists (M.R., K.R.) from the Scottish School of Herbal Medicine. Digital voucher specimens were recorded with a Motorola Droid Mini 10 MP camera and deposited for reference in the Mobile Discovery plant collection of the Plants for Human Health Institute, NC State University, Kannapolis, NC. When warranted by the ethnobotanical knowledge (and availability), separate samples were collected from different parts of the same plant (stem, leaf, root, bulb, seed, bark, fruit, resin, or flower).

2.2. Ethnobotanical survey of the *Physicians of Myddfai* manuscript

John Pughe's 1861 English translation of the *Physicians of Myddfai* text was used to determine which plants to survey and screen *in situ*. This translation included a separate index of Welsh and Latin names of each plant mentioned in the text. Since the Myddfai herbals were recorded in Welsh, translated into pre-Linnaean Latin, and then into English (post Linnaeus), we performed additional checks to ensure proper plant name identification. Those included data from (1) historical Welsh dictionary (Thomas et al, 2002), (2) Gaelic language sources concerning similar ailments, (3) common names listed in the English edition, (4) consultations with local herbalists, and (5) modern plant systematics including biogeographic origin of species in question. The modern glossary of Nicholas Culpeper's 17th century AD herbal text was consulted to aid in the understanding of various terminologies pertaining to diseases (Culpeper, 1975). Even though the Welsh were unaware of microbes at the time, the text provided clear and appropriate descriptions that are recognizable as microbial infections or related conditions (abscess, ague (fever), bites (snake, spider, dog and/or "mad" dog), colds (catarrh), cough (including whooping cough), diarrhea, erysipelas, eye problems (cloudiness, opacity, redness), fetid breath, fevers (such as typhoid), leprosy, mouth sores, plague, pneumonia, proud flesh (excessive granulation), scabs (and scabies), scrofula, skin eruptions, sore throat, stomach sores (including ulcers and wounds)). On many occasions, these plants were listed as ingredients of a more complex mixture that contained upwards of 10 components, including animal and mineral additives. A list of 138 plants was compiled based on these indications (Table 1). From this list, we successfully located, identified, and tested 83 plants (103 samples) for their antimicrobial properties.

2.3. Incorporation of modern ethnobothanical knowledge

An additional 18 plants (27 samples) not listed in the *Physicians of Myddfai* text were added to the study for comparison purposes. These included remedies listed in "Healing Threads" (Beith, 1995), a popular modern compilation of the Gaelic medical tradition (Table 2), and plants that grow on Arran and are routinely used to treat microbial infections or related conditions by the modern herbalists from the Scottish School of Herbal Medicine (Table 3).

2.4. Mobile Discovery assay for antibacterial activity

The *in situ* antibacterial activity of the small samples (50-100 mg) of the fresh plants was determined by the Mobile Discovery kits against human saliva (C.W.) as a source of bacteria for screening. Plant samples were collected in the morning hours before 13:00, transported in the plastic pouches, and tested within 1 h of collection. The Mobile Discovery approach ensured an ethical, non-destructive, and safe (no external bacterial cultures were used or shipped) method to test samples from the local environment for their antimicrobial properties without associated biosafety risks to local biodiversity, population, or environment. Mobile Discovery kits were requested through the program website (http://MobileDiscovery.org) follow the Mobile Discovery Kit link), where they are available for scientists and the general public interested in antimicrobial discovery research. The antibacterial activity was determined according to the instructions from the Mobile Discovery manual shipped with the kit and visually scored against untreated and saliva-treated controls as "0" (no bacterial colonies, high score), "1" (few bacterial colonies, medium score), "2" (some bacterial colonies, low score), and "3" (many bacterial colonies, low score).

2.5. Extraction of high scoring plant materials

In a follow-up proof-of-concept study, juniper "berries" (*Juniperus communis* L.) and garlic cloves (*Allium sativum* L.), two of the high scoring hits recorded in the Mobile Discovery tests, were obtained from Frontier Co-Op (Norway, IA), freeze dried (Labconco Freezone 18, Kansas City, MO), ground to powder (IKA A11 mill, Wilmington, NC) and subjected to sequential hexane, ethyl acetate, butanol, and aqueous fractionation (60 g of plant powder, 360 ml of solvent, repeated twice, batches were combined and evaporated to dryness using Buchi Rotavapor R210, Flawil, Switzerland). Fractions and respective bioactive compounds were then tested in the laboratory settings for their ability to inhibit Gram-positive and Gram-negative bacterial growth. All chemicals and solvents (anhydrous and ACS grade) were purchased from Sigma-Aldrich (Saint Louis, MO), unless specified otherwise.

2.6. Bacterial strains and culture conditions

Gram-positive *Staphylococcus aureus* ATCC 25923 (Manassas, VA) and Gram-negative *Escherichia coli* DH5a (Promega, Madison, WI) were incubated under aerobic conditions on the LB medium at 37 ºC, with or without shaking (100 rpm, New Brunswick Innova 43 shaker, Enfield, CT).

2.7. Disk diffusion

Disk diffusion was used to determine the antibacterial activities of high scoring plants in the laboratory setting. For each 100 mm Petri/LB agar plate surface spread with the appropriate bacterial culture, 6 filter paper disks (6 mm) were imprinted with 10 µl of vehicle (DMSO, negative control) or three concentrations of the test articles (10, 3, and 1 µg), tested in duplicate.

Antibiotics tetracycline and ampicillin (5 µg) were used as reference drugs for Gram-positive and Gram-negative bacteria, respectively. After an overnight incubation at 37 °C, the diameter of the inhibition zones was measured with a digital caliper in mm.

2.8. Minimum inhibitory concentration (MIC)

Different doses of the test articles (50 mg/ml stocks in DMSO) or reference drugs (5 mg/ml in DMSO) were serially 3x diluted and 20 µl of each dilution were added in triplicate to a 96 well plate containing 180 µl of diluted bacterial culture in the LB medium. The plates were incubated overnight at 37 °C, and bacterial growth was measured by absorbance read at 600 nm on the Synergy H1 microplate spectrophotometer (BioTek, Sunnyvale, CA). The MIC was determined as the lowest concentration inhibiting at least 80% of the growth.

2.9. Minimum bactericidal concentration (MBC)

MBC was determined by serial sub-cultivation of 100 µl of each dilution onto plates containing LB agar and further incubation overnight at 37 °C. The lowest concentrations without visible growth were defined as MBCs.

2.10. Cell culture

Human HT-29 cells of epithelial origin (ATCC HTB-38, Manassas, VA) were routinely passaged every 3-4 days and maintained in high glucose DMEM containing 10% fetal bovine serum FBS (Life Technologies, Carlsbad, CA), and 1% penicillin-streptomycin (Fisher Scientific, Pittsburg, PA) at 37 °C and 5% CO_2. Cell viability was estimated using the 96 well

MTT assay (Mosmann, 1983) by absorbance read at 570 nm on a Synergy H1 microplate spectrophotometer (BioTek, Sunnyvale, CA).

2.11. Statistical analysis

Statistical analyses were performed using Prism 6.0 (GraphPad Software, San Diego, CA). Data were analyzed by one-way ANOVA with treatment as a factor. Post hoc analyses of differences between individual experimental groups were made using the Dunnett's multiple comparison tests. Significance was set at $p < 0.05$. Values are reported as means ± SEM.

3. Results

3.1. Diversity of plants from the *Physicians of Myddfai* manuscript

The ethnobotanical survey of some 500 remedies described in the *Physicians of Myddfai* text was focused on plants indicated for treating diseases recognizable as microbial infections or related conditions. A total of 138 plant species that fit this description were classified into 51 families and 125 genera (Table 1). The most used families were Apiaceae, Asteraceae, Lamiaceae, and Rosaceae that contributed 55 medicinal plant species (40%) with putative antimicrobial properties (Figure 2A). Important medicinal genera were *Allium* and *Artemisia* (3 species each). Several genera represented non-native plants that were either imported to the region or cultivated for their medicinal properties, for example *Crocus*, *Aloe, Piper*, *Citrus, Afromomum, and Cinnamomum*. Myddfai herbalists used different plant parts or their combinations, depending on the target plant species, however the distinction was made clear only for root, bark, juice, and fruit/seed parts of the plant. When not specified, we collected and

analyzed aerial parts of the plant (leaf, stem, and/or flower) available at the time of collection. Among 20 health conditions possibly related to microbial infections, wounds, fevers, eye problems, and pneumonia were most prominent (Figure 2B).

3.2. Modern ethnobothanical knowledge in the region

Review of Beith's "Healing Threads" and consultations with modern herbalists from the Scottish School of Herbal Medicine (Arran) resulted in the inclusion of an additional 9 genera from the former (Table 2) and 9 genera from the latter source (Table 3). Two species of lichens and an endemic species, the Arran whitebeam (*Sorbus arranensis* Hedl.), were reported to be used as antimicrobial remedies in the modern traditional practice on the island. At least 26 medicinal plant species recognized as antimicrobial in the *Physicians of Myddfai* text were listed for the similar indications in Beith's herbal. In addition to common health conditions like cough, fevers, and wounds, modern ethnobotanical knowledge recognized and directly specified microbial infections as a direct target for traditional plant use.

3.3. Antimicrobial activity of the tested plants

About 52 (50.5%) of the plants tested were single parts, predominantly leaves (34.6%), although roots, stems, seeds and other organs were also sampled when indicated and available (Figure 3A). In many instances, a combination of plant parts was used, with an aerial sample (leaf and stem) being the most frequent. Mobile Discovery assay using human saliva as a source of bacteria for screening confirmed high (score of 0) antimicrobial activity of 37 *Myddfai* plants tested (44.6%). Additionally, 13 plants (15.7%) were measured as medium (score of 1) and further 17 plants (20.5%) were noted to have low (score of 2) antimicrobial activity. Altogether,

out of 83 *Myddfai* plants tested, 67 plants (80.7%) showed any level of measurable antimicrobial properties (Table 1). We also noted the evidence of an increased level of modern ethnobotanical knowledge regarding the anti-infective plants of the region, as 14 modern leads (77.8%) showed any level of antimicrobial activity, with 10 (55.6%) plants measured as high scores (Tables 2-3). Notable exceptions were 3 plants from the order Lamiales which showed no antimicrobial activity even though indicated in the Beith's herbal (Table 2). Traditional knowledge from the modern Scottish School of Herbal Medicine (Arran) was confirmed in 8 out of 9 species tested, with majority of the plants producing high or medium scores (Table 3).

Original health indication had a major impact on confirmation of the antibacterial activity. Plants indicated for bites, pneumonia, and erysipelas were confirmed as antimicrobial in >75% tests. Bioactivity of remedies targeting sores, scrofula, eye problems, and wounds was confirmed in >65% of the cases (Figure 4A). Notable exceptions were diarrhea (0/2, 0%), scabs (1/3, 33%), and leprosy (3/7, 43%). Plants indicated for bites also showed the highest antibacterial scores (0.31±0.24), three times above the average for other health indications (1.01±0.42, $p<0.05$) (Figure 4B).

3.4. Bioactivity-guided fractionation of the high scoring plants

In a follow-up proof-of-concept study, antibacterial activity of juniper "berries" (*Juniperus communis* L.) and garlic cloves (*Allium sativum* L.) fractions were measured by disk diffusion method in the dose range of 1-10 μg. Gram-positive *S. aureus* showed dose-dependent susceptibility to the hexane fraction from juniper (10-13 mm inhibition zone diameter) and the water fraction from garlic (8-25 mm inhibition zone diameter). Gram-negative *E. coli* showed

dose-dependent susceptibility from the hexane fraction of juniper (7-12 mm inhibition zone diameter) and the water fraction from garlic (6-18 mm inhibition zone diameter) (Figure 5).

The antimicrobial activity was attributed to sabinene and alliin, the major bioactive compounds found in these fractions. MICs were determined for extracts and respective bioactive compounds after 24 h and ranged from 215 μg/ml for juniper to 54 μg/ml for sabinene against *S. aureus*; and from 108 μg/ml for garlic to 27 μg/ml for alliin against *E. coli* (Figure 6). MBCs were lower for *S. aureus* (sabinene, 215 μg/ml) than for *E. coli* (alliin, 108 μg/ml).

4. Discussion

The spread of antibiotic-resistant pathogens, combined with decades of little success in discovering new antibiotics, has become an increasing problem for modern medical interventions and community health (Lewis, 2012). The current portfolio of new antimicrobial drugs in clinical trials consisted largely of already known chemical classes for which resistance has already emerged. High throughput screening of individual molecular targets (genes and proteins) against synthetic chemical libraries has had very limited success as well (Payne et al., 2007). Therefore, there is still a great need for discovering new antimicrobial compounds, but the strategy of antibiotic discovery needs to be modified to capture unexploited biodiversity and identify new natural lead compounds for further antimicrobial development (Harvey, 2000).

Traditional knowledge, such as that captured in *Physicians of Myddfai*, provided an initial source of screening targets containing biologically relevant chemical spaces and pharmacophores, all with a recorded history of prior human use. Mobile Discovery approach, offered a simple, low-cost, safe, and effective method to identify and validate antimicrobial hits with no geographical, technological, or ethical constraints. It relied on bacteria naturally present

in human saliva, thus allowing for phenotypical, non-targeted, whole cell antibacterial screens. Bacterial flora of the human saliva contains both Gram-positive and Gram-negative facultative cocci and rods (Socransky and Manganiello, 1971), therefore Mobile Discovery assay interrogated all targets in their physiological context simultaneously. The assay used fresh, whole plant parts rather than extracts or other preparations, thus capturing *in situ* chemical diversity and bioactivity of natural products, which is often lost during collection, storage, or separation of synergistic interactions.

Of some 500 remedies described in the Myddfai text, the largest number were purely herbal and are still in use (Beith, 1995). Many of these plants were indicated for treating diseases recognizable as microbial infections or related conditions (Figure 2). Overall, high antibacterial activity was demonstrated in 37 Myddfai plants (44.5%), while any level of antibacterial activity was detected in 67 species (80.7%). Such high hit rates are explained by extensive Celtic ethnobotanical knowledge of local plants and centuries of continuous practical use. Several plants showed high antimicrobial scores in the majority of the parts tested, including juniper (*Juniperus communis* L.), wild garlic (*Allium ursinum* L.) and its cultivated relative (*Allium sativum* L.), hemp-agrimony (*Eupatorium cannabinum* L.), and herb Robert (*Geranium robertianum* L.). In other cases, the antimicrobial activity was clearly restricted to one major part of the plant tested, for example, the root of dandelion (*Taraxacum officinale* L.) or the seed of caraway (*Carum carvi* L.). High antimicrobial scores were also found in certain plants that are of particular importance to traditional Celtic medicine of the Western Isles, including bog myrtle (*Myrica gale* L.), wood sage (*Teucrium scorodonia* L.), and white oxe eye daisy (*Leucanthemum vulgare* Lam.). Widespread medicinal plants traditionally used to fight infections were well represented among the high scoring hits (Figure 3), including wormwoods (*Artemisia* spp.),

garlic and onion (*Allium* spp.), and sage (*Salvia officinalis* L.). Herbs or spices traditionally used to prepare meals and home remedies, such as cumin, fennel, saffron, dittany, rosemary, thyme, and bay laurel, were also confirmed as high scoring antimicrobial hits, thus validating the *in situ* Mobile Discovery approach (Table 1).

Many of the *Myddfai* plants were investigated for antibacterial properties previously, and the bioactive constituents responsible for the antibacterial effects were isolated and identified in some cases. For example, antimicrobial effects were attributed to terpenoids such as eucalyptol, thujones, camphor, borneol, and myrtenol present in the essential oil of feverfew (*Tanacetum vulgare* L.) (Judzanentiene and Mockute, 2005; Muresan, 2015), caffeic acid derivative plantamajoside from the leaves of plantain (*Plantago major* L.) (Stanisavljevic et al., 2008), terpenoids such as patchoulol, pinene, humulene, valerenic acid, and camphene in the essential oil of valerian roots (*Valeriana officinalis* L.) (Letchamo et al., 2004), and alkaloid berberine in barberry (*Berberis vulgaris* L.) (Dashti et al., 2014). However, the review of literature suggested that in many cases, while the antimicrobial properties of the plant or plant extract were reported, the specific bioactive constituents responsible for these effects were not identified, for example in case of beets (*Beta vulgaris* L.) (Bucur et al., 2015) and other plants.

Wounds, including bites and sores, were very common at the time, and Celtic knowledge of plants effective for treating these conditions seems to be very relevant today. *Myddfai* plants indicated for treating bites were confirmed to contain antibacterial activity in more than 75% of remedies, with highest antibacterial potency three times above the average for other health indications analyzed in this study (Figure 4). This could be possibly explained by the diverse and complex polymicrobial nature of the bite wound infections (Abrahamian and Goldstein, 2011). Pneumonia, skin infections (erysipelas), halitosis (fetid breath), and sores of various origins were

other indications that resulted in high rates of antimicrobial discovery. On the other hand, plants indicated for health conditions of mixed etiology, for example, a combination of bacterial infection and acute nonmicrobial inflammation due to direct injury (wounds) or eye disorders, while producing some high scoring hits such as thyme (*Thymus serpyllum*), resulted in lower overall antibacterial hit rates. In other instances, however, when the eye disease is described in sufficient details to recognize a microbial infection, the laboratory follow-up confirmed the antimicrobial properties of the traditional medicine. This is the case with a remedy for sty from the Anglo-Saxon text, *Bald's Leechbook,* from the 10th century AD, that included a garlic (*Allium sativum* L.), onion (*Allium cepa* L.), and leek (*Allium ampeloprasum* L.) decoction in wine (Harrison, 2015). This makes a very interesting parallel between medicinal traditions of Celts and Anglo-Saxons, that co-inhabited the British Isles at that time and likely shared knowledge of local medicinal plants and their traditional uses.

Focusing on novel and diverse chemical structures – and not biological targets – that show good preclinical antibacterial activity, seems to be the most attractive approach to improve antibacterial discovery (Payne et al., 2007). Plants, due to their intrinsic chemical complexity, synergistic, multi-target, and environmental flexibility, are poised to provide such diversity. For example, an endemic Arran whitebeam (*Sorbus arranensis* Hedl.) was measured as a high scoring hit in this study, however its phytochemical and bioactivity profiles remain unknown, likely due to rarity and environmental isolation of this species. In a follow-up proof-of-concept study, juniper "berries" (*Juniperus communis* L.) and garlic cloves (*Allium sativum* L.), two of the high scoring hits recorded in the Mobile Discovery assay, were further fractionated and confirmed to contain compounds with high antimicrobial activity, sabinene and alliin (Choi et al., 2007; Pepeljinjak et al., 2005). Although not novel, these leads proved a high applicability of

Mobile Discovery *in situ* approach to the early screening stage. Alliin also showed excellent *in vitro* safety margin (no human cell toxicity up to 108 μg/ml, Figure 6). However, the parent structure does not follow Lipinski's rule of five (Lipinski et al., 2001) and is hydrolyzed to bioactive allicin that has stability concerns (Lawson and Gardner, 2005). When leads like this are identified, it may still take years and synthesis of numerous derivatives to ensure an antibacterial molecule with desirable pharmaceutical profile, broad spectrum activity, stability, acceptable safety at high blood levels, and a competitive dosing regimen. This is not going to happen until more antibiotic discovery work is done in academic, industrial, and citizen science settings.

5. Conclusions

Despite the historical successes of traditional medicines and early drug discovery efforts based on plants, it is likely that the vast majority of plant species have not been investigated and their bioactive constituents have not been determined. The knowledge of medicine possessed by the Celts of the British Isles was fixed in the *Physicians of Myddfai* manuscript in the form of a culture-specific, detailed knowledge of local medicinal plants and their traditional uses. Our study demonstrated that most of the *Myddfai* medicinal plants indicated for treating diseases recognizable as microbial infections showed an antibacterial effect *in vitro*, and justified at least in part their use in traditional medicine. These results encourage further investigations to extract and identify the active chemical compounds responsible for the antibacterial effects.

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7. Tables and Figures

Table 1. *The Physicians of Myddfai* plants indicated for treating diseases recognizable as microbial infections and their antibacterial activity measured with the Mobile Discovery approach.

Genus	*Species*	Common name	Plant part(s)	Score	Traditional use
Angelica	sp.	Angelica	Leaf and stem	3	Fetid breath
Apium	*graveolens*	Celery	Leaf and stem	2	Ague, eye problems
			Stem	2	
Conium	*maculatum*	Hemlock	--	--	Abscess, erysipelas, pneumonia
Carum	*carvi*	Caraway	Seed	0	Fevers, pneumonia
			Leaf and stem	2	
Conopodium	*majus*	Pignut, kippernut	--	--	Fevers
Coriandrum	*sativum*	Coriander	Leaf and stem	2	Ague
Cuminum	*cyminum*	Cumin	Seed	0	Cough, wounds
Foeniculum	*vulgare*	Fennel	Leaf and stem	0	Bites, cough, erysipelas, eye problems, fevers, pneumonia
Petroselinum	*marinum*	Parsley	Leaf	3	Ague, eye problems, wounds
Pimpinella	*anisum*	Anise	--	--	Cough, fevers, pneumonia
Ilex	sp.	Holly	Leaf and stem	3	Wounds
			Bark	3	
Allium	*sativum*	Cultivated garlic	Bulb	0	Abscess, bites, cough, proud flesh, plague, sore throat, wounds
Allium	*ampeloprasum*	Leek	Bulb	1	Bites, cough, pneumonia
Allium	*cepa*	Onion	Bulb	1	Cough, eye problems, pneumonia, skin eruptions, wounds (also Beith)
Hyacinthoides	*non-scripta*	Bluebell, wild hyacinth	Leaf and stem	2	Leprosy
Ruscus	*aculeatus*	Butcher's broom	--	--	Fevers
Crocus	*sativus*	Saffron	Stigma (dry)	0	Ague
Hyacinthoides	*non-scripta*	Bluebell, wild hyacinth	Fruit	2	Leprosy
Iris	*pseudocorus*	Yellow flag	Root	3	Ague, erysipelas, skin eruptions, sore throat (also Beith)
			Leaf and stem	2	
Aloe	sp.	Aloe	Leaf	3	Eye problems
Achillea	*millefolium*	Yarrow, milfoil	Leaf and stem	3	Eye problems, fevers, scrofula, wounds (also Beith)
Arctium	*lappa*	Burdock	Root	0	Fevers, skin eruptions
			Leaf	1	
Artemisia	*vulgaris*	Mugwort	Leaf and stem	0	Abscess, ague, bites, erysipelas, fevers, pneumonia, scrofula, skin eruptions

Table 1 continued.

Artemisia	*absinthium*	Wormwood	Leaf and stem	0	Ague, fevers, pnuemonia
Artemisia	*abrotanum*	Southernwood	--	--	Fevers
Bellis	*perennis*	Bruisewort	Leaf and stem	3	Wounds
			Root	2	
			Flower	1	
Calendula	*officinalis*	Marigold	Leaf and flower	2	Fevers
Centaurea	sp.	Knapweed	Leaf, stem and flower	0	Bites, eye problems, fevers, scrofula, skin eruptions
Chrysanthemum	*leucanthemum*	Common daisy	Leaf, stem and flower	3	Eye problems, fevers, pneumonia, scrofula, skin eruptions, wounds (also Beith)
Eupatorium	*cannibinum*	Hemp-agrimony	Leaf and stem	0	Cough, wounds
			Root	0	
Inula	*helenium*	Elecampane, elfwort	Leaf	0	Cough, leprosy, scabs, scrofula, skin eruptions, sore throat (also Beith)
Leucanthemum	*vulgare*	White oxe eye daisy	Leaf, stem and flower	0	Cough, plague, wounds (also Beith)
Matricaria	*chamomilla*	Chamomile	Leaf, stem and flower	0	Abscess, scrofula, wounds
Tanacetum	*parthenium*	Feverfew	Leaf and stem	0	Bites, fetid breath, skin eruptions, sore throat, wounds
Tanacetum	*vulgare*	Tansy	Leaf and stem	0	Eye problems, pneumonia, wounds
Taraxacum	*officinale*	Dandelion	Leaf and stem	3	Fevers
			Flower	3	
			Root	1	
Tragopogon	*dubius*	Yellow goat's beard	--	--	Fevers, pneumonia, proud flesh
Borago	*officinalis*	Borage	Leaf and flower	3	Fevers, wounds
Cerinthe	*major*	Honeywort	--	--	Wounds
Cynoglossum	*officinale*	Hound's tongue	--	--	Wounds
Symphytum	*officinale*	Comfrey	Leaf	3	Abscess
Brassica	*oleracea* var. *rubra*	Red cabbage	Leaf	1	Eye problems, fevers, wounds
Brassica	sp.	Mustard	--	--	Bites, cough, wounds
Cardamine	*pratensis*	Lady's smock	--	--	Wounds
Raphanus	*sativus*	Radish	Leaf and stem	3	Bites
			Root	2	
Cochlearia	*officinalis*	Scurvygrass	Leaf, stem and flower	2	Wounds
Beta	*vulgaris* subsp. *maritima*	Sea beet	Leaf and stem	0	Pneumonia

Table 1 continued.

			Root	0	
Agrostemma	*githago*	Corn cockle	--	--	Pneumonia
Persicaria	*amphibian*	Amphibious persicaria	--	--	Fevers
Rumex	*obtusifolia*	Common dock	Leaf	3	Abscess, leprosy, pneumonia, skin eruptions, wounds
Rumex	*crispus*	Yellow dock	--	--	Abscess, leprosy, pneumonia, skin eruptions, wounds
Byronia	*alba*	White bryony	--	--	Wounds
Sambucus	*nigra*	Elder	Stem and Flower	0	Colds, fevers, mouth sore, sore throat, wounds
Sambucus	*ebulus*	Dwarf elder	--	--	Fevers, mouth sore, sore throat, wounds
Knautia	*arvensis*	Field scabious	--	--	Bites, eye problems
Lonicera	sp.	Honeysuckle	Leaf	0	Eye problems, leprosy, sore throat, wounds (also Beith)
Succisa	*pratensis*	Devil's bit	--	--	Skin eruptions, wounds
Valeriana	*officinalis*	Valerian	Root	0	Abscess, wounds
			Leaf, stem and flower	1	
Calluna	*vulgaris*	Heather	Leaf, stem and flower	3	Fevers, pneumonia (also Beith)
Empertrum	*nigrum*	Crowberry, crake berry	--	--	Fevers, pneumonia
Anagallis	*arvensis*	Pimpernel	--	--	Abscess, fevers, wounds
Samolus	sp.	Brook weed	--	--	Wounds
Caesalpinia	*bonduc*	Bonduc bean, nickernut	--	--	Diarrhea
Cytisus	*scoparius*	Broom	Leaf, stem and flower	1	Ague, leprosy, wounds
Glycyrrhiza	*glabra*	Liquorice	--	--	Cough
Quercus	*robur*	English oak	Bark	2	Fevers, sore throat, wounds (also Beith)
			Leaf	1	
Centaurium	*erythraea*	Centaury	--	--	Eye problems, fevers, wounds
Geranium	*robertanium*	Herb Robert	Leaf	0	Erysipelas, pneumonia, wounds (also Beith)
			Flower	0	
Galium	*aparine*	Cleavers, goosegrass	Leaf and stem	2	Colds, fevers, leprosy, scabs, scrofula, skin eruptions, wounds
Galium	*odoratum*	Woodruff	--	--	Fevers, pneumonia, wounds (also Beith)
Ajuga	*reptans*	Bugle	--	--	Leprosy, skin eruptions, wounds
Glechoma	*hederacea*	Ground ivy	--	--	Ague, bites, cough, eye problems, fevers, skin eruptions
Hyssopus	*officinalis*	Hyssop	--	--	Sore throat, wounds
	Lamium	*purpeum*	Red dead nettle	--	--	Fevers, plague, scrofula, wounds
	Marrubium	*vulgare*	Horehound	Leaf, stem and flower	0	Cough, pneumonia (also Beith)
	Melittis	*melissophylum*	Bastard balm	--	--	Fevers

Table 1 continued.

Mentha	sp.	Mint (local)	Leaf and stem	2	Fevers
Origanum	*dictamnus*	Dittany	Leaf and stem	0	Bites
Rosmarinus	*officinalis*	Rosemary	Leaf and flower	0	Colds, fetid breath, fivers, mouth sores, sore throat
Salvia	*officinalis*	Sage	Leaf, stem and flower	0	Ague, bites, cough, fetid breath, fevers, mouth sores, plague, sore throat, wounds
Salvia	*sclarea*	Clary sage, clear eye	Leaf and stem	3	Wounds
Stachys	*officinalis*	Wood betony	Leaf, stem and flower	0	Ague, bites, eye problems, fevers, wounds (also Beith)
Teucrium	*scorodonia*	Wood sage	Leaf and stem	0	Abscess, colds, fevers, proud flesh
Thymus	*officinalis*	Thyme	Leaf, stem and flower	0	Eye problems
Thymus	*serpyllum*	Creeping thyme	Leaf, stem and flower	0	Colds, eye problems
Vitex	*agnus-castus*	Chastetree, vitex	Bark	3	Wounds
			Leaf	1	
Ligustrum	*vulgare*	Privet	--	--	Fevers, wounds
Olea	*europea*	Olive	--	--	Bites, scrofula, wounds
Digitalis	*purpurea*	Foxglove	Leaf and flower	2	Abscess (also Beith)
Plantago	*major*	Greater plantain	Leaf	0	Ague, bites, erysipelas, eye problems, scrofula, skin eruptions, wounds (also Beith)
Plantago	*lanceolata*	Ribwort plantain	--	--	Ague, bites, erysipelas, eye problems, scrofula, skin eruptions, wounds (also Beith)
Veronica	*chamaedrys*	Germander speedwell	--	--	Abscess, wounds
Verbena	*officinalis*	Vervain	Leaf	0	Eye problems, scrofula, wounds
Cinnamomum	*verum*	Cinnamon	Bark (dry)	2	Cough
Laurus	*nobilis*	Bay laurel	Leaf and stem	0	Plague, scrofula, wounds
Hypericum	*perforatum*	St. John's wort	Leaf and stem	3	Abscess, fevers, wounds (also Beith)
Hypericum	*androsaemum*	Tutsan	--	--	Fevers
Salix	sp.	Willow	Bark	1	Eye problems
Viola	sp.	Violet	--	--	Scrofula, wounds
Althaea	*officinalis*	Marshmallow	Leaf and stem	2	Cough, erysipelas, fevers, mouth sores, scrofula, sore throat, wounds
Tilia	*europaea*	Lime, linden	--	--	Wounds
Oxalis	*acetosella*	Wood sorrel	Leaf	2	Ague, colds, fevers, scabs, skin eruptions, sore throat, wounds (also Beith)
Juniperus	*communis*	Juniper	Leaf and stem	0	Bites, stomach sores, wounds (also Beith)

Table 1 continued.

			Fruit	0	
Aristolochia	*rotunda*	Round birthwort	--	--	Bites, fevers, pneumonia
Asarum	*europaeum*	Asarabacca	--	--	Pneumonia
Piper	*nigrum*	Black pepper	Fruit (dry)	3	Ague, cough, erysipelas, eye problems, proud flesh, wounds
Cyperus	*longus*	Galingale	--	--	Cough
Avena	*sativa*	Oats	--	--	Abscess, wounds
Sparganium	sp.	Burr reed	--	--	Eye problems, wounds
Berberis	*vulgaris*	Barberry	Leaf and stem	0	Wounds
			Bark	3	
Ranunculus	*ficaria*	Lesser celandine	--	--	Eye problems, scabs, scrofula, skin eruptions
Cannabis	*sativa*	Hemp	--	--	Fevers, wounds
Ficus	*carica*	Fig	Leaf and stem	2	Cough
Agrimonia	*eupitoria*	Agrimony	Leaf and stem	0	Bites, fevers, pneumonia, scrofula, wounds
Argentina	*anserina*	Silverweed	Leaf and stem	2	Diarrhea, fevers, plague (also Beith)
Crataegus	*monogyna*	Hawthorne	Leaf, stem and flower	3	Eye problems, sore throat (also Beith)
Filipendula	*ulmaria*	Meadowsweet	Leaf	1	Pneumonia
Fragaria	sp.	Wild strawberry	Leaf	0	Eye problems, wounds
Geum	*rivale*	Water avens	--	--	Erysipelas, fevers, leprosy, pneumonia, wounds
Potentilla	*erecta*	Tormentil	Leaf and stem	3	Diarrhea, fevers, plague (also Beith)
			Flower	2	
Potentilla	*reptans*	European cinquefoil	--	--	Diarrhea, fevers, plague (also Beith)
Prunus	*spinosa*	Blackthorn, sloe	--	--	Diarrhea
Rosa	*rubiginosa*	Sweetbrier	--	--	Ague, fevers, mouth sores
Rubus	*idaeus*	Raspberry	Leaf	0	Ague, eye problems, fevers, mouth sores, wounds
Rubus	*fruticosus*	Blackberry, bramble	Leaf and stem	1	Ague, erysipelas, eye problems, fevers, mouth sores, wounds (also Beith)
Sorbus	*aucuparia*	Rowan, mountain ash	--	--	Cough, fevers (also Beith)
Ulmus	sp.	Elm	--	--	Erysipelas
Urtica	*dioica*	Common nettle	Leaf	3	Sore throat, wounds (also Beith)
Pistacia	*lentiscus*	Mastic	--	--	Wounds
Boswellia	*sacra*	Frankincense	Resin	1	Erysipelas, mouth sores, scrofula, wounds
Commiphora	*myrrh*	Myrrh	--	--	Fetid breath, wounds
Citrus	sp.	Orange	Fruit (juice)	3	Mouth sores
			Fruit (peel)	1	

Table 1 continued.

Ruta	*graveolens*	Rue	--	--	Abscess, bites, cough, fetid breath, fevers, mouth sores, plague, wounds
Sedum	*telephium*	Orpine	--	--	Fetid breath, fevers, scrofula
Sempervivum	sp.	Houseleek	--	--	Erysipelas, eye problems, wounds
Solanum	*dulcamara*	Bitter nightshade	--	--	Bites, skin eruptions
Sphagnum	*cymbifolium*	Moss	All	0	Fevers, wounds (also Beith)
Aframomum	*melegueta*	Grains of paradise	Seed (dry)	3	Ague, cough

Table 2. Plants indicated for treating diseases recognizable as microbial infections in Beith's Herbal (1995).

Family	Genus	*Species*	Common name	Plant part(s)	Score	Traditional use
Asteraceae	*Solidago*	*virgaurea*	Goldenrod	Leaf and stem	1	Wounds
Brassicaceae	*Capsella*	*bursa-pastoris*	Shepherd's purse	Leaf, stem and flower	1	Wounds
	Sisymbrium	*officinale*	Hedge mustard	Flower	0	Colds
				Leaf	1	
Carophyllaceae	*Stellaria*	*media*	Chickweed	Leaf, stem and flower	0	Abscess
Myricaceae	*Myrica*	*gale*	Bog myrtle	Leaf	0	Parasites
				Bud	0	
Lamiaceae	*Prunella*	*vulgaris*	All heal	Leaf, stem and flower	3	Wounds
Orobanchaceae	*Euphrasia*	*officinalis*	Eyebright	Leaf, stem and flower	3	Eye problems
				Stem and root	3	
Scrophulariaceae	*Scrophularia*	*nodosa*	Figwort	Leaf	3	Scrofula
				Flower	3	
Rosaceae	*Alchemilla*	*vulgaris*	Lady's mantle	Leaf, stem and flower	0	Mouth sores, wounds

Table 3. Modern plants used to treat bacterial infections on the Island of Arran (2015)

Family	Genus	*Species*	Common name	Plant part(s)	Score	Traditional use
Apiaceae	*Smyrnium*	*olusatrum*	Alexanders	Leaf, stem and flower	0	Infections
Amaryllidaceae	*Allium*	*ursinum*	Wild garlic	Leaf	1	Wounds
				Flower	1	
				Bulb	0	
				Fruit	0	
				Stem	3	
Ericaceae	*Vaccinium*	*myrtillus*	Blaeberry, bilberry	Leaf	1	Diarrhea
Lamiaceae	*Origanum*	*vulgare*	Oregano	Leaf and stem	0	Infections, wounds
		majorana	Marjoram	Leaf, stem and flower (dry)	3	Infections, wounds
Cladoniaceae	*Cladonia*	sp.	Lichen	All	0	Wounds
Parmeliaceae	*Usnea*	sp.	Lichen	All	0	Infections, wounds
Rosaceae	*Sorbus*	*arranensis*	Arran whitebeam	Leaf	1	Cough, fevers
				Fruit	1	
Grossulariaceae	*Ribes*	*nigrum*	Black currant	Leaf and fruit	0	Infections

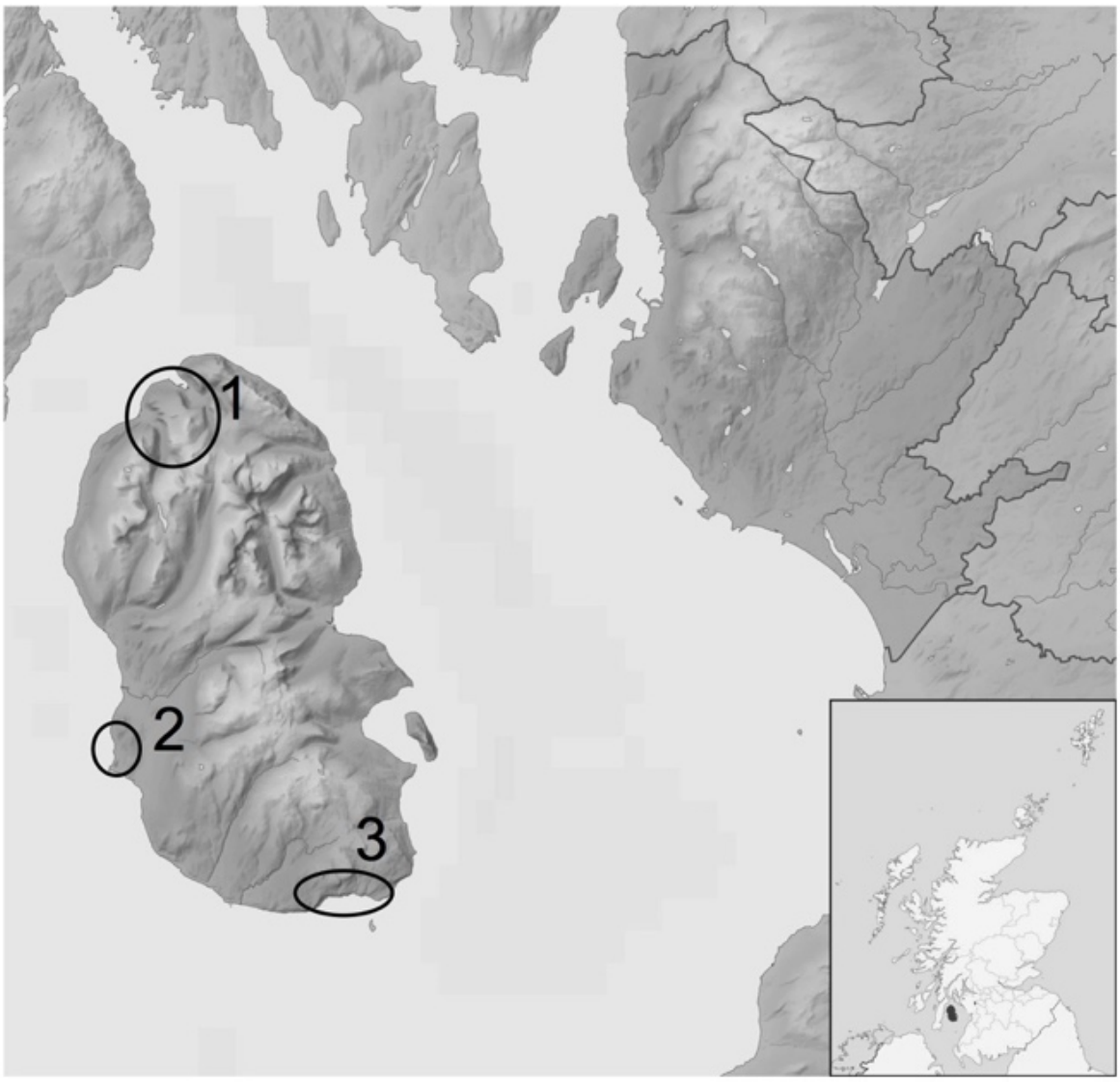

Figure 1. Study sites on the Island of Arran, Scotland. (1) Northern site near Lochranza village, (2) Western site between Blackwaterfoot and Tormore villages, and (3) Southern site between Shannochie and Kildonan villages. Small fresh plant samples were tested *in situ* for antibacterial activity using Mobile Discovery approach.

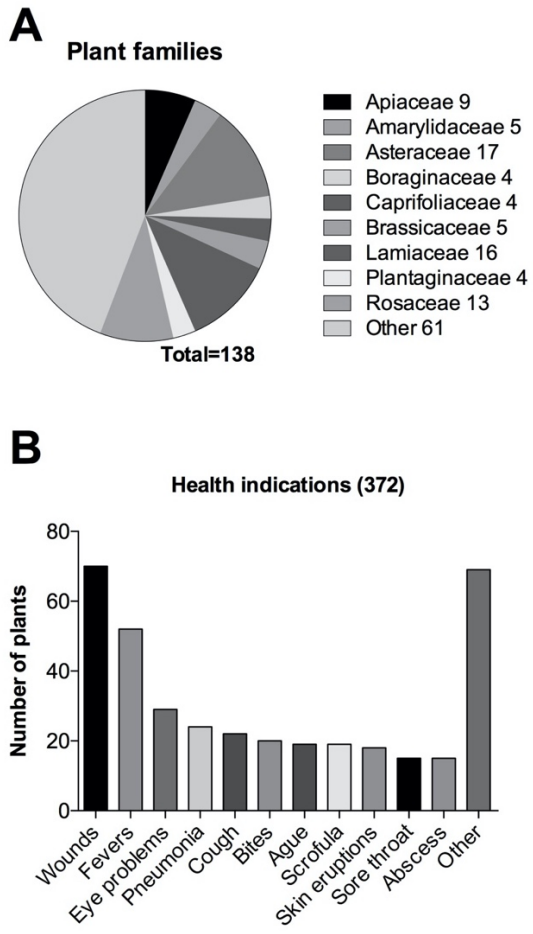

Figure 2. Botanical profile of *The Physicians of Myddfai* plants indicated for treating diseases recognizable as microbial infections. (A) Distribution of plant species across taxonomic plant families. (B) Frequency of health indications attributed to Celtic traditional use of these plants.

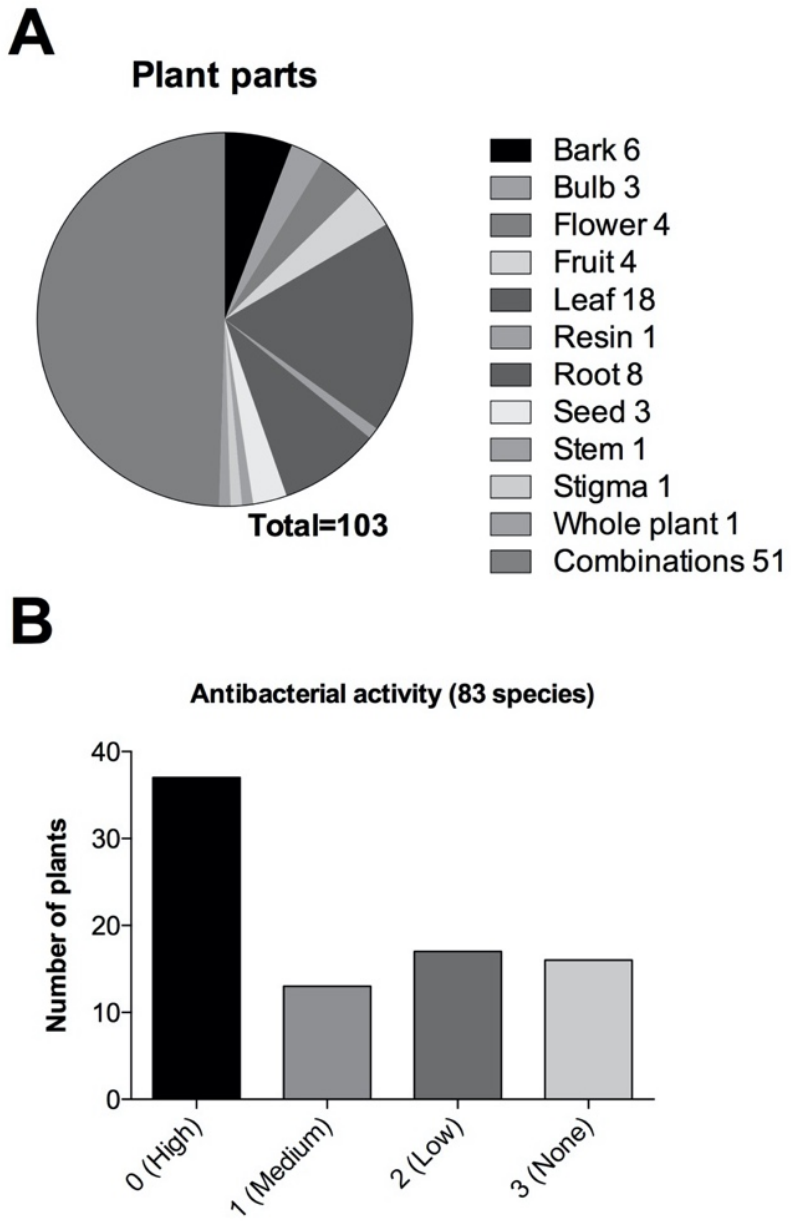

Figure 3. Plant parts analyzed for antibacterial activity. (A) Distribution of single and combinational plant parts used in this study. (B) Antibacterial activity scores of *Myddfai* plants quantified using Mobile Discovery approach against bacteria naturally present in human saliva.

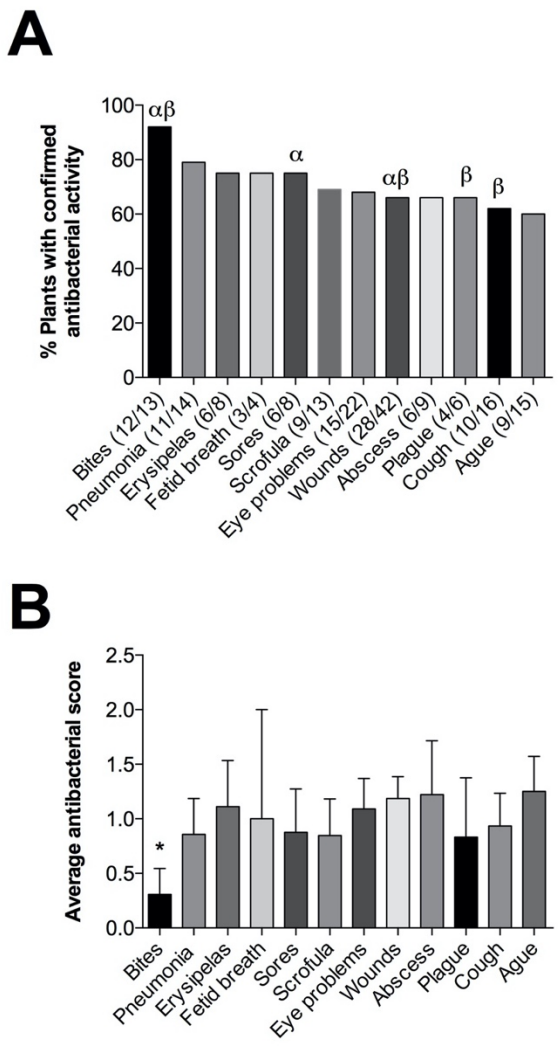

Figure 4. Confirmed antibacterial activity of *Myddfai* plants. (A) Rates of successful confirmation of antibacterial activity of plants according to the original health indication for their traditional use, including high scoring hits juniper (α) and garlic (β). (B) Average antibacterial scores of plants indicated for a particular health condition. Plants indicated for treating bites showed the highest antibacterial potency, 3 times above the average for other health indications (* $P < 0.05$ by one-way ANOVA).

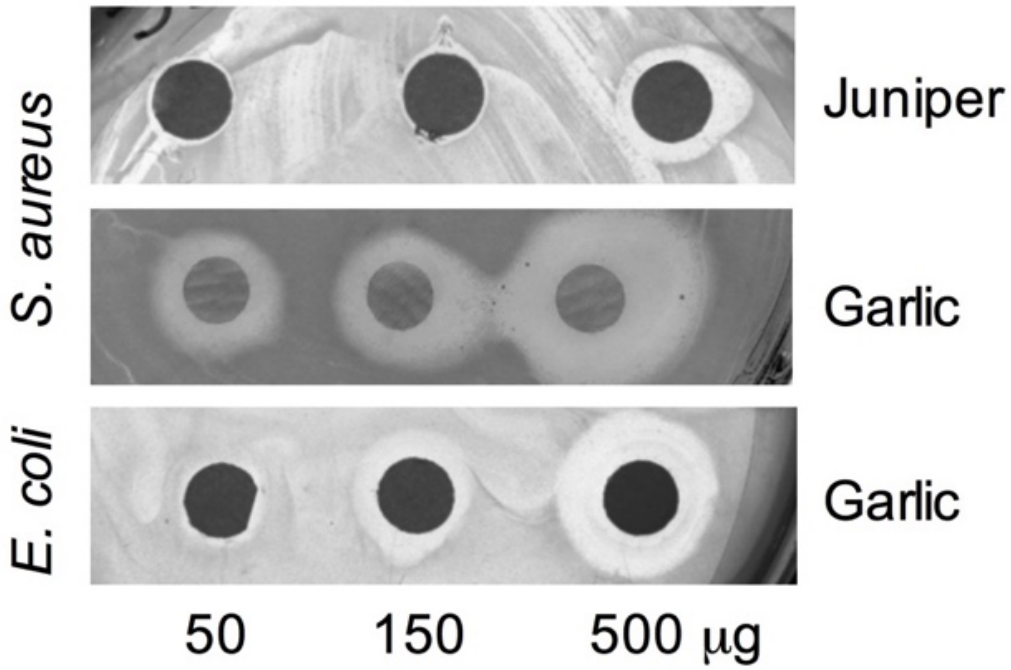

Figure 5. Dose-dependent quantification of antibacterial activity of two high scoring *Myddfai* plants using disk diffusion method. *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) bacteria were surface spread on agar plates and filter paper disks imprinted with three concentrations of juniper (hexane) and garlic (ethyl acetate) fractions were tested in duplicate.

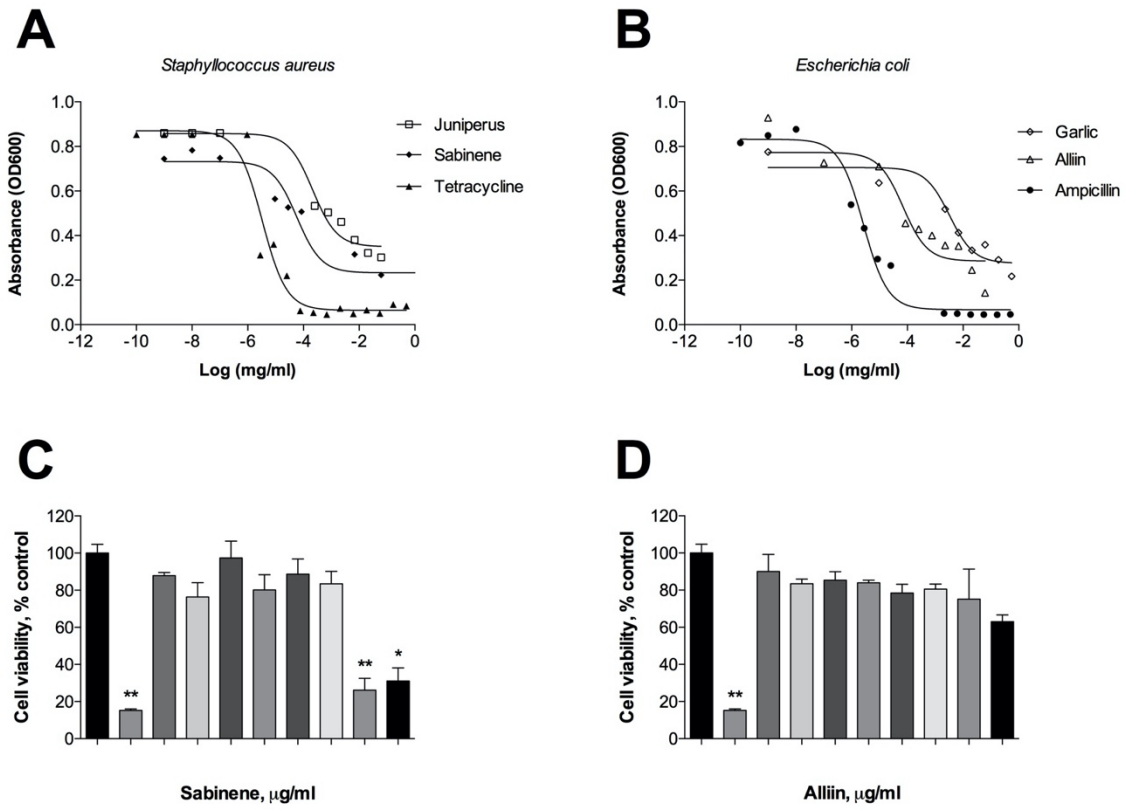

Figure 6. Direct comparison of antibacterial activity and human cell toxicity of bioactive constituents from juniper (sabinene) and garlic (alliin). MIC curves of (A) sabinene against *S. aureus* (A) and (B) alliin against *E. coli* relative to the reference antibiotics. Human HT-29 cells of epithelial origin were treated with vehicle (0.1% DMSO), toxic control (10% DMSO), and different doses of (C) sabinene and (D) alliin, then cell viability was measured as a function of redox potential using MTT dye. Data are the mean ± SEM (n=3) normalized to vehicle control (* $P<0.05$, ** $P<0.01$ by Dunnett's test subsequent to one-way ANOVA).

CHAPTER 3: A MEDIEVAL HERBAL REMEDY ATTENUATES DSS INDUCED COLITIS IN MICE

Charles Wagner, Mickey Wilson, Thirumurugan Rathinasabapathy, and Slavko Komarnytsky

Abstract

Inflammatory bowel disease (IBD) has a complex etiology and pathogenesis. Characterized by a dysfunctional gut epithelial barrier, bacterial colonization, and upregulation of the immune effector cells, which contribute to onset and propagation of the disease, IBD currently lacks efficient multi-target, safe therapeutic options. In this study, we reconstituted a centuries old remedy from the 14th century Welsh *Meddygon Myddfai* manuscript for inflammation of the gut and evaluated its therapeutic potential in the dextran sodium sulfate (DSS) model of murine ulcerative colitis. The *Myddfai* remedy improved DSS induced colitis symptoms in mice, improved histological score of colon tissue, modulated the gut microbiome in a beneficial manner, and increased cell viability in an *in vitro* model. The effect of the recipe depended upon the unique combination of *Artemisia vulgaris* L. (mugwort), *Lamium album* (white dead nettle), and *Plantago major* (greater plantain) aerial parts boiled in goat's whey, which additively utilized their well-recognized, anti-inflammatory biochemical properties. Furthermore, a significant anti-inflammatory effect was attributed to the *Myddfai* remedy based on gene expression results both *in vivo* and *in vitro*, preforming in a similar fashion as the current clinical treatment (5-ASA). Taken together, these data warrant the further investigation of historical botanical interventions to improve human health outcomes and further substantiates the potential use of antique medical texts as sources of novel therapeutics.

1. Introduction

Inflammatory bowel disease (IBD) is a group of complex multifactorial chronic conditions primarily affecting the intestinal walls of the gastrointestinal tract. It encompasses Crohn's disease (CD), which can affect nearly all parts of the gastrointestinal system, and ulcerative colitis (UC), which primarily affects the colon and rectum. The primary symptoms of IBD are abdominal pain, diarrhea mixed with blood, weight loss, fever, and anemia (Seyedian et al., 2019). While the exact cause of IBD is not fully understood, it is well accepted that pathogenesis occurs due to interaction between genetic factors, altered microbiome, environment (especially diet), and the immune dysfunction of the intestine (Seyedian et al., 2019). Under normal conditions, the intestinal mucosa is in a state of "controlled" inflammation regulated by a delicate balance of proinflammatory (tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1, IL-6, IL-12) and anti-inflammatory cytokines (IL-4, IL-10, IL-11) (Ardizzone and Porro, 2005). A combination of the dysfunctional epithelial barrier, bacterial colonization, and upregulation of the immune effector cells contribute to onset and propagation of the disease that currently lacks efficient and safe therapeutic options (Weisshof et al., 2018). Moreover, IBD is also associated with a multitude of downstream molecular pathways including Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling, cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS) (Araújo et al., 2016).

The 14th century Welsh *Meddygon Myddfai* is a medieval manuscript held as a part of the Red Book of Hergest (Bodleian MS 111, shortly after 1382). It is believed to incorporate the healing tradition of Rhiwallon Feddyg, a physician to the Medieval Welsh prince Rhys Gryg ("Rhys the Hoarse") who ruled the Cymry kingdom of Deheubarth (ca. 1234). The manuscript is divided in two parts: *Myddfai I* (recipes considered original to 1382) and *Myddfai II* that

incorporates recipes allegedly drawn by Iolo Morganwg from the continental herbal tradition (Luft, 2018). While Medieval European medicine has generally been dismissed as backwards or superstitious, recent studies have suggested that among the remedies there may be methods and recipes that indicate a more empirical application (Harrison et al., 2015; Wagner et al., 2017). Many of *Meddygon Myddfai* recipes of both plant and animal origin included remedies for various gastrointestinal ailments (Wagner et al., 2020). Two remedies directed at healing the gastrointestinal inflammation of the throat (*for hoarseness*) and gut (*for stomach ache or colic*) shared the following recipe: "Take mugwort, plantain, and red nettle; boil in goat's whey, strain through linen, and administer to the patient."

To a modern biologist, the plant ingredients of the remedy are well recognized as effective anti-inflammatory interventions in the evidence-based botanical research (Wagner, 2020). Mugwort (*Artemisia vulgaris* L.) and its close relative wormwood (*Artemisia absinthium* L.) are a staple of traditional European medicine, especially with regard to treating digestive disorders. Substantial *in vitro* data suggested the potent anti-inflammatory and anti-oxidant activities of mugwort (Bora and Sharma, 2011). In an animal model, mugwort at 500 and 1000 mg/kg significantly inhibited abdominal contortions by 65% and 23%, with rutin and caffeic acid derivatives found to be the major components (Pires et al., 2009). In a placebo controlled clinical trial, patients with active Crohn's disease received powdered wormwood for 6 weeks. More than 80% of patients receiving treatment achieved remission as compared to 20% of patients in the control group, and their serum TNF-α levels were suppressed from 24.5 pg/ml to 8 pg/ml (Krebs et al., 2010).

Greater plantain (*Plantago major* L.), also called waybread in European tradition, is another staple medicinal plant used in treating a wide variety of wounds and inflammatory

conditions. Aqueous plantain extract reduced rat paw edema and pleurisy induced by carrageenan, demonstrating their anti-inflammatory and analgesic activities (Guillén et al., 1997). *Plantago major* L. aqueous extract (1000 and 2000 mg/kg) also decreased writhing induced by application of acetic acid by 37% and 43% in mice (Guillén et al., 1997). Verbacoside, a caffeoyl phenylethanoid glycoside found in *Plantago lanceolata* L. significantly improved colon length and histopathological scores in the dextran sodium sulfate (DSS) model of rodent colitis in the dose range of 120-600 ug/mouse/day. Verbacoside treatment was associated with down regulation of IFN-γ secretion and inhibition of oxidative burst activity of macrophages, which subsequently reduced mucosal tissue damage (Hausmann et al., 2007).

Red nettle (*Lamium purpureum* L.), also called red dead nettle or purple dead nettle, is a common medicinal and food plant native throughout Europe. The name "dead" refers to its inability to sting. Similar to plantain, this plant contains phenylpropanoid glycosides such as verbascoside and isoverbascoside, which can make up 55% of the total phenolic compounds (Salehi et al., 2019). Numerous antioxidant, antimicrobial, and anti-inflammatory effects of *Lamium* extracts have been described *in vitro* (Salehi et al., 2019). *Lamium* spp. *n*-butanol extracts also showed the anti-inflammatory activity in the carrageenan- and PGE(2)-induced rodent hind paw edema models, and the antinociceptive activity in the p-benzoquinone (PBQ)-induced writhing test at doses of 200 mg/kg (Akkol et al., 2008). Interestingly red nettle also contains manninotriose, a prebiotic oligosaccharide with bifidogenic properties (dos Santos et al., 2013).

Whey is a common byproduct of cheese making in many different cultures around the world (Zotta et al., 2020). The increased use of whey and ashes was identified as a unique herbal preparation signature (a traditional vehicle for formulation of the herbal remedies) used by Celtic

and Medieval Welsh healers in the past (Wagner et al., 2020). Goat whey, goat milk, and goat milk yogurt were respectively shown in three different studies to ameliorate both DNBS and acetic acid induced colitis in rats by reducing malondialdehyde levels and increasing total glutathione content. These treatments were associated with a reduction in colonic tissue TNF-α, LTB4, IL-Iβ, COX-2, iNOS, and matrix metalloproteinase-9, along with an increased expression of suppressor of cytokine signaling-1 (Araújo et al., 2016, 2017; Assis et al., 2016). Potent antioxidant peptides have been also identified from goat milk proteins (Ahmed et al., 2015).

In view of the above, there is a portfolio of strong evidence-based botanical research to support the use of the *Myddfai* polyherbal formulation encompassing mugwort, plantain and dead nettle in goat's whey as an alternative approach to promote gastrointestinal health and ameliorate pathological conditions associated with inflammation of the gastrointestinal walls. In this study, we investigated the original recipe according to the instructions laid down by the medieval Welsh physicians, and its individual botanical components in a DSS-induced murine model of colitis for their ability to remit the acute signs of ulcerative colitis and associated inflammation, including the changes in microbiome profiles associated with these treatments.

2. Materials and Methods

2.1 Chemicals and Reagents

Dexamethasone (DEX), lipopolysaccharide (LPS), 5-aminosalicylic acid (5-ASA), and dextran sodium sulfate (DSS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM, RPMI media, fetal bovine serum, TrypLETM, TRIzol reagent, and cDNA kit were purchased from Life Technologies (Grand Island, NY, USA). Penicillin and streptomycin were

obtained from Fisher Scientific (Atlanta, GA, USA). All other solvents and chemicals used in this investigation were obtained from VWR International (Suwanee, GA, USA).

2.2 Remedy Materials and Formulation

Goat milk whey was purchased from Split Creek Farm (Anderson, SC, USA). Goat milk was obtained from a mixed herd of all breeds recognized by the American Dairy Goat Association, including Nubians, Alpines, Nigerian Dwarf, Saanen, Lamancha, Oberhasli, and Toggenburg breeds. Goats were over day 107 of lactation cycle and were fed a local custom grain blend, fresh hay, with no added hormones or pesticides. After culture and rennet were added to pasteurized milk, the cheese curds were dipped into straining containers to then be pressed. Whey was strained out during the process of dipping the curds, chilled to 4C for transportation and stored at -20C until used.

Dried plant materials of *Artemisia vulgaris* L. and *Plantago major* L. were purchased from Starwest Botanicals, Inc. (Sacramento, CA, USA), while dried *Lamium album* L. was purchased from a wild harvester (Ontario, Canada) due to the fact that *Lamium purpureum* L. was not available. Approximately 60 g of each dried plant was placed in 3.6 L of either goat whey or water, brought to a boil, and reduced at low heat until the final volume of 1.2 L. Vehicle control whey was reduced in a similar manner. Reduction of mixtures down to 1/3 volume was the common practice described in *Meddygon Myddfai,* along with the use of "handfuls" for dry materials, roughly 30 g, which is also equivalent to an apothecary's ounce. Mixtures were then centrifuged at 3000 RPM for 10 minutes to simulate straining, and stored at -20C. Aliquots of 20 ml were freeze dried and taken for use in cell culture assays and chemical analysis. Cell culture

stocks (100 mg/ml) were serially diluted in a three-fold manner to generate a dose response curve (100, 33.3, 11.1, 3.7, 1.23 mg/ml).

2.3 HPLC-UV Analysis

HPLC-UV analysis was performed using a Shimadzu HPLC system equipped with a pump (LC-20AT), an autosampler (SIL-20A), a diode array detector (SPD-M20A) and an automatic column temperature control oven (CTO-20A). Separation was performed on Restek Ultra C18 column (250 x 4.6 mm, 5 μ) at a column temperature of 30°C. The binary mobile phase consisted of 0.1% formic acid in water (Eluent A) and acetonitrile (Eluent B) in a gradient as follows: 0–5 min, 10% acetonitrile; 5–35 min, 10–55% acetonitrile; 35–37 min, 55-95% acetonitrile; 37–39 min, 95% acetonitrile; 39–40 min, 95-10% acetonitrile; 40–45 min, 10% acetonitrile. Each run was followed by equilibration time of 10 min. Ultraviolet (UV) spectra were monitored at 340 nm, and the flow rate was 1.0 mL/min. The data were collected and analyzed with LC solution (Shimadzu) software. Peaks were identified based on comparison of retention times and UV spectra with those of authentic standards.

2.4 Experimental design of colitis model

All animal experiments were performed according to procedures approved by the NC Research Campus Institutional Animal Care and Use Committee in the David H. Murdock Research Institute, an AAALAC accredited animal care facility. Male, 6-week-old C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed four animals per cage under controlled temperature (24 ± 2 °C) and light (12 h light-dark cycle, lights on at 0700 h). Immediately upon arrival, animals were allowed to adapt to new conditions for 7 days, and

daily animal handling was performed during this time to reduce the stress of physical manipulation.

Mice (n=72) were then randomized into 9 groups (n=8/group): healthy naïve control, DSS disease control, 5-ASA reference drug control, goat whey vehicle control, original recipe (all 3 herbs combined and extracted in goat's whey), aqueous extract (all 3 herbs combined and extracted in water to eliminate the gastrointestinal effects of whey), and aqueous extracts of individual botanical herbs in water to estimate individual contribution of each herb to the gastrointestinal health outcomes. Healthy animals received acidified water only (pH 3), while all other groups received 3.5% DSS water (pH 3) for the first 5 days of the study, to induce colitis. Mice had *ad libitum* access to food and liquid treatments. The body weight, incidence of diarrhea, bleeding, and water and food consumption were monitored daily throughout the experiment. Disease development was assessed using the Disease Activity Index (DAI), which considers three parameters: weight loss, stool consistency, and either blood in the perianal region or occult blood in the stool.

At the end of the study, the animals were euthanized by cardiac puncture after CO_2 inhalation. The colon was then removed aseptically and washed with saline solution (0.9%), and the length from the ileocecal junction to the anal margin was measured. Histology was performed on paraffin embedded, 5 μm-thick cross sections stained with hematoxylin and eosin. The microscopic score of inflammation was calculated as previously described (Erben et al., 2014). Briefly, this score comprised the sum of changes in mucosal architecture, neutrophil infiltration, mononuclear cell infiltration, goblet cell loss and epithelial defects. The histological active disease score comprised the sum of neutrophil infiltration and epithelial defects, reflecting the activity of disease.

2.5 Dosing calculations

Whey solids contributed on average 97 mg/ml dry matter to the original recipe (122.7 mg/ml dry matter). The remaining dry matter (25.7 mg/ml and 36.9 mg/ml for whey and water vehicles, respectively) was contributed by each botanical herb extracted in water in the following proportion: mugwort (17.5 mg/ml), plantain (9 mg/ml), and dead nettle (6.1 mg/ml). The average water intake did not differ significantly between the control (8.7 ml/mouse/day) and treatment groups (8.4-9.6 ml/mouse/day). When adjusted for liquid intake, mice consumed on average 8.9 ml of the original recipe or 1177 mg/mouse/day recipe dry solids, with 936 mg/mouse/day contributed by whey solids and 240 mg/mouse/day of dry solids contributed by three herbs used in the extraction. When translated to humans using a 12.3 HED human/mouse conversion factor (Reagan-Shaw et al., 2008), this treatment was equivalent to consuming 3.8 grams of herbal aqueous dry solids (52% mugwort, 27% plantain, 21% dead nettle) in 31.2 ml of goat whey daily for an average adult.

2.6 RNA extraction and qPCR

Total RNA was extracted from snap frozen colon using TRIzol reagent (Life Technologies) following the manufacturer's instructions. RNA was quantified using the Synergy H1/Take 3 spectrophotometer (BioTek, Winooski, VT, USA). The cDNAs were synthesized using 1 μg of RNA for each sample using a commercially available high-capacity cDNA Reverse Transcription kit (Life Technologies), following the manufacturer's protocol on an ABI GeneAMP 9700 (Life Technologies). Real-time RT-PCR was performed for murine COX2, iNOS, TNF-a, and IL6. Murine β-actin was used as housekeeping gene. PCR amplification for each sample was performed in duplicate wells. Quantitative PCR (qPCR) amplifications were

performed on an ABI 7500 Fast real-time PCR (Life Technologies) using 1 cycle at 50 °C for 2 min and 1 cycle of 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The dissociation curve was completed with 1 cycle of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C. mRNA expression was analyzed using the ΔΔCT method and normalized with respect to the expression of the β-actin housekeeping genes using 7500 Fast System SDS software v1.3.0 (Life Technologies). A value of <1 indicates transcriptional down-regulation (inhibition of gene expression) compared with LPS, which shows maximum genetic induction. Values >1 imply overexpression of the particular gene in excess of LPS stimulation. Amplification of specific transcripts was further confirmed by obtaining melting curve profiles. To avoid interference due to genomic DNA contamination, only intron-overlapping primers were selected using Primer Express version 2.0 software (Applied Biosystems, Foster City, CA, USA) as follows: β-actin, forward primer 5′-AAC CGT GAA AAG ATG ACC CAG AT-3′, reverse primer 5′-CAC AGC CTG GAT GGC TAC GT-3′; COX2, forward primer 5′-TGG TGC CTG GTC TGA TGA TG-3′, reverse primer 5′-GTG GTA ACC GCT CAG GTG TTG-3′; iNOS, forward primer 5′-CCC TCC TGA TCT TGT GTT GGA-3′, reverse primer 5′-TCA ACC CGA GCT CCT GGA A-3′; IL6, forward primer 5′-TAG TCC TTC CTA CCC CAA TTT CC-3′, reverse primer 5′-TTG GTC CTT AGC CAC TCC TTC-3′; IL1β, forward primer 5′-CAA CCA ACA AGT GAT ATT CTC CAT G-3′, reverse primer 5′-GAT CCA CAC TCT CCA GCT GCA-3′; TNF-α, forward primer 5′-GTT CTA TGG CCC AGA CCC TGA CA-3′, reverse primer 5′-TAC CAG GGT TTG AGC TCA GC-3′. TLR4, forward primer 5′ -CCA GAG CCG TTG GTG TAT CT-3′, reverse primer 5′- TCA AGG CTT TTC CAT CCA AC-3′.

2.7 Microbiome Analysis

Fecal pellets were collected from all mice at three time points (0,5,9 days after DSS administration). Genomic DNA was extracted from mouse fecal samples using QIAamp Fast DNA Stool Minikits (Qiagen, Germantown, MD, USA), quantified using Take3 plate and Synergy H1 microplate spectrophotometer (BioTek, Sunnyvale, CA, USA), and adjusted to 1 ng/μL. Quantitative real-time PCR was performed on an ABI 7500 Fast (Life Technologies, Carlsbad, CA, USA) in a total volume of 20μL containing 10μL 2×SYBR Green PCR Master Mix, 1μL of each primer, 4.4μL of nuclease-free water and 3.6μL of template DNA. The amplification program consisted of 50◦C for 2 min; 95◦C for 10 min; 40 cycles of 95◦C for 15s and 60◦C for 1 min; and a dissociation curve (95◦C for 15s, 60◦C for 15s, then increasing to 95◦C at 2% rate). The mean Ct-value was determined based on a set threshold value of 0.2 and using the automatic baseline correction. Differences in Ct-values for each bacterial target (N0-normalization) were calculated between those obtained with the universal and target-specific primers and log-transformed. Fold-changes for target amplicons were calculated as the (log 2) ratio of normalized abundances at different time points. Bacterial DNA concentrations were estimated by quantitative PCR assays using primers for the specific bacterial phylum Firmicutes, Bacteriodetes, Actinobacteria, Proteobacteria, Verrucomicrobia, and Euryarchaeota. After initial screening of phylum, substratum of these groups were tested to include *Lactobacillus, Clostridium clusters I, IV, XIVa, Eubacterium, Roseburia, Dorea, Ruminococcous cluster IV, Enteroccocus, Blautia, Faecalibacterium, Bacteroides, Parabacteroides, Prevotella, Alistipes, Bifidobacterium, Enterobacteriaceae, Esherichia, Desulfovibrio, Akkermansia,* and *Methanobrevibacter.*

2.8 Cell Culture

The mouse macrophage cell line RAW 264.7 (ATCC TIB-71, obtained from American Type Culture Collection, Livingstone, MT, USA) was maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, NY, USA), supplemented with 100 IU/mL penicillin/100 μg/mL streptomycin (Fisher) and 10% fetal bovine serum (Life Technologies) at a density not exceeding 5 × 105 cells/mL and maintained at 37 °C in a humidified incubator with 5% CO2. Human colorectal adenocarcinoma cells (HT-29), obtained from American Type Culture Collection (Manasses, VA), were cultured and maintained in DMEM supplemented with 10% FBS and 1% antibiotic in a 5% CO2 environment at 37°C. Cells were routinely passaged every three to four days at <90% confluence and only passages 2-16 were used for cell proliferation studies.

2.9 Nitric oxide (NO) radical inhibition assay

The abilities of test samples to inhibit nitric oxide radical formation in activated macrophages were determined by a colorimetric assay. The cells were seeded in 96-well plates 24h prior to treatment and then treated with extracts at 200, 100, 33.3, 11.1, 3.7, 1.23 μg/mL for 1h before elicitation with LPS at 1 μg/ml for an additional 4h. For every experiment, one positive control (dexamethasone at 10 μM) and one negative control (DMSO at a final concentration of 0.1%) were included. Three replicates were made for both the treatments and the controls. Subsequently, 100 μL of culture media was mixed with 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthyl-ethylenedia-mine in 2% phosphoric acid), and the mixture was incubated at room temperature for 10 min and then read on a microplate reader at 540 nm.

Absorbance was compared against a calibration curve created with serial dilutions of sodium nitrite ($R^2 = 0.999$.)

2.10 Cell Proliferation Assay

The abilities of test samples to inhibit the growth of HT-29 human colon cancer cells formation was determined by a colorimetric assay utilizing SRB staining. The cells were seeded in 96-well plates 24h prior to treatment and then treated with extracts at 100, 33.3, 11.1, 3.7, 1.23 µg/mL once a day for 72 hours. For every experiment, one positive control (PBS) and one negative control (DMSO) was included. Three replicates were made for both treatments and the controls. HT-29 cells were seeded at a concentration of 1x104 cells per well in a 96 well plate with 100µL of complete media. Cells were incubated for 4 hours to allow cell treatments to be prepped and digested. The extracts were administered at a concentration of 100µg/mL in a 100µL vehicle of 0.7% DMSO, 1.17% double distilled water plus enzyme and complete media. A DMSO only vehicle control and a paclitaxel positive control, (Sigma- Aldrich, St. Louis, MO,) were used to validate each assay and compare to test samples. Cells were allowed to grow uninhibited for 72 hours in a 5% CO2 environment at 37°C.

Following the incubation period, the media was removed and living cells were fixed to the plate with 10% trichloroacetic acid (TCA) (Sigma-Aldrich, St. Louis, MO,) for 1 hour at 4°C. Plates were then washed four times with H2O to remove dead cells and dried overnight. The colorimetric assay was performed by adding 100µL of 0.056% SRB dye Sigma-Aldrich, St. Louis, MO, in 1% acetic acid treatment for 30 minutes to stain the remaining cells. Dye solution was then removed and plates were washed with 1% acetic acid four times to remove any residual dye. The remaining SRB dye bound to cells could then be extracted with addition of 200µL of

10mM Trizma (Sigma-Aldrich, St. Louis, MO), solution and rotation for 30 minutes. Extracted dye was moved to a new plate and measured at 510nm with a BioTek Synergy H1 spectrophotometer.

2.11 Statistical Analyses

Statistical analysis was performed using Prism v7 (Graphpad Software). Data were analyzed by one-way ANOVA with treatment as a factor. Post hoc analyses of differences between individual experimental groups were made using Dunnett's multiple-comparison tests. Significance was set at $p < 0.05$. Values are reported as means ± SEM

3. Results

3.1 Characterization of the original Medieval Welsh herbal remedy

Myddfai herbal does not specify the exact amounts of herbs and goat's whey used in both the throat (*for hoarseness*) and gut (*for stomach ache or colic*) recipes. Based on the other recipes present in the manuscript, a standard dosing was assumed that translated to an equal amount (*two handfuls*) of each herb in a gallon of whey (60 g dry polyherbal mixture in 3.6 L of whey), reduced by boiling (*boil down to a third*) to 1.2 L and strained. This procedure was performed twice with two different vehicles, goat whey and water, to investigate the changes in phytochemical profiles obtained with each solvent, and the contribution of each herb to the mixture (**Figure 1**). Whey solids contributed on average 97 mg/ml dry matter to the original recipe (122.7 mg/ml dry matter). The remaining dry matter (25.7 mg/ml and 36.9 mg/ml for whey and water vehicles, respectively) was contributed by each botanical herb extracted in water

in the following proportion: mugwort (17.5 mg/ml), plantain (9 mg/ml), and dead nettle (6.1 mg/ml). The HPLC profiles confirmed the polyherbal nature of the mixture and an additive nature of the aqueous extraction with all major phytochemical peaks present both in the individual extracts and the combined recipe in a similar proportion.

3.2 Anti-inflammatory activity of the recipe and its components

In murine RAW264.7 macrophages, the *Myddfai* remedy showed a dose dependent inhibition of the LPS-induced nitric oxide levels, achieving a maximum of 31.78% reduction at 100 μg/ml (**Figure 2**). The additive effect of mugwort (-14.03%), red nettle (-13.41%), and whey (-8.45%) contributed to the observed effect, while plantain remained largely ineffective in this in vitro assay (-3.24%).

3.3 Gastrointestinal epithelial cell survival

In HT-29 human colon epithelial cells, the *Myddfai* remedy and its individual components showed a set of opposing effects. While aqueous extracts of the botanical herbs and their combinations was anti-proliferative in a dose dependent manner with maximum inhibition of 74.49% at 200 μg/ml, this effect was largely counteracted by goat whey. Alone, goat whey showed a potent dose-dependent response on gastrointestinal cell survival in the dose range of 1.23-200 μg/ml (**Figure 3**).

3.4 Medieval Welsh herbal remedy ameliorates symptoms of DSS-induced colitis in mice

To evaluate the potential effect of the *Myddfai* remedy in DSS-induced colitis, the animals were treated with the herbal mixture in whey, vehicle control (whey), disease control

(DSS), or drug control (5-ASA). When compared to healthy mice (naïve control group), animals that received DSS in drinking water lost 9.5% bodyweight by day 5 and this extended up to 13.7% weight loss at day 9. The same holds for the DAI (a function of body weight), where the DSS group presented visible blood in the stool (diarrhea), as well as a colon length reduction (24.2%), when compared to the naïve control group (**Figure 4**).

The *Myddfai* remedy significantly reduced the body weight loss and DAI from day 5 (9.9%) to day 9 (1.6%). The *Myddfai* remedy also prevented the reduction of colon length (average 8.9 cm) when compared to DSS disease control group (average 5.8 cm) and diminished the presence of occult blood in the feces when compared to DSS group. The vehicle control group (whey) reduced body weight loss less effectively from day 5 (8.28%) to day 9 (3.50%) when compared to the *Myddfai* remedy group, and similarly was less effective in preventing colon shortening (average 7.3 cm). Thus, the addition of herbs to whey provides an enhanced effect against DSS induced colitis.

The histological evaluation of colon specimens confirmed that intestinal anti-inflammatory effects were exerted by the *Myddfai* remedy. When compared to normal colonic architecture, the DSS group showed signs of intense transmural inflammation involving severe leukocyte infiltration of both the mucosa and submucosa layers. Ulceration was noted, especially in colonic crypts in which abscesses were present. Blood and erosion were present along the lumen. Mice treated with the *Myddfai* remedy, showed partial recovery with lower levels of inflammatory infiltrate and improved colonic architecture resulting in a significantly lower histopathological score than the DSS control group. Whey alone exhibited improvement in epithelial architecture more than improvement of inflammatory infiltrate, resulting in a higher histological score when compared to the *Myddfai* remedy group, suggesting that the plants in the

recipe have anti-inflammatory properties and that whey may contribute more to improve cell survival.

3.5 Contribution of individual components to the observed effects

Upon observation of anti-inflammatory effects and colitis amelioration of the *Myddfai* remedy, we sought to discern whether the activity of the remedy was due to one ingredient or due to some combination of more than one ingredient. The animals were treated with mugwort, red nettle, and plantain in water, as well as the mixture of all three plants in water to be compared to the naïve and DSS control groups (**Figure 5**).

When compared to DSS mice (13.7% body weight loss), each individual plant showed some ability to reduce body weight loss and DAI. Mugwort appeared to be the most active of all plants in the recipe in preserving bodyweight (2% weight loss) potentially due to its hemostatic activities (Ohkura et al., 2015). Plantain (4.58% weight loss) and red nettle (5.5% weight loss) were less effective. The water mix (5.4% weight loss) was comparable to plantain and red nettle, suggesting that mugwort had become somehow muted. It is possible that some of these plants could also be exerting diuretic effects resulting in water weight loss in the mice. Taken together with whey (3.50% weight loss), mugwort seems to make up the active principle for preventing weight loss in the original whey-based *Myddfai* mixture (1.6% weight loss).

Each individual plant extract and the water-based mixture improved colon length. Plantain (9.0 cm) and red nettle (8.8 cm) exerted the strongest effect, comparable to the whey based *Myddfai* remedy (8.9 cm). Mugwort (8.0 cm) was less effective, as was the water-based mixture (8.4 cm), suggesting that whey may contribute to the improvement of colon length when combined with red nettle and/or plantain.

Histological scoring and microscopy analysis indicated a strong differential effect between plants in the recipe. The *Myddfai* remedy (score 2), the mix in water (score 2.33), and plantain (score 2) all showed similar scores, indicating that plantain was the main contributor to reducing inflammatory cell infiltration to the mucosa and damage to both the epithelial and mucosal architecture of the colon. *Plantago* spp. leaves and seeds can contain up to 30% insoluble mucilage by weight. Mucilage may improve lower bowel transit time by adding bulk to the stool (Samuelsen, 2000).

3.6 Reduced expression of anti-inflammatory mediators

The intestinal anti-inflammatory effect of the *Myddfai* recipe was also confirmed by qPCR of snap frozen colonic tissue. Increased mRNA expression of COX-2, iNOS, IL6, and TLR4 was observed in the disease control group as compared to naïve mice. After receiving treatment with the *Myddfai* remedy, expression of these inflammatory factors decreased by 2-4 fold. The expression of TNF-a, IL1B were not increased in the disease control suggesting that the early response inflammatory signaling pathways have been downregulated in disease and treatment groups by day 9 of DSS exposure and recovery (**Figure 6 & 7**).

3.7 Changes in microbiome profiles associated with colitis and the *Myddfai* remedy

The microbial community profiles observed in individuals with IBD is characterized by certain bacterial species (Swidsinski et al., 2009). To evaluate the effect of the *Myddfai* remedy on the microbiome, fecal bacterial DNA was quantified by real time PCR (**Figure 8**). At the phylum level, the induction of colitis by DSS shows an increased presence of Firmicutes in all groups (day 5) at the expense of other intestinal flora, especially the Bacteroidetes.

Bacteroidetes, specifically the butyrate producing genus *Bacteroides,* have been implicated to protect the colon from DSS induced colitis in multiple studies (Rath et al., 1996; Chiu et al., 2014; Lee et al., 2018; Delday et al., 2019). After receiving treatment with *Myddfai* remedy and 5-ASA members of the Bacteroidetes were restored beyond the starting population on day 0. Groups receiving whey alone showed no restoration of Bacteroidetes, demonstrating that whey is more beneficial by protecting epithelial cells. This would indicate some microbiome modulatory activity of the *Myddfai* remedy and provide some explanation into a cursory mechanism of action in protecting the colon from acute damage. The expansion and contraction of Firmicutes groups could be primarily associated with changes in *Clostridium Cluster XIV* bacteria.

4. Discussion

Due to the fact that medieval Welsh physicians formulated recipes utilizing plant derived compounds that can attenuate clearly recognizable gastrointestinal health issues, it is reasonable to conclude that some medieval physicians used observation and experience to design or choose effective remedies, creating an empirical tradition of scholarship and rational methodology often overlooked by modern science (Harrison et al., 2015; Wagner et al., 2017, 2020). While these physicians were certainly unaware of the molecular mechanism of action of these plants, in this case, it is clear that they were aware of the beneficial effects of mugwort, red nettle, plantain, and goat's whey to improve epithelial health outcomes. This is further evidenced by the usage of the same recipe for hoarseness, or inflammation of the larynx.

Generalized anti-inflammatory activity is a not a sufficient mechanism for explaining the anti-colitic effect of a substance. For true amelioration of a multi-faceted disease, multiple

symptoms and likely multiple targets must be addressed. To start, it is likely that weight loss and diarrhea are mediated by an increase in butyrate producing bacteria of the gut microbiome spurred on by plant pectic poly- and oligosaccharides.

Studies utilizing germ free mice have confirmed that while the intestinal microbiome is not necessary for the induction of acute DSS induced epithelial damage, it is however a crucial component in the development of colitis as a classical disease (Ahmad, 2000; Kitajima et al., 2001). Interestingly, germ free mice often die from hemorrhage, without showing signs of inflammation or other symptoms while conventional mice show rapid onset of symptoms but a lower rate of mortality (Kitajima et al., 2001; Yu, 2018). From these studies it can be surmised that commensal flora aid in the turnover of injured epithelial cells and possibly protect from chemical irritants, but likely also cause transmural inflammation and from there localize host immune factors such as macrophages, leukocytes, and T cells. DSS, a sulfated polysaccharide, may increase colonic availability of sulfate and may stimulate sulfate reducing bacteria (phylum Deltaproteobacteria and Firmicutes) to generate higher sulfide levels, which are eventually toxic to the colonic mucosa and its bacterial contents. Similarly, carrageenan, a sulfated polysaccharide, induces colitis in normal but not in germ free mice. Sulfate reducing bacteria are increased in the colon of patients with ulcerative colitis (Ahmad, 2000).

Abnormal butyrate oxidation has been noted in mice treated with DSS, leading to a large reduction in cell energy, and preferential use of glucose, which may cause further inflammation due to mitochondrial stress. Reducing sulfur compounds, principally sulfide, impair butyrate oxidation by human and rat colonocytes. Impaired short chain fatty acid (SCFA) metabolism also leads to dysbiosis of the colon and causes an increase in the Firmicutes and a decrease in Bacteroides (Tingirikari, 2018). A host of beneficial effects have been attributed to butyrate,

including dose dependent anti-inflammatory activity, and thus the levels of bacteria that produce them can help indicate the severity of disease state (Chen et al., 2018; Onyszkiewicz et al., 2019). Butyrate producing bacteria such a *Clostiridum* have been implicated to help protect against colitis and sodium butyrate helps maintain intestinal barrier integrity via homeostasis of the epithelium (Lopetuso et al., 2013). Diarrhea associated with ulcerative colitis (UC) occurs primarily as a result of reduced Na+ absorption. Colonic Na+ absorption is mediated by butyrate (Rajendran et al., 2015). Notably, In the western population often lacking adequate dietary fiber, the ratio of SCFAs is 60 : 25 : 15 for acetate, propionate, and butyrate, respectively (Neis et al., 2019).

SCFA synthesis utilizing oligosaccharides occurs in the proximal colon, while polysaccharides must be broken down and transported before SCFA synthesis can occur in the distal colon (Tingirikari, 2018). It has been noted that the highest blood levels of SCFA are found in the inferior mesenteric vein (Cummings et al., 1987). Slow fermenting fibers that increase SCFAs specifically in the distal colon are expected to have higher potential for influencing host metabolism given the much higher SCFA release by the distal colon (den Besten et al., 2013). The more complex the substrate, the greater the chance it will reach the distal part of the colon and stimulate the growth of Firmicutes and Bacteroides, producing SCFAs, to exert a beneficial effect on the health of the host.

Colon shortening could be mediated by increased blood perfusion to the colonic epithelium via mesenteric blood supply. Roughly 25% of the body's blood supply is in the intestines at any given moment (Mortillaro et al., 1980). Changes in mesenteric artioles cause fluctuating blood flow. Mesenteric strangulation and/or vascular occlusion may lead to increased intraluminal pressure and ultimately shortening of the bowel in the condition known as

ischemic colitis. Interestingly, DSS has been shown to cause ischemic conditions in the lamina propria of the colon and subsequent expression of iNOS in myenteric neurons, the major nerve supply of the gastrointestinal tract (Saijo et al., 2015).

The lactokinins found in whey are peptides that have demonstrated angiotensin converting enzyme (ACE) inhibitory activity (FitzGerald and Meisel, 1999; Brandelli et al., 2015; Ibrahim et al., 2017). Goat whey peptides such as b-lactoglobulin fragments compare favorably with the activity of captopril on weight basis (Ibrahim et al., 2017). While it is unlikely that whey protein reaches the colon intact, it may influence the mesenteric blood supply and vascular resistance, as has been demonstrated by other peptides (and also butyrate) in man (Bremholm et al., 2009). Whey likely does not improve microbiome profile, but instead works to improve blood flow to the colon and protect the cells of the mucosa (Brandelli et al., 2015). Whey protein has been demonstrated to induce greater mucosal surface area and protection of epithelial cells, perhaps due to strong anti-oxidant activity demonstrated by peptides (Ahmed et al., 2015; Brandelli et al., 2015). Amino acids derived from dietary protein in general, are needed for mucosal healing after an inflammatory episode, as well as for energy substrates to maintain body weight.

While the anti-inflammatory mechanism of 5-ASA has not been fully elucidated, several have been proposed (Punchard et al., 1992). It is likely diverse and multi-targeted, most often involving the inhibition of the expression of classical inflammatory mediators, particular the cyclooxygenase-prostaglandin axis. Aminosalicylates are also known inhibitors of lipoxygenase, platelet-activating factor, interleukin-1, nuclear factor jB, tumor necrosis factor activation, B cells, and oxygen radical production, and a scavenger of reactive oxygen species (Hanauer, 2004). We have demonstrated that each individual plant, as well as both whey and water based

mixtures can down regulate some of these factors, and produce highly similar results to 5-ASA, especially with regard to the *Myddfai* remedy, red nettle, and plantain for attenuating colon length reduction, disease index, and histological score.

5. **Conclusions**

In summary, we have reconstituted a centuries old remedy for inflammation of the gut and have shown that it can improve the underlying causes of the condition it was designed to treat. The *Myddfai* remedy improved DSS induced colitis symptoms in mice, modulated the microbiome in a beneficial manner, and increased cell viability in an established *in vitro* model. This effect depended upon the combination of several ingredients specified in the recipe in goat's whey and their unique biochemical properties. Furthermore, a significant anti-inflammatory effect can be attributed to the *Myddfai* remedy based on colonic gene expression results, histological evaluation, and an *in vitro* model of inflammation, preforming in a similar fashion as the current clinical treatment (5-ASA).

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7. Figures

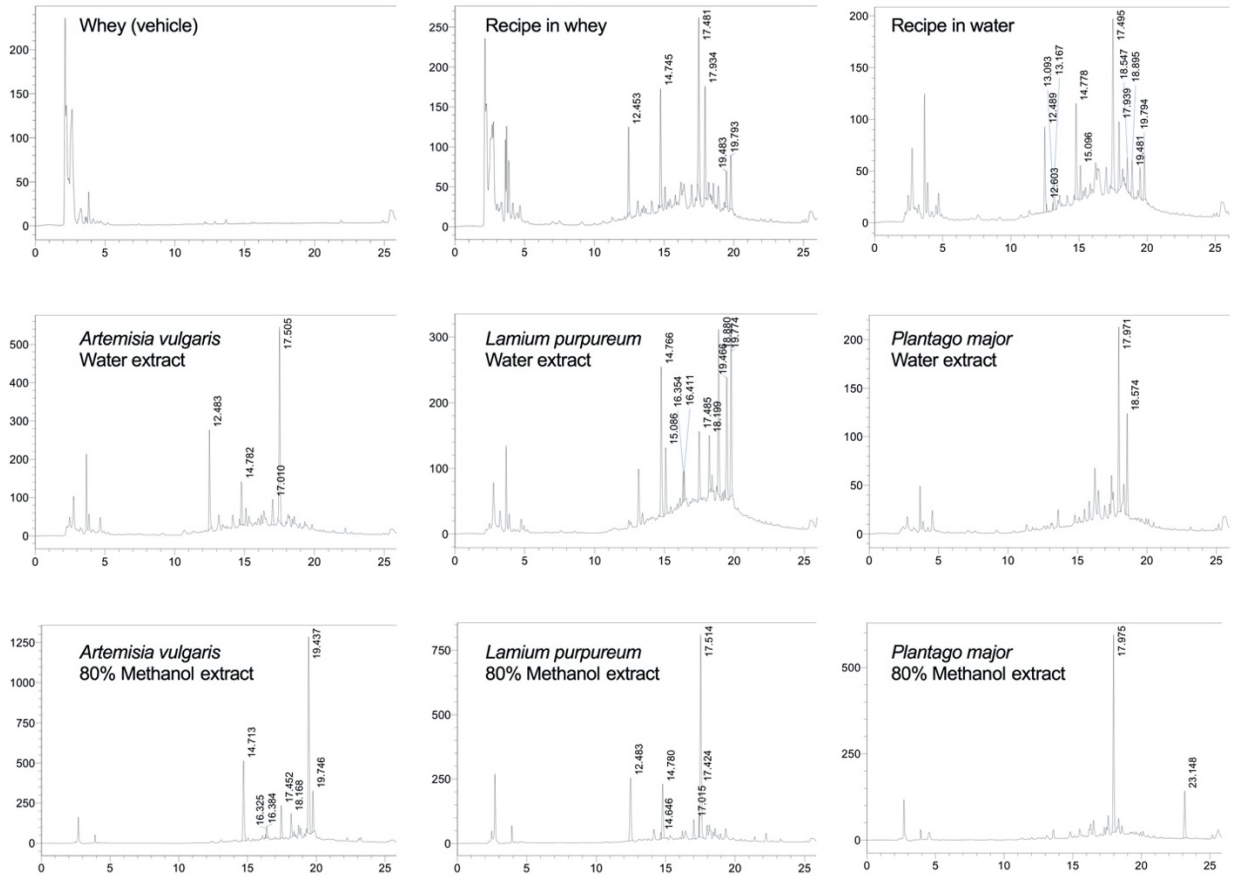

Figure 1. HPLC profiles of the *Myddfai* remedy and constituents. Spectral data confirmed the polyherbal nature of the mixture and an additive nature of the aqueous extraction with all major phytochemical peaks present both in the individual extracts and the combined recipe in a similar proportion. Note the difference in scale between whey and water extracts.

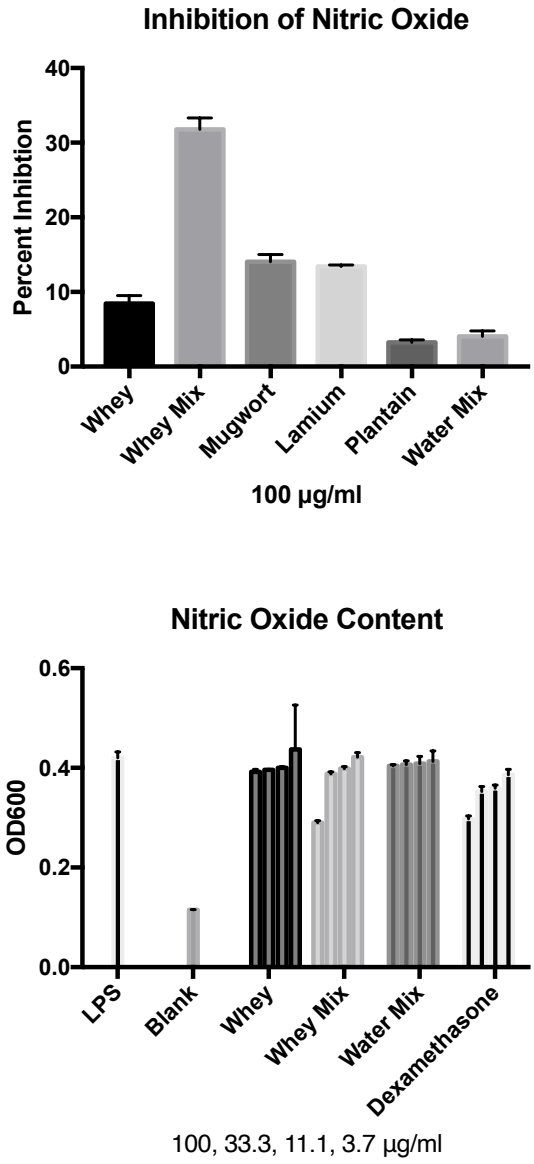

Figure 2. Dose dependent inhibition of LPS-induced nitric oxide levels exerted by the *Myddfai* remedy in murine RAW264.7 macrophages.

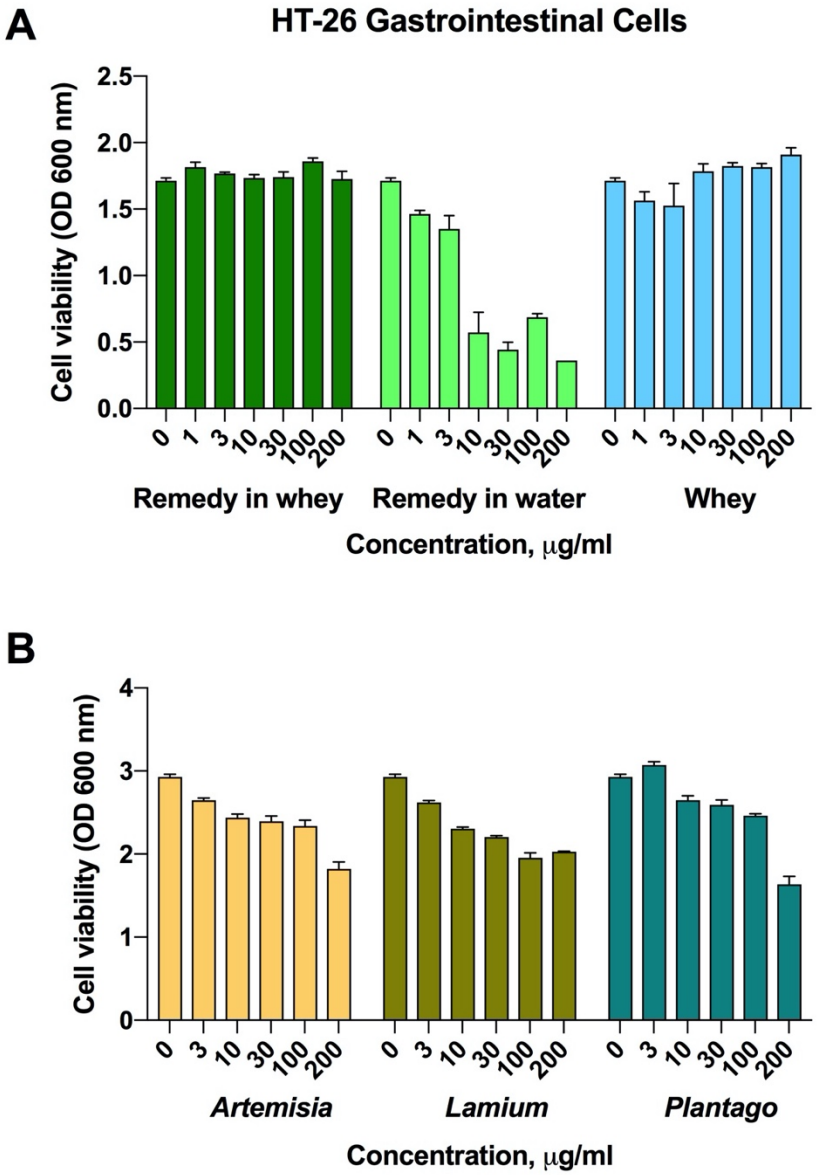

Figure 3. Differential effects of the *Myddfai* remedy and constituents in HT-29 human colon epithelial cells. Aqueous extracts of the herbs and their combinations was anti-proliferative in a dose dependent manner while goat whey showed a potent dose-dependent response on gastrointestinal cell survival.

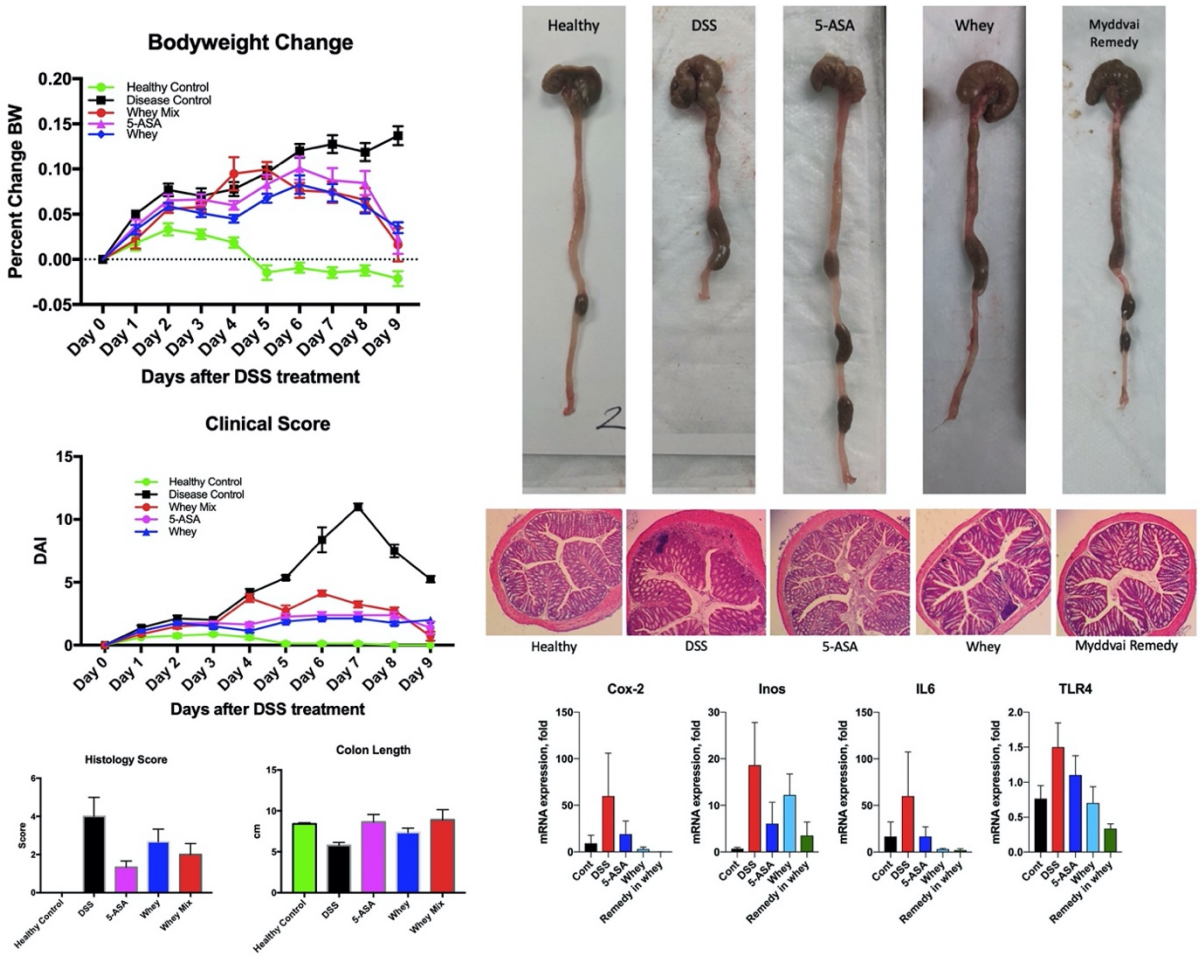

Figure 4. Effect of the *Myddfai* remedy on DSS-induced colitis in mice. Bodyweight change, disease activity index (DAI), histology score, and colon length constituted a panel of metrics by which the treatments were evaluated.

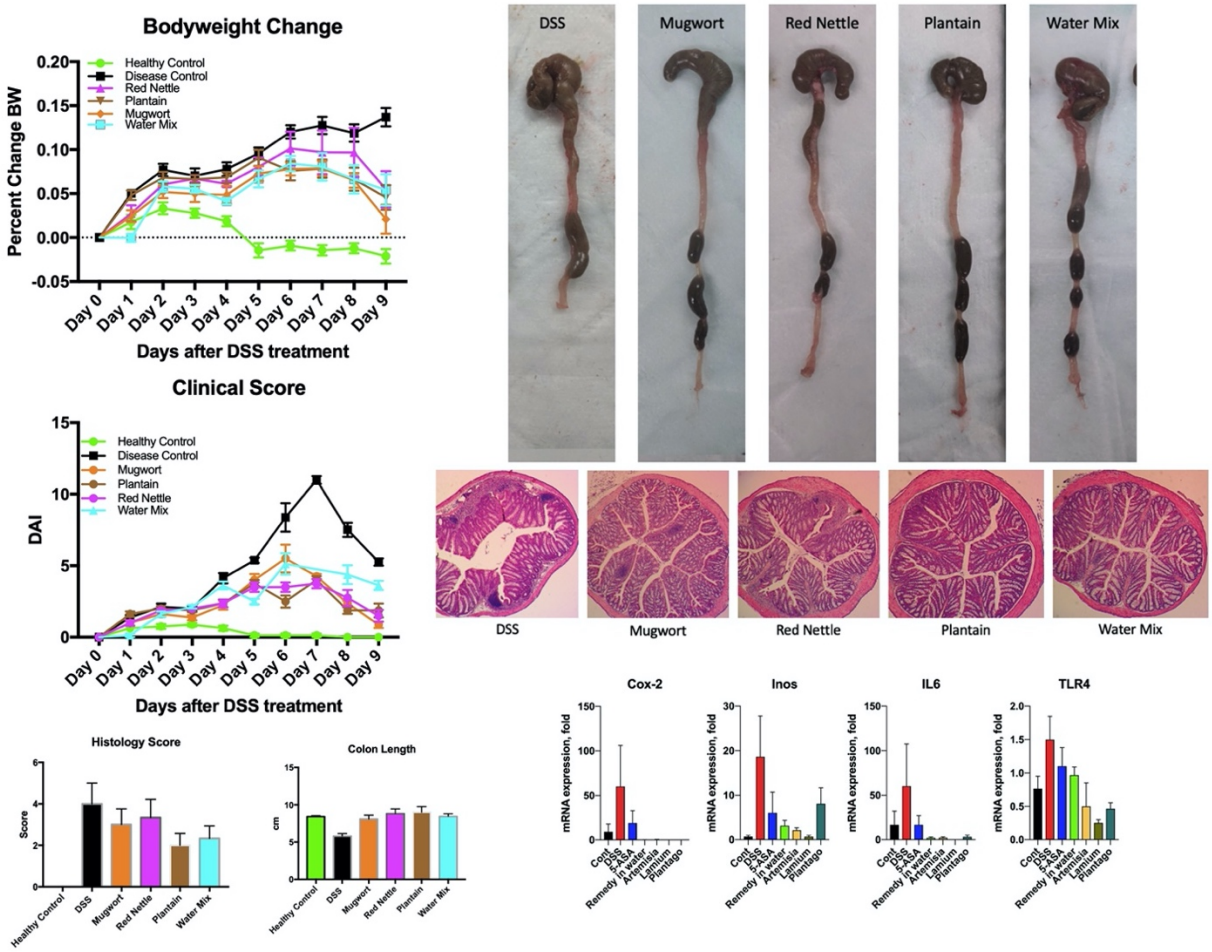

Figure 5. Effect of the individual components on DSS-induced colitis in mice. Bodyweight change, disease activity index (DAI), histology score, and colon length constituted a panel of metrics by which the treatments were evaluated.

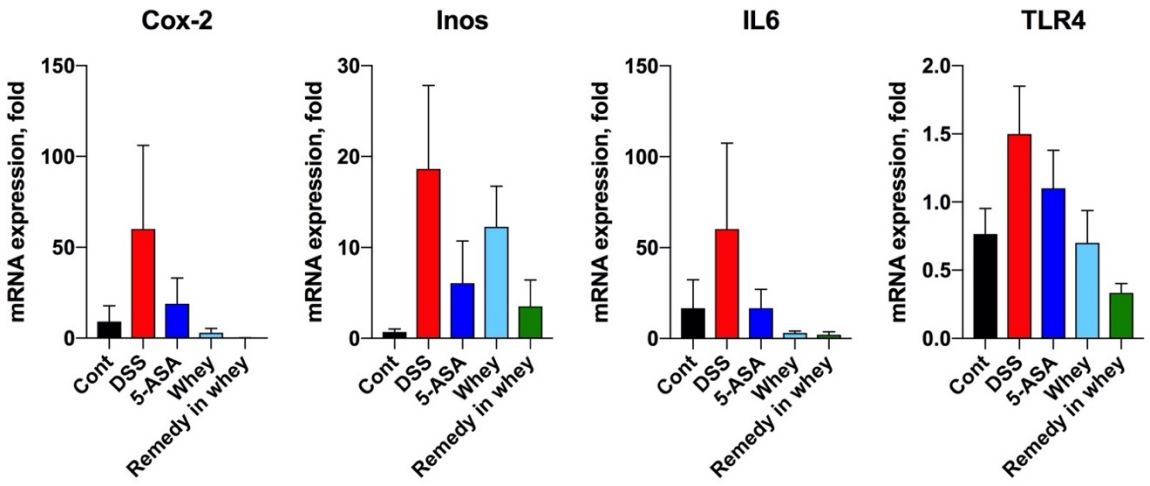

Figure 6. mRNA expression of anti-inflammatory mediators in response to the *Myddfai* remedy.

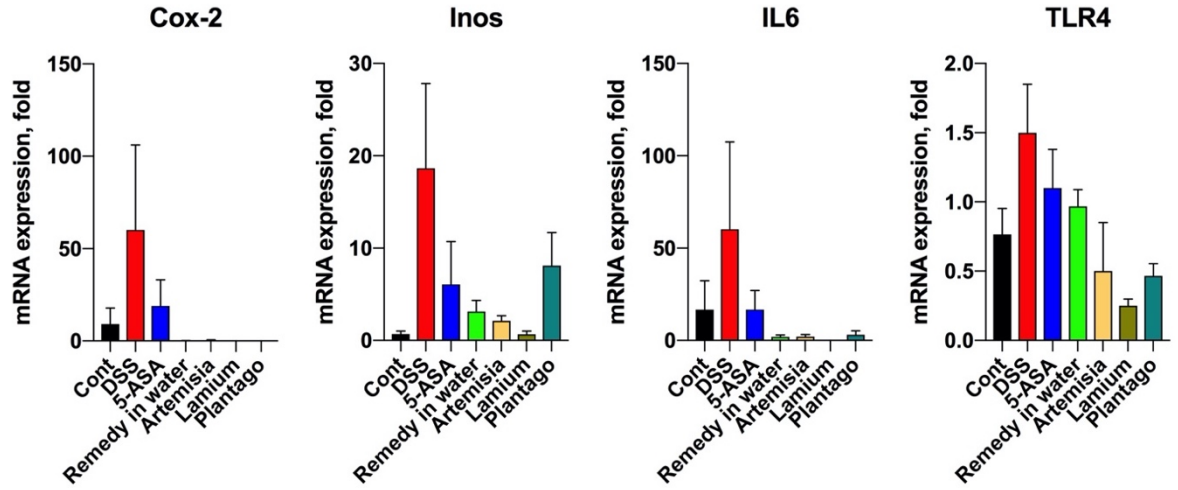

Figure 7. mRNA expression of anti-inflammatory mediators in response to individual components.

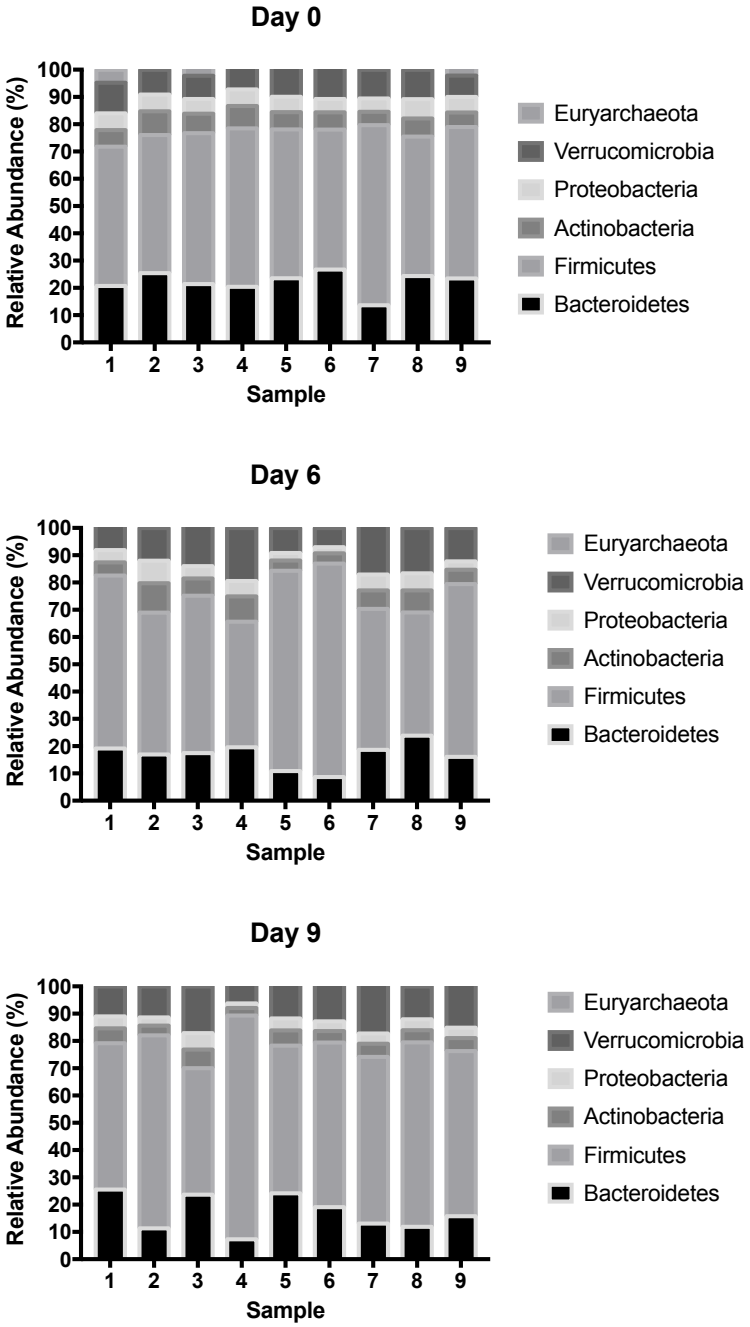

Figure 8. Changes in microbiome profiles associated with colitis and the remedy. Note the expansion of Firmicutes and decease in Bacteroidetes associated with DSS induced colitis. Whey alone did not influence the recovery of the Bacteroidetes.

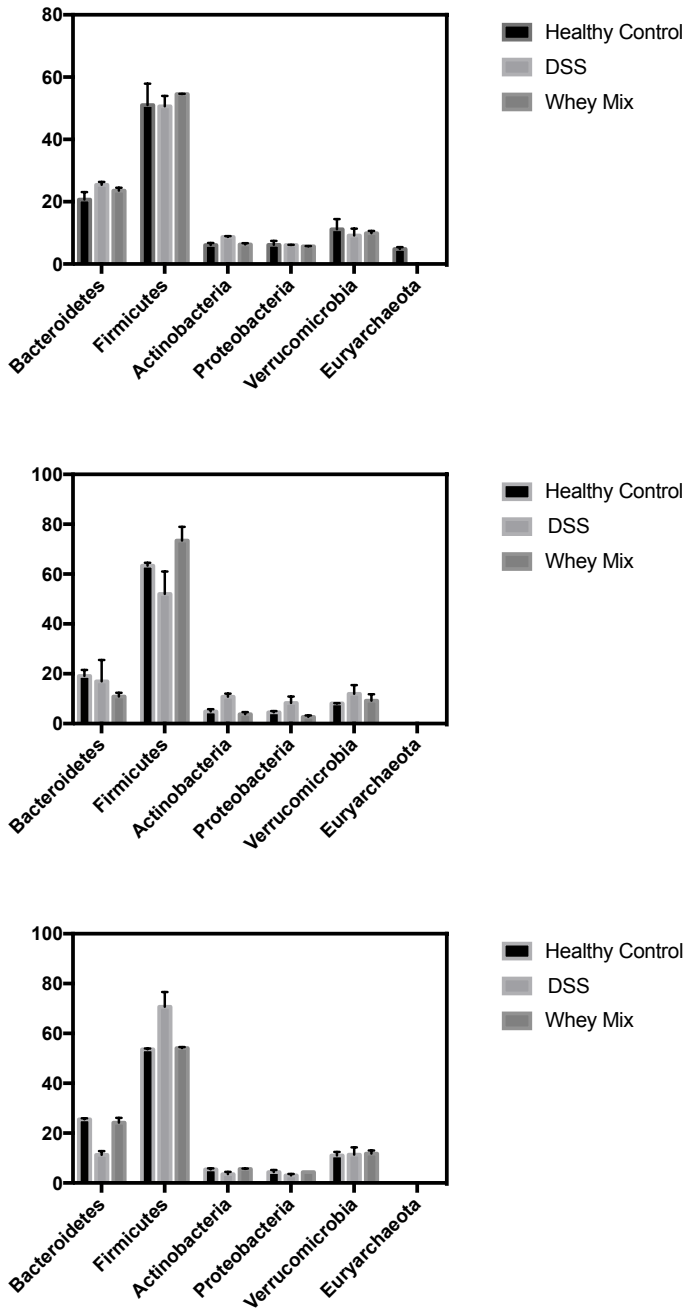

Figure 8 continued.

CHAPTER 4: ANTI-HYPERGLYCEMIC BITTER BIOACTIVES FROM OATS (*AVENA SATIVA* L.)

Charles Wagner, Xueying Xie, Thirumurugan Rathinasapabathy, and Slavko Komarnytsky

Abstract

Oats (*Avena sativa* L.) is an annual cereal grain with a long history of use as both food and medicine in Northern Europe. While claims that consumption of oat fiber can reduce the risk of heart disease have been substantiated, oats is also host to bitter compounds such as avenanthramides (AVNs) and phenolic acids (PAs) (ferulic, caffeic, p-coumaric, and chlorogenic acids). Bitter compounds have been suggested to improve glucose metabolism in humans, but this effect has not been confirmed using oat bitter bioactives. In this study, we quantified free, bound, and digested AVNs and PAs in 242 oat flour samples consisting of 109 accessions from the AFRI CORE diversity oat panel grown at two different locations (Lacombe, Alberta, CA and Aberdeen, Idaho, US.) We then evaluated a panel of oat varieties containing low, medium, and high levels of AVNs in the polygenic C57BL/6J mouse model of diet-induced obesity and diabetes. Acute oral administration of oat flour at 50-200 mg/kg resulted in a modest dose-dependent decrease in fasting blood glucose in a manner consistent with AVN content. Together with previous data from our laboratory, these data suggest oats containing high levels of AVNs stimulate T2R signaling in the GI tract to control acute postprandial hyperglycemia associated with metabolic syndrome by preventing glucose absorption and stimulating satiety hormone secretion following carbohydrate consumption, as demonstrated in the STC-1 cell culture model of intestinal glucose uptake. These results strengthen the case for the development of novel functional ingredients from oat varieties that preserve bitter tasting constituents to achieve immediate improved control over acute postprandial hyperglycemia.

1. Introduction

Over 26 million tons of oats (*Avena sativa* L.) were produced globally in 2017; 75% of which were used in animal feed applications (FAOSTAT, 2017; Nations, 2004). However, oats are a relatively well-known human health-supporting food, due to high levels of protein (avenalin, avenins) and fiber (β-glucan), as well as the presence of bioactive secondary metabolites such as avenanthramides (AVNs), avenacosides, avenacin, phenolic acid (PAs) derivatives (ferulic, caffeic, p-coumaric, and chlorogenic acids), vitamins, and minerals (Martínez-Villaluenga and Peñas, 2017). While the FDA allows the claim that consumption of soluble fiber in oats reduces the risk of heart disease, many of the other health supporting compounds in oats impart a bitter taste to the consumer and have subsequently been removed or reduced by the food industry through selective breeding and other debittering processes (Drewnowski and Gomez-Carneros, 2000; Gaudette and Pickering, 2013). With growing interest in functional foods and whole food supplementation, in which food products are consumed in a manner that preserves their natural health promoting properties, the selection and processing of oat varieties with high levels of evidence backed secondary metabolites such as AVNs is of principal importance for improving human health (Drewnowski and Gomez-Carneros, 2000; Gaudette and Pickering, 2013).

Oats have a long history of use as both food and medicine, mainly in Europe, where the oldest cultivated oats (*A. sativa*) were found in caves in Switzerland dating back to the Bronze Age (Murphy and Hoffman, 2015). From a medicinal standpoint, oats have long been used in improving gastrointestinal and skin health outcomes, as mentioned by Roman authors such as Pliny the Elder and Dioscorides, who otherwise scorned oats as a “barbarian” food (Dioscorides; The Natural History of Pliny, 1856). Favoring a cool and wet climate, oat production is mainly

concentrated between 35-65 degrees North of the equator, with successful production recorded as far as 70 degrees North (Nations, 2004). The staple consumption of oats (which was not common in continental Europe) has long been associated with the robust health of 17th through 19th century Scottish peasants (Martin et al., 1703; McNeill, 1957). Famously, Samuel Johnson noted in his dictionary (1755), "Oats. A grain, which in England is generally given to horses, but in Scotland supports the people." In another quote he states, "…such food makes men strong like horses, and purges the brain of pedantry."

Because yield is the primary factor influencing the selection of crop varieties (especially animal feeds), and bitterness is the primary factor influencing food rejection by consumers, the health modifying properties of oats have likely undergone dramatic changes due to human selection (Drewnowski and Gomez-Carneros, 2000; Pingali, 2012). Black oats (*Avena strigosa* Schreb.) were likely introduced to the north of Scotland in the Bronze Age and formed the staple crop throughout the medieval period increasing during the Viking Age, while common oats were introduced as late as the 18th century in some areas (Alldritt, 2003). Interestingly black oats have been shown to have higher levels of avenanthramides and phenolic acids than some common oat varieties (Smitterberg, 2018).

Even in the 19th and 20th century, intellectuals and doctors in Scotland lamented the new industrial processing of oats and the ever-growing preference for wheat. "Up until the middle of the last [nineteenth] century," lamented Lord Boyd Orr, director of the Rowett Nutrition Institute at Aberdeen in the early 1920s, "the people of Scotland were eating natural foodstuffs. With the introduction of machinery, this has been changed... Natural foods have been changed into artificial foodstuffs, with the very best substances purified away that the Almighty put there to keep us in perfect health." Similarly, Sir James Crichton-Browne, writes in 1901 in *Stray Leaves*

from a Physician's Portfolio, "At one time it [oats] was the mainstay of the Scottish peasants' diet and produced a big-boned, well-developed and mentally energetic race, but it is so no longer, having given way to less useful and economic foods, and in the case of children in the large towns. . . to tea and [wheat] bread with dripping, margarine or jam." Orcadian Marian McNeill also notes the preference for wheat as a societal problem, "…threatened by wheaten flour, the victory of which would be regarded by many as a national disaster." According to the WHO, the United Kingdom is now the most overweight country in Western Europe.

The selection of less bitter, more processed or refined foods, typified by the preference for white bread and sweets over whole grains, has been linked to increased risk for cardiovascular disease (CVD), type 2 diabetes (T2D), and obesity (Serra-Majem and Bautista-Castaño, 2015; Gaesser, 2019). One of the largest contributors to overall health, especially metabolic health, is the maintenance of proper glucose metabolism – or the body's ability to properly absorb and quickly transport dietary carbohydrates to the proper destination (Aronoff et al., 2004; American Diabetes Association, 2014). Poor glucose metabolism is characterized by elevated levels of fasting blood glucose and high postprandial glucose spikes due to the severely diminished first-phase insulin response as well as an increased rate of gastric emptying – these high postprandial glucose spikes also cause inflammation, neuropathy, CVD, blindness, and orthopedic problems (Aronoff et al., 2004; Collier et al., 2008).

Research has shown that bitter compounds, such as oat avenanthramides, can improve the oral glucose tolerance in both humans and mice, without the undesirable side effects and cost of conventional treatments (Kim et al., 2009; Habicht et al., 2011; Joseph and Jini, 2013; Street et al., 2013; Hou et al., 2015; Bozzetto et al., 2016; Banihani and Banihani, 2017; Cárdenas-Ibarra et al., 2017). Previous work by our lab has confirmed the anti-hyperglycemic effect of oat flours

and individual avenanthramides and phenolic acids (with and without bitter receptor inhibitors) in an STC-1 cell culture model of intestinal glucose uptake (Xie, 2019) (**Figure 1**). Phenolics from whole grain oat products have also been shown to modulate maltose hydrolysis and glucose transport by human intestinal cells leading to attenuation of glucose transport (Li et al., 2017).

Therefore, the goal of this study was twofold, with the first objective being to quantify free, bound, and digested avenanthramides (AVNs) and phenolic acids (PAs) in 242 oat flour samples consisting of 109 accessions from the AFRI CORE diversity oat panel grown at two different locations (Lacombe, Alberta, CA and Aberdeen, Idaho, US.) The second objective was to evaluate the effect of selected oat varieties in the C57BL/6J mouse model of diet-induced obesity to determine the anti-hyperglycemic potential of oat flours and correlate it to their metabolite composition.

2. Materials and Methods

2.1 Reagents

All chemicals were obtained from Sigma (St. Louis, MO), unless otherwise specified.

2.2 Oats samples and preparation

A set of 242 samples consisting of 109 accessions from the AFRI CORE world diversity oat panel grown at two different locations – Lacombe, Alberta, CA (1) and Aberdeen, Idaho, US (2) – was kindly provided by Dr. Eric Jackson as a part of the Collaborative Oat Research Enterprise (CORE) initiative22 in the form of de-hulled and uniformly milled whole grain flour and stored at -20 °C until extraction. Leading commercially available oatmeals were purchased

to be used as references, with preparation following the same procedures as the AFRI CORE oats. Oat beta glucan content ranged from 3-6% dry weight in both locations across all cultivars.

2.3 Extraction of free avenanthramides and phenolic acids

Oat flours (200 mg) were milled with IKA Analytical Mill into a fine powder and extracted with 1 ml of 95% methanol in a phosphate buffer (pH=2.8) using vertical shaking (20 27 min, RT, 300 rpm) and ultrasonication (30 min). The samples were centrifuged at 3000 rpm and the exhausted pellets were extracted 2 more times following the same procedure and discarded. Combined supernatants were concentrated under vacuum at a temperature not exceeding 40 °C (Buchi R210 rotavapor, Flawil, Switzerland) and freeze-dried (Labconco Freezone18, Kansas City, MO). Resulting powders were dissolved in methanol, filtered through a syringe filter, and analyzed by HPLC.

2.4 Extraction of bound avenanthramides and phenolic acids

Bound phenolic acids were extracted from freeze-dried pellets as previously described (Multari et al., 2018). Briefly, pellets were suspended in NaOH and stirred at room temperature. The pH was reduced to 2 with HCL and the samples were extracted into ethyl acetate. This was repeated twice. The ethyl acetate extracts were combined and concentrated under vacuum at a temperature not exceeding 40°C and freeze-dried. Resulting powders were dissolved in methanol, filtered through a syringe filter, and analyzed by HPLC.

2.5 *In Vitro* gastrointestinal digestion of oat flours

Simulated oral, gastric and small intestinal digestion of oat flours was performed following an established protocol with minor modifications (Minekus et al., 2014). Briefly, SSF (simulated salivary fluid), SGF (simulated gastric fluid) and SIF (simulated intestinal fluid) were made up of the respective electrolyte solutions, enzymes, water and calcium chloride. A-amylase from human saliva (type IX-A) or porcine pancreas (type VI-B) was prepared as 1500 U/ml stock solution in the SSF fluid. Porcine pepsin was prepared as 25000 U/ml in the SGF fluid. Pancreatin stock solution was prepared based on the trypsin activity as 800 U/ml in the SIF fluid. Fresh bile preparations were made as 160 mM stocks in the SIF solution. Calcium chloride stocks were prepared as 0.3 M solution (0.44 g CaCl2x2H2O in 10 ml water).

In the oral digestion phase, 100 mg of oat flour sample was added to 300 µl of water and boiled for 5 minutes, then 280 µl of SSF electrolyte stock solution and 40 µl of salivary α-amylase solution (1500 U/ml) were added. Following the addition of 2 µl of 0.3M CaCl2 and 78 µl water, the reaction was mixed by vortexing to simulate food processing in human mouth for 2 min. The pH of solution was controlled at 6.8 where salivary α-amylase has a highest activity.

In the gastric digestion phase, 600 µl of SGF electrolyte stock solution, 128 µl of porcine pepsin (3850U/mg), 4 µl of 0.3M CaCl2, 16 µl of 1M HCl and 52 µl of water were added to the sample and incubated for 2 hours at 37°C.

In the intestinal digestion phase, 880 µl of SIF electrolyte stock solution, 400 µl of pancreatin (800U/ml), 200 µl of fresh bile (160mM of taurocholic acid), 3.2 µl of 0.3M CaCl2, 12 µl of 1M NaOH and 104.8 µl of water were added to the gastric chyme and digested for additional 2 hours at 37°C. After digestion was completed, the solution was water bathed for 15 minutes in 80°C to inactivate the enzymes and then centrifuged at 2000 rpm for 10 minutes.

Supernatant liquid was transferred to a new vial, frozen at -80°C, and freeze-dried to obtain a powder sample of water-soluble metabolites released from the digested oat flour.

2.6 HPLC quantification of avenanthramides (AVNs) and phenolic acids (PAs)

HPLC-UV analysis was performed using a Shimadzu HPLC system equipped with a pump (LC-20AT), an autosampler (SIL-20A), a diode array detector (SPD-M20A) and an automatic column temperature control oven (CTO-20A). Separation was performed on Restek Ultra C18 column (250 x 4.6 mm, 5 μ) at a column temperature of 30°C. The binary mobile phase consisted of 0.1% formic acid in water (Eluent A) and acetonitrile (Eluent B) in a gradient as follows: 0-5 min, 20% acetonitrile; 5-25 min, 20-65% acetonitrile; 25-26 min, 65% acetonitrile; 26-29 min, 65-95% acetonitrile; 29-32 min, 95% acetonitrile; 32-44 min, 95-20% acetonitrile. Each run was followed by an equilibration time of 10 min. Ultraviolet (UV) spectra were monitored at 340 nm, and the flow rate was 1.0 ml/min. The data were collected and analyzed with LC solution (Shimadzu, Nakagyo-ku, Kyoto, Japan) software. Peaks were identified based on comparison of retention times and UV spectra with those of authentic AVNs standards. The same method was repeated for phenolic acid quantification, with UV spectra monitored at 324 nm (caffeic acid), 310 nm (p-coumaic acid), and 323 nm (ferulic). Peaks were identified based on comparison of retention times and UV spectra with those of authentic PA standards.

2.7 Gastrointestinal STC-1 cell culture model

The mouse neuroendocrine intestinal cell line STC-1 (CRL-3254), an established model for glucose absorption and hormone secretion, was obtained from ATCC (Manassas, VA). Cells

were routinely passaged every 3-4 days and maintained in high glucose DMEM containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA) and 1% penicillin-streptomycin (Fisher Scientific, Pittsburg, PA) at 37 °C and 5% CO2. Cells were sub-cultured into 24-well plates and, once confluent, changed to induction medium that contained glucose-free DMEM supplemented with 2 mM sodium pyruvate to induce fluorescent glucose uptake. Treatments with oat flour digests were administered for 2 hours before cells were exposed to fluorescent 2-NBDG analog of glucose. 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) is a fluorescently labeled 2-deoxyglucose analog for monitoring glucose uptake in living cells. It is transported according to Micharelis-Menten kinetics, similar to glucose, and it is compatible with fluorescence techniques such as flow cytometry. Intracellular 2-NBDG shows a strong fluorescence at 542 nm and is excited at 467 nm. It was used in this project to directly detect and quantify glucose transport in STC-1 cell culture model. Oat flour digests prepared as 50mg/ml stocks in PBS were added into 1-ml batches of STC-1 cells in duplicate and incubated at 37℃ for 30 minutes. Next, 25µl of 2-NBDG from a 5mM (1.71mg/ml) master stock in PBS were added to the control (baseline vehicle blank) or digest-treated STC-1 cells, incubated for 30 min, centrifuged and washed to removed excessive 2-NBDG, and the fluorescence of intracellular 2-NBDG was measured using flow cytometry (BD Accuri C6, San Jose, CA). Absolute glucose fluorescence intensity values obtained from FL1 fluorescent channel were normalized to baseline blank fluorescence signal and compared between cells in the treatments and baseline blank groups.

2.8 Animal study and diets

All animal experiments were performed according to procedures approved by the NC

Research Campus Institutional Animal Care and Use Committee in the David H. Murdock Research Institute, an AAALAC accredited animal care facility. Male, 6-week-old C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed four animals per cage under controlled temperature (24 ± 2 °C) and light (12 h light-dark cycle, lights on at 0700 h). Immediately upon arrival, animals were allowed to adapt to new conditions for 7 days, and daily animal handling was performed during this time to reduce the stress of physical manipulation. Mice (n=32) were then randomized into a low-fat diet (LFD) (n=16) containing 10% fat-derived calories (D12450B; Research Diets, New Brunswick, NJ) or a high fat diet (HFD) (n=16) containing 60% fat-derived calories (D12492, Research Diets) for 9 weeks. Mice had ad libitum access to food and water.

Animal weight and food intake (accounting for spillage) were recorded weekly for the duration of the study. All animal diets were kept at -80C for long-term storage and stability, and freshly thawed food was dispensed to animals every 3-4 days to limit phytochemical degradation in food matrix. For long-term fasting blood glucose levels, mice were tested after 16 h overnight fast on weeks 4 and 6 of the study. At the end of the study, blood was collected by cardiac puncture after CO2 inhalation. Gastrointestinal (stomach, duodenum, jejunum, ileum, cecum, and colon) and metabolic (liver, gastrocnemius muscle, and epididymal fat) tissues were collected and stored at -80C.

2.9 Oral glucose tolerance tests with selected oat flours

Acute oral glucose tolerance tests were performed on a randomized subset of HFD animals with an orally administered selection of AFRI CORE and commercial oat flours (50-200 mg/kg) 30 minutes prior to glucose gavage. Mice were fasted overnight (16 h) and received oral

gavage of D-glucose (1.5 g/kg body weight, Sigma). Blood glucose concentrations were measured at 0, 15, 30, 60, and 120 minutes after glucose challenge in blood samples obtained from tail-tip bleedings using a glucometer (True Result, Trivida, FL).

2.10 Statistical analysis

Statistical analyses were performed using Prism 7.0 (GraphPad Software, San Diego, CA) and expressed as means ± SEM. Two tailed t-test or two-way ANOVA were applied at a significance level of $p<0.05$. Post-hoc analyses of differences between individual experimental groups were made using the Bonferroni's or Dunnett's multiple comparison test.

3. Results

Oat cultivars from the AFRI CORE worldwide diversity panel differed markedly not only in total and individual AVNs and phenolic acid content, but also in the amounts of metabolites produced depending on the geographical location of the field. Two leading store-bought oat cultivars (location marked "X") showed lower levels of both metabolite groups as compared to cultivars grown in Lacombe, AB (**Figure 2)**.

3.1 Quantification of free and bound avenanthramides (AVN)

Total levels of free AVNs ranged from 3.3 to 227.3 μg/g in Lacombe, AB location, and from 1.0 to 65.9 μg/g when grown in Aberdeen, ID. Similarly, total levels of bound AVNs ranged from 0.45 to 42.6 μg/g in Lacombe, AB location, and from 0.3 to 7.99 ug/g when grown in Aberdeen, ID **(Figure 3)**.

Cultivar UFRGS 881971 grown in Lacombe showed the highest total free AVN content of 227.3 μg/g, with levels of the individual free AVNs at 68.1 μg/g (AVNA), 91.4 μg/g (AVNB), and 67.8 μg/g (AVNC). UFRGS 881971 also showed the highest total bound AVNs content of 42.6 μg/g, with levels of the individual bound AVNs at 15.6 μg/g (AVNA), 20.4 μg/g (AVNB), and 6.7 μg/g (AVNC).

Cultivar Ajax grown in Aberdeen showed the lowest total free AVN content of 1.025 μg/g, with levels of the individual free AVNs at 0.48 μg/g (AVNA), 0.445 μg/g (AVNB), and 0.1 μg/g (AVNC). Ajax also showed one the lowest total bound AVNs content of 0.405 μg/g, with levels of the individual bound AVNs at 0.32 μg/g (AVNA), .085 μg/g (AVNB), and 0 μg/g (AVNC).

The total content of free AVNs differed markedly across the samples ($p<0.05$) and locations ($p<0.01$), with up to 75-fold difference among the cultivars grown in the northern location, 33-fold difference among the cultivars grown in the southern location, and 5-fold difference among the average means for the cultivars grown in each location (54.9±2.3 versus 11.1±0.8 μg/g whole flour for Lacombe and Aberdeen, respectively). These numbers are consistent with total free AVNs ranges reported for nine oat cultivars from China (5-175 μg/g), eight oat cultivars from Finland (27-185 μg/g), and four oat cultivars from US (17-116 μg/g).

3.2 Quantification of free and bound phenolic acids (PA)

Total levels of free PAs ranged from 1.035 to 9.58 μg/g in Lacombe, AB location, and from 1.79 to 8.61 μg/g when grown in Aberdeen, ID. Similarly, total levels of bound PAs ranged from 12.585 to 89.65 μg/g in Lacombe, AB location, and from 3.345 to 28.56 μg/g when grown in Aberdeen, ID **(Figure 4)**.

Cultivar Dominik grown in Lacombe showed the highest total free PA content of 9.58 µg/g, with levels of the individual free PAs at 0 µg/g (coumaric), 4.11 µg/g (ferulic), and 5.47 µg/g (caffeic). Dominik also showed one of the highest total bound PA content of 32.6 µg/g, with levels of the individual bound PAs at 0.155 µg/g (coumaric), 31.4 µg/g (ferulic), and 1.055 µg/g (caffeic).

Cultivar Z614-4 grown in Lacombe showed the lowest total free PA content of 1.035 µg/g, with levels of the individual free PAs at 0 µg/g (coumaric), 0.38 µg/g (ferulic), and 0.655 µg/g (caffeic). Cultivar MAM 17-5 grown in Aberdeen showed the lowest total bound PA content of 3.345 µg/g, with levels of the individual bound PAs at 0 µg/g (coumaric), 3.345 µg/g (ferulic), and 0 µg/g (caffeic).

3.3 Bioaccesibility of avenanthramides following in vitro digestion

The presence of avenanthramides in the aqueous fraction of the three-stage in vitro digestion system following their release from the wet cooked whole oat flour was quantified in all oat cultivars subjected to free AVNs analysis. Total levels of AVNs released from whole flours upon digestion ranged from 1.9 to 67.2 µg/g whole flour in Lacombe, AB location, and from 1.475 to 14.7 µg/g when grown in Aberdeen, ID **(Figure 5)**.

Cultivar UFRGS 881971 grown in Lacombe showed the highest total bioaccesible AVNs content of 67.2 µg/g, with levels of the individual AVNs at 31.0 µg/g (AVNA), 36.2 µg/g (AVNB), and 0 µg/g (AVNC). Cultivar Matilda grown in Aberdeen showed the lowest total bioaccessible AVNs content of 1.475 µg/g, with levels of the individual AVNs at 1.25 µg/g (AVNA), 0.225 µg/g (AVNB), and 0 µg/g (AVNC).

The complete loss of AVNC as well as all PAs from the digested oat samples can be explained both by inherent instability of caffeic acid and the respective avenanthramide AVNC (2c) under both neutral and alkaline conditions, with and without heating, encountered during oat flour processing and digestion (Dimberg et al., 2001).

Therefore, the total content of bioaccessible AVNs differed markedly across the samples ($p<0.01$) and locations ($p<0.001$), with up to 35-fold difference among the cultivars grown in Lacombe, 9-fold difference among the cultivars grown in Aberdeen, and a 5-fold difference among the average means for the cultivars grown in each location (15.3±0.8 versus 3.8±0.2 μg/g whole flour for Lacombe and Aberdeen, respectively). Relative average bioaccessibility of total AVNs varied in the range of 40-45% between locations, and 5-92% among cultivars in the same location.

3.4 Fluorescent glucose uptake in STC-1 cells in response to oat flour digests

Aqueous digests of 109 accessions from the AFRI CORE oats diversity panel, grown in two locations (244 total samples) were lyophilized and applied as treatments to the *in vitro* STC-1 cell culture model of gastrointestinal glucose uptake. Total AVN content showed a weak significant correlation ($r=-0.32$, $p<0.001$) to reduction of fluorescent glucose uptake, suggesting that increasing this trait in oat cultivars is beneficial to enhance their glucose modulation properties (**Figure 6**.). The weak correlation suggested a large environmental influence that drove these responses, as well as the likely presence of other secondary metabolites that contributed to the overall biological activity of the digested oat flours such as a group of oat saponins known as avenacosides, which are two to three times more bitter than AVN (Günther-Jordanland et al, 2016) . Literature reports quantities up to 900+ μg/g flour, with other plant

saponins known to increase glucose homeostasis by stimulating glycogen synthesis and GLUT4 protein expression (Kim et al., 2009; Xu et al., 2018).

3.5 Acute anti-hyperglycemic effect of selected oat flours

Obesity and hyperglycemia were induced by feeding a HFD to C57BL/6J mice for 4 weeks before the animals were tested for oral glucose tolerance. During this time, HFD mice developed significant body weight gain (50-60 g final bw) and fasting hyperglycemia (150 mg/dl glucose) as compared to LFD controls. When tested by oral glucose tolerance test (OGTT), a panel of oat varieties representing high (HiFi, 173.1 μg/g AVN), medium (Coker234 71.8 μg/g), and low (H927 3.3 μg/g AVN) AVNs showed modest efficiency at lowering blood glucose levels in C57BL/6J mice in a manner consistent with their respective AVN contents at a 100 mg/kg dose **(Figure 7)**. When tested by oral glucose tolerance test (OGTT), the selected high AVN oat variety HiFi, showed modest efficiency at lowering blood glucose in C57BL/6J mice in a dose dependent manner from 50-200 mg/kg (**Figure 8).** Similarly, a panel of commercial oat varieties Comm var 1 (store bought, 10.42 μg/g AVN), Comm var 2 (44.385 μg/g AVN), and Comm var 3 (157.275 μg/g AVN) were tested by OGTT and showed modest efficiency at lowering blood glucose levels in a manner consistent with their respective AVN content at a 100 mg/kg dose (**Figure 9).** Oat varieties with the highest levels of AVNs, HiFi (173.1 μg/g AVN) and Comm var. 3 (157.275 μg/g AVN) showed the largest decrease in blood glucose levels.

4. Discussion

Whole grains (those containing the fiber rich bran and nutrient rich germ) used in traditional diets have markedly lower carbohydrate densities than white flour. Furthermore,

refinement of grains into flours often removes a host of beneficial compounds present in the outer layers of the seed. Oat groats, while hulled, are unique among grains in that they are commonly consumed as a whole grain. Mechanical processing such as grinding or rolling preserves the bran and germ, however steaming (used to make rolled and quick oats) likely reduces the presence of compounds such as avenanthramide C, as demonstrated by this research and others (Dimberg et al., 2001). This may be one reason why commonly available commercial oat varieties do not contain high levels of AVNs or PAs and why industrial processing was scorned by historical physicians.

It has been suggested that Type 2 Bitter Receptors (T2Rs) in the gastrointestinal tract respond to naturally bitter plant compounds, such as those found in oats, to affect digestion and absorption of carbohydrates (Dotson et al., 2008). When stimulated, TAS2R activate phospholipase C β2, which lead to the activation of Inositol triphosphate (IP3) release of Ca2+ (Dotson et. al 2008). These events result in action potential generation allowing the nervous system to communicate with the endocrine system to release hormones and small peptides that are appropriate to return the body back to blood glucose homeostasis. These molecules include: glucagon-like peptide-1 (GLP-1), glucagon, neuropeptide Y, peptide YY (PYY), cholecystokinin (CCK), vasoactive intestinal peptide, and ghrelin (Kinnamon, 2012). The body must control the amount of these molecules as some are antagonistic toward each other in their roles as blood glucose modulators, and as molecules that can either promote satiety or hunger. The exact mechanism from the point of TAS2R's initial binding, up until its signal cascade is not fully understood (Dotson et al., 2008; Kinnamon, 2012). While many plant extracts have been demonstrated to inhibit intestinal glucose uptake, their active compounds have very few structural similarities – yet interestingly, many of these plants (such as bitter melon, chicory,

olive oil, radish, aloe vera) have a reported bitter taste and a long history of use in controlling diabetes (Joseph and Jini, 2013; Street et al., 2013; Bozzetto et al., 2016; Banihani and Banihani, 2017; Cárdenas-Ibarra et al., 2017). Use of probenecid (which inhibits TAS2R activation) potentiates hyperglycemic effect of furosemide (Greene et al., 2011), while use of denatonium benzoate (a TAS2R ligand) lowers blood glucose levels after oral glucose administration in mice, through increased secretion of GLP-1 (Kim et al., 2014).

The selected AFRI CORE world diversity oat panel cultivars differed greatly in their AVN composition with up to a 75-fold difference among the cultivars grown in the northern location, a 33-fold difference among the cultivars grown in the southern location, and a 5-fold overall difference in favor of the more northern location (Lacombe, AB). Cultivars UFRGS 881971, ProFi CDC, HiFi and Centennial showed the highest content of AVNs. Along with the fact that oat phenolics have been shown to inhibit starch digestion in human cells, these cultivars could be specifically utilized in the development of novel nutritional and dietary formulations to supplement human diet with high levels of bitter bioactives such as AVNs (Li et al., 2017).

The digestive release of AVNs in the simulated model of upper gastrointestinal digestion, critically depended on cultivar and growth location of the crop. AVNC and all PAs, however, were completely lost from the target digests, possibly due to its inherent instability under neutral and alkaline conditions. In spite of the loss of AVNC and all PAs, high AVN oats showed more pronounced hyperglycemic effects *in vivo* independent of beta-glucan content (4-5% dry weight for HiFi, Coker, and H927). Free phenolic acid content did not appear to influence the anti-hyperglycemic outcome, as PAs do not survive *in vitro* digestion and as such do not likely survive digestion *in vivo*.

5. Conclusion

We quantified free, bound, and digested avenanthramides and phenolic acids in 242 oat flour samples consisting of 109 accessions from the AFRI CORE diversity oat panel grown at two different locations (Lacombe, Alberta, CA and Aberdeen, Idaho, US.) We confirmed acute hypoglycemic effects of high AVN oat flour varieties such as HiFi. Together with previous data from our laboratory, these data suggest oats containing high levels of avenanthramides stimulate T2R signaling in the GI tract to control acute postprandial hyperglycemia associated with metabolic syndrome by preventing glucose absorption and stimulating satiety hormone secretion following carbohydrate consumption. These results strengthen the case for the use of nutritional interventions as an alternative choice for managing postprandial hyperglycemia in individuals with metabolic syndrome.

6. Acknowledgements

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8. Figures

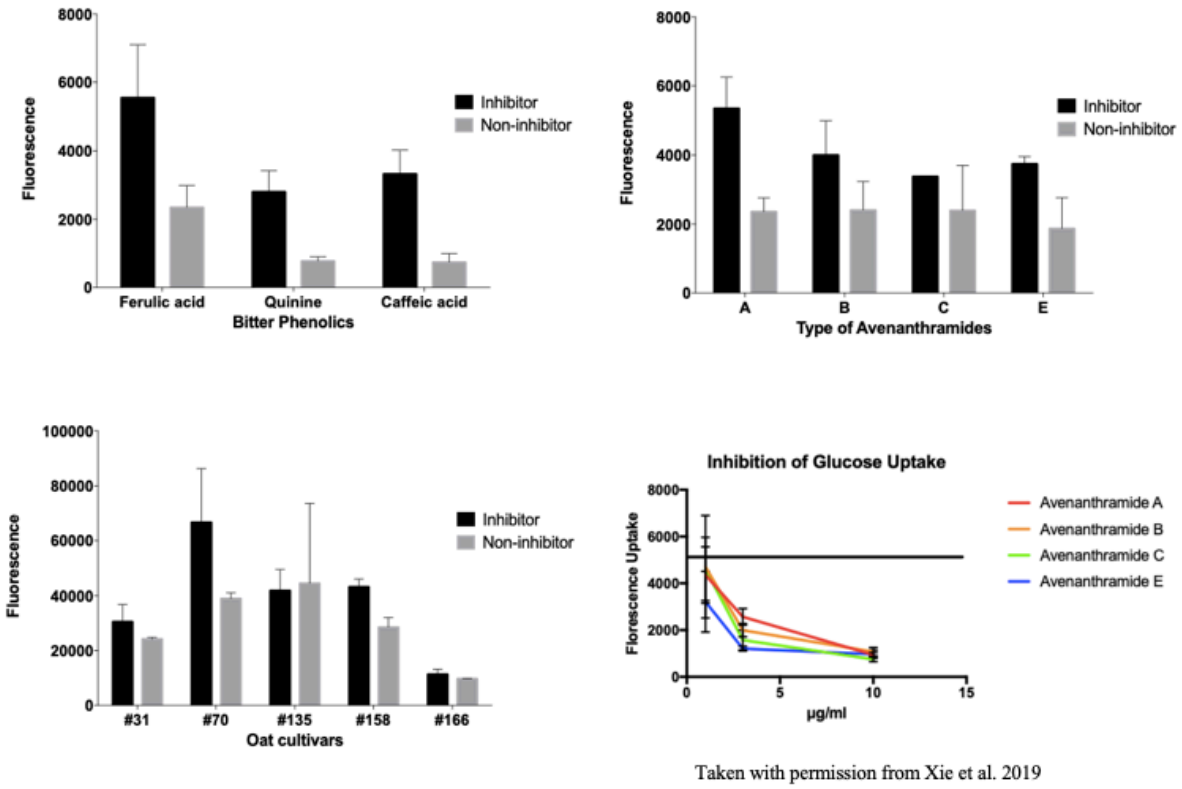

Figure 1. Anti-hyperglycemic effect of oat flours and their bioactives in the STC-1 cell culture model of intestinal glucose uptake. Fluorescent 2-NBDG glucose uptake in the STC-1 intestinal cells treated with individual AVNs, PAs, and oat flours. Glucose absorption effects of oat bioactives were inhibited by co-exposure to an allosteric inhibitor of bitter receptors probenecid (10 μM).

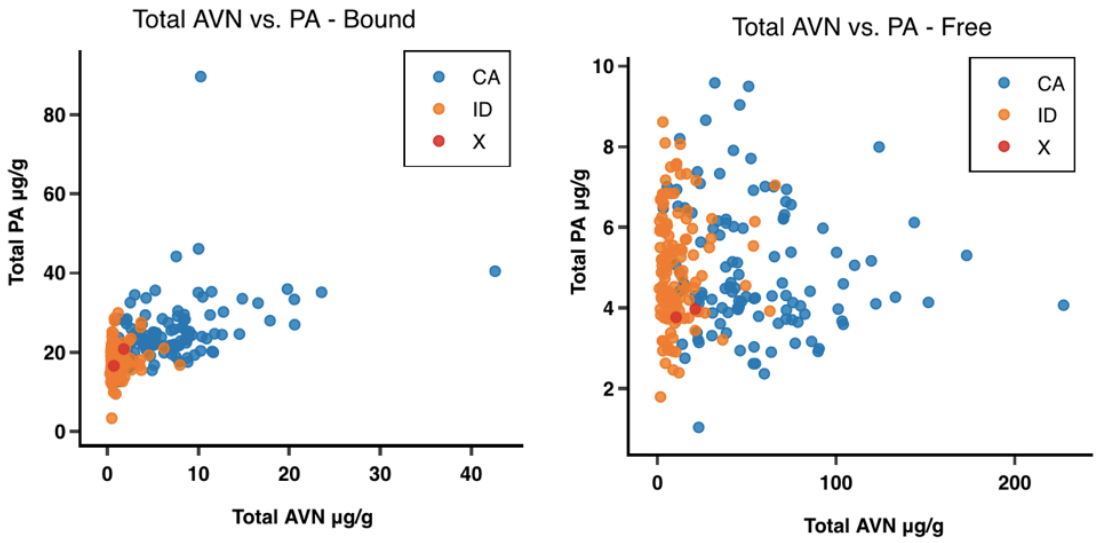

Figure 2. Distribution of free and bound metabolites in oat diversity panel. "X" indicates commercial oat varieties.

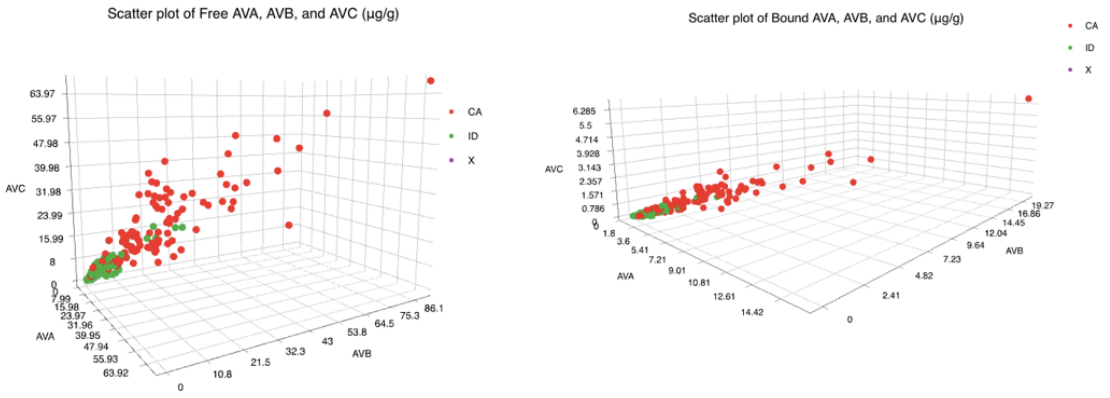

Figure 3. Distribution of free and bound avenanthramides in oat diversity panel. "X" indicates commercial oat varieties.

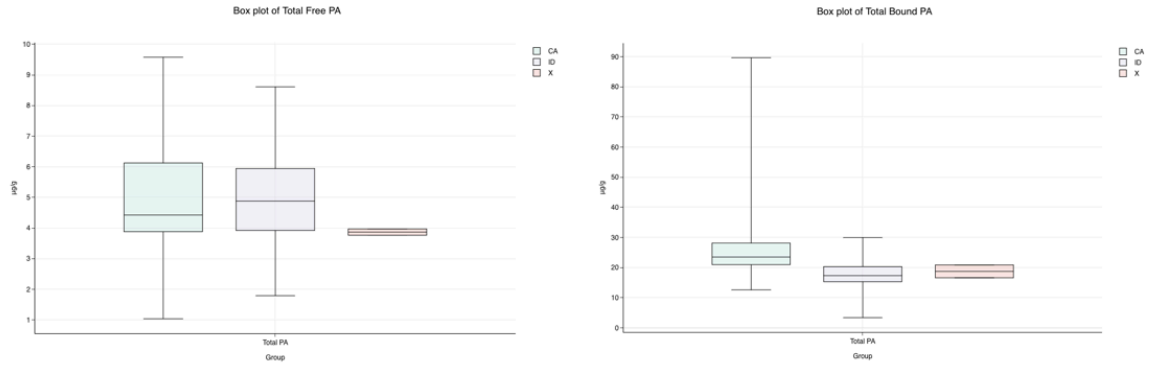

Figure 4. Distribution of free and bound phenolic acids in oat diversity panel. “X” indicates commercial oat varieties.

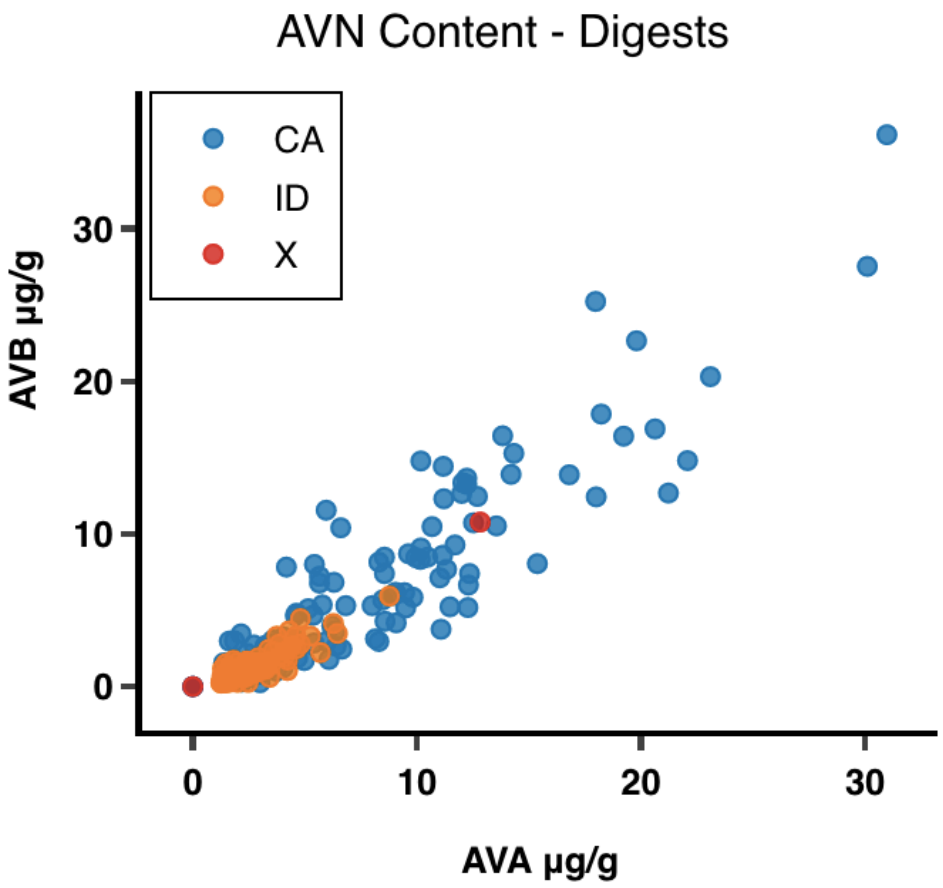

Figure 5. Distribution of digested avenanthramides in oat diversity panel. "X" indicates commercial oat varieties.

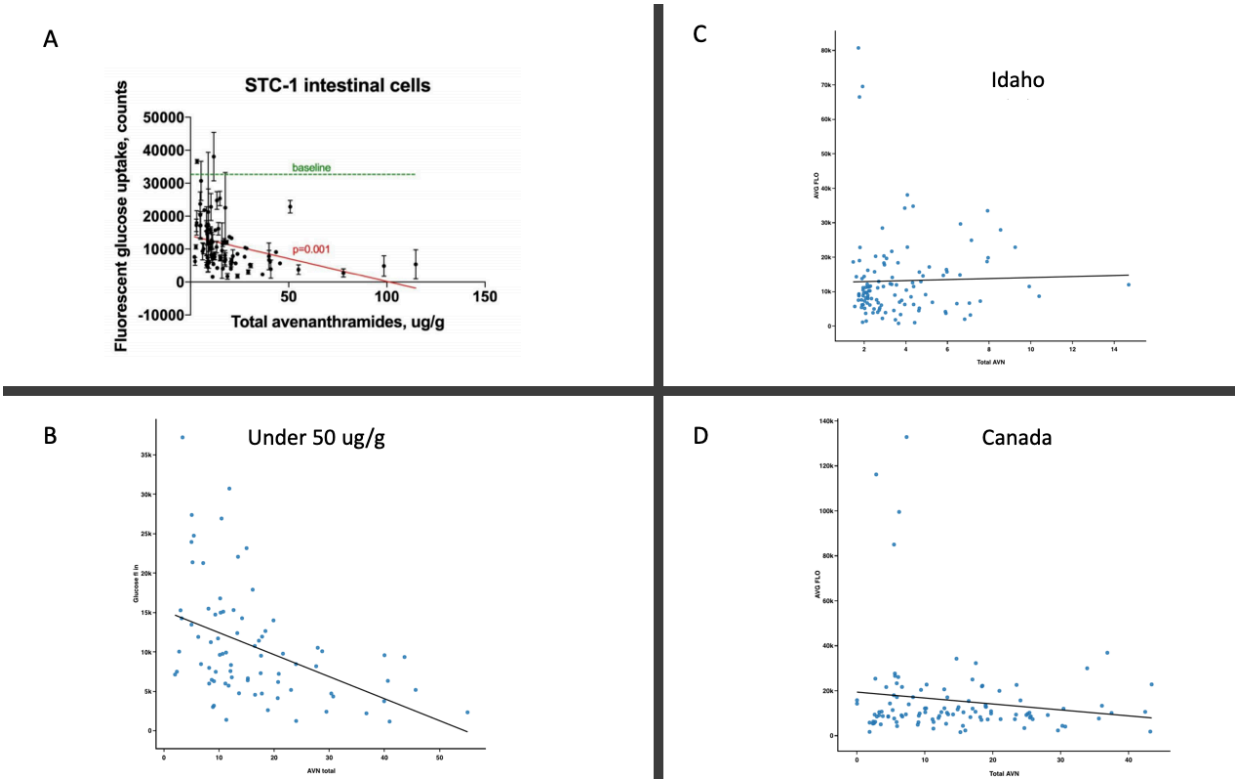

Figure 6. Fluorescent 2-NBDG glucose uptake in the STC-1 cell culture model. Unstratified total AVN content (A) showed a weak significant correlation ($r=-0.32$, $p<0.001$) to reduction of fluorescent glucose uptake, suggesting the likely presence of other secondary metabolites that contributed to the overall biological activity. This notion is supported by total AVN content under 50 µg/g (B), which removed extremely high AVN cultivars. Data stratified by location (C,D) suggested an environmental influence that drove responsesthat may not be associated with AVN content.

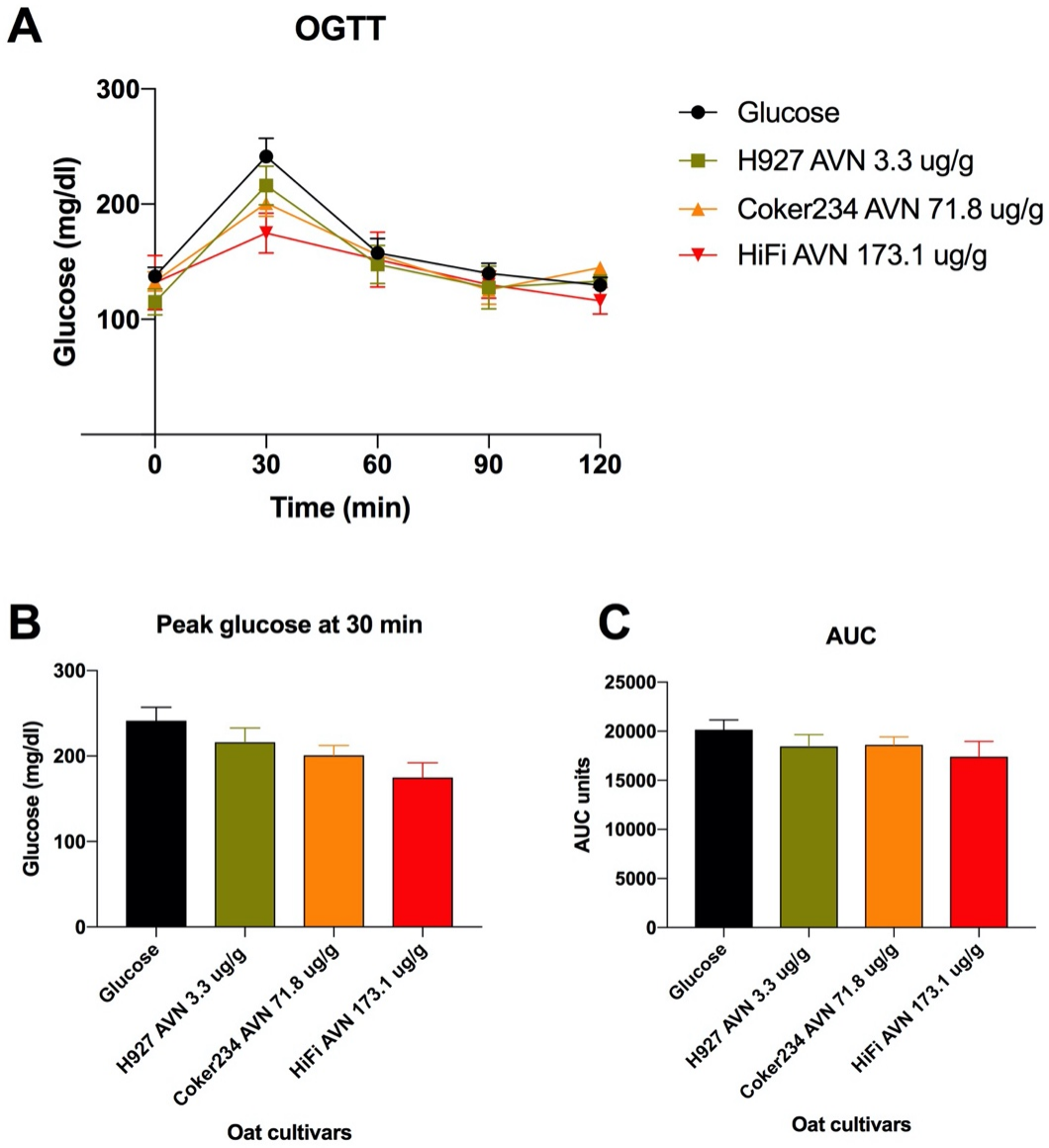

Figure 7. Anti-hyperglycemic effect of three oat varieties in the C57BL/6J mouse model of diet-induced obesity oral glucose tolerance test (OGTT).

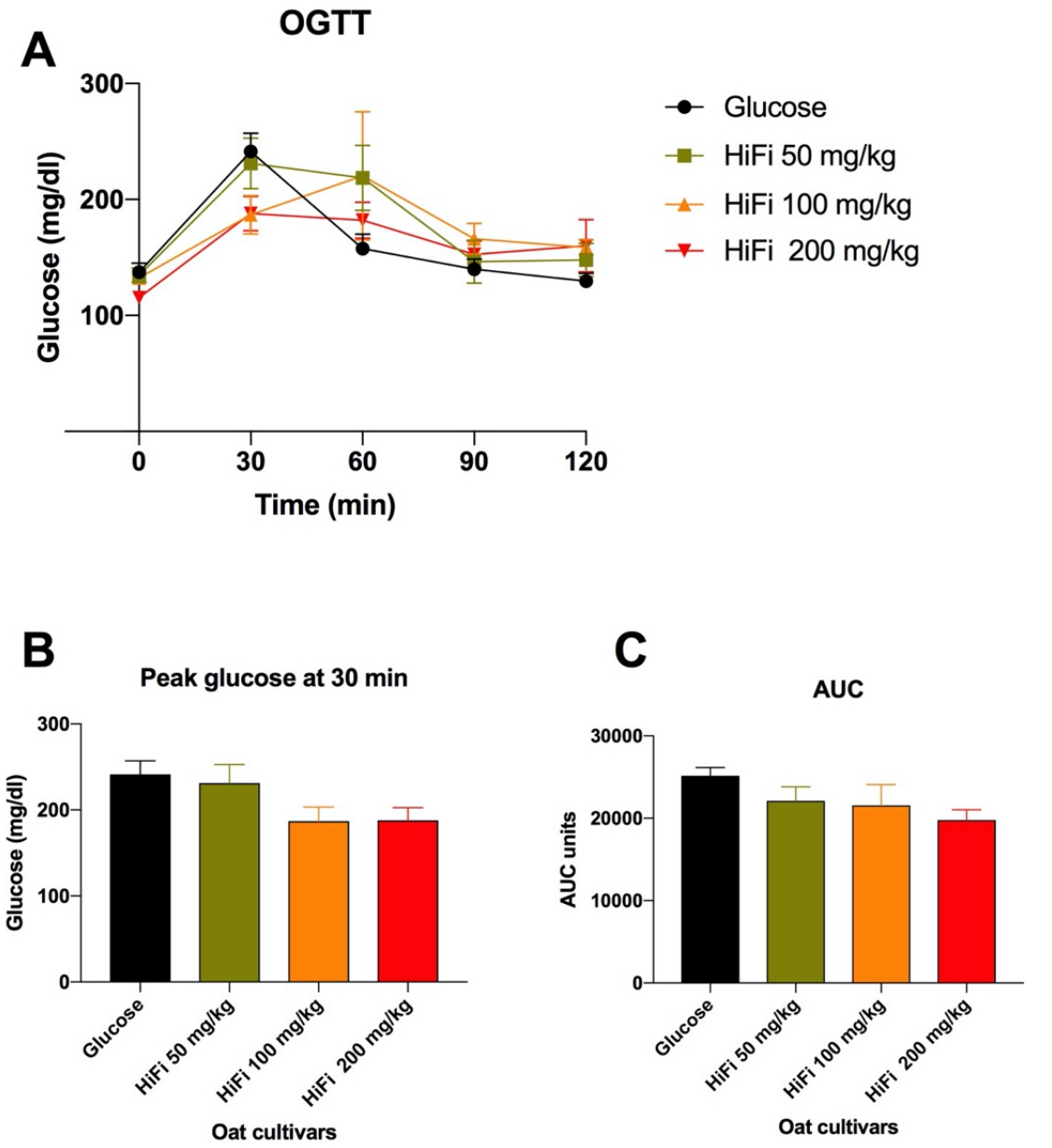

Figure 8. Dose dependent anti-hyperglycemic effect of HiFi oat variety in the C57BL/6J mouse model of diet-induced obesity oral glucose tolerance test (OGTT).

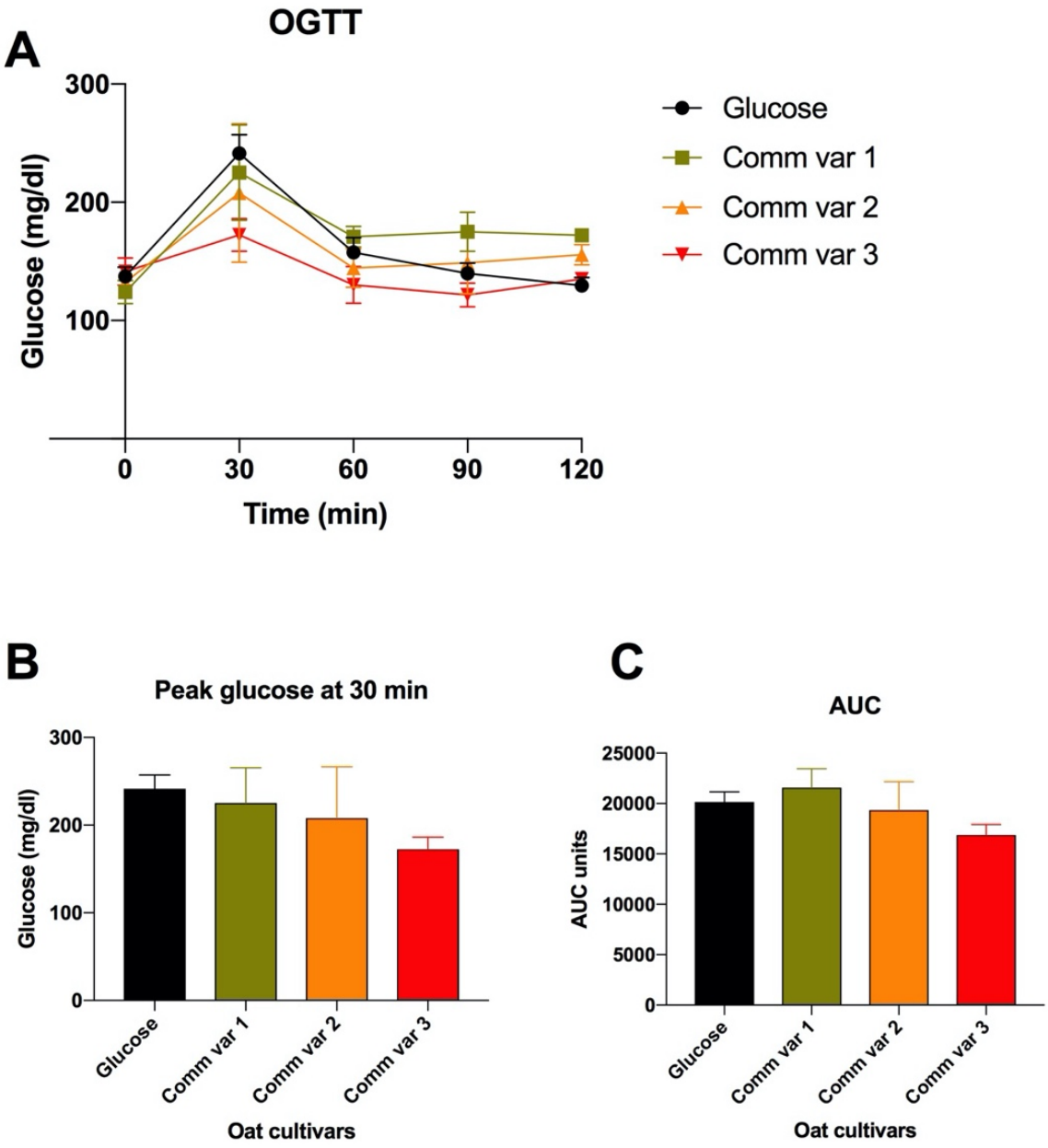

Figure 9. Anti-hyperglycemic effect of commercial oat varieties in the C57BL/6J mouse model of diet-induced obesity oral glucose tolerance test (OGTT).

CHAPTER 5: CONCLUSIONS

Charles Wagner

1. Conclusions

> **“**There’s a popular saying among doctors: There’s no such thing as alternative medicine; if it works, it’s just called medicine.” – Ed Yong

From this dissertation, it is reasonable to conclude that some medieval physicians used observation and experience to design or choose effective remedies against a variety of symptoms and illnesses, creating an empirical tradition of scholarship and rational methodology often overlooked by modern science. Demonstrated herein lies the untapped potential of premodern remedies for yielding novel therapeutics at a time when chronic lifestyle-based inflammatory and metabolic diseases account for the bulk of deaths worldwide, and new antibiotics are desperately needed. When evidence-backed botanical interventions can be integrated into conventional practice by healthcare providers, patients can benefit from the best of both worlds.

Human beings’ use of natural products for health purposes dates back to prehistoric times. Archaeological evidence suggests that the Neanderthals used more than 60 taxa of plants for food, medicine, and ritual uses (Shipley and Kindscher, 2016). Despite tremendous advances in science and technology over the years, our reliance on plants and fungi has remained. During the early 1800s, morphine was the first natural product isolated from a plant; however, some 5,000 years earlier, the opium poppy, the source of morphine, was mentioned on a Sumerian clay tablet (Norn, Kruse, and Kruse 2005). Historical texts continue to be a source of bioactive interventions for improving human health. **Chapter 1** demonstrates that throughout Classical Antiquity and the Middle Ages, a distinct healing tradition emerged in the British Isles, especially at its fringes in Scotland and Wales**.** For the first time, evidence has been provided for traces of a Celtic healing tradition within this milieu, with undescribed chemical biodiversity

warranting further investigations of bioactivity and clinical applications of the described plant leads and formulations (**Chapters 2-4)**.

Between 1981 and 2010, up to 50 percent of the drugs approved by the FDA have been derived in some way from natural products (Newman and Cragg, 2012). The World Health Organization's (2019) list of essential medicines compiles the drugs that experts deem to be the most cost-effective options for key health problems globally. About 10 percent of the drugs on the list are derived exclusively from flowering plants, yet less than 10 percent of the world's biodiversity has been screened for medicinal properties (Veeresham, 2012). The historical botanical interventions of Scotland and Wales, such as those found in *Meddygon Myddfai,* proved to be an extensive source of potential leads for antimicrobial screening efforts with 67 historical plants (80.7%) and 14 modern plants (77.8%) found to have detectable levels of antimicrobial activity when tested using Mobile Discovery kits, including plants with uncharacterized chemical composition or biological activity **(Chapter 2).**

When backed by evidence and proper clinical integration, historical botanical interventions have great potential for preventing chronic disease, managing symptoms and side effects, and enhancing patient health and quality of life (Wagner, 2020). One such example is the reconstituted Welsh remedy for ulcerative colitis (**Chapter 3).** The *Myddfai* remedy was shown to improve the underlying causes of the condition it was likely designed to treat, by dramatically improving DSS induced colitis symptoms in mice and by modulating the microbiome in a beneficial manner. The rather unique media for the recipe, goat's whey, increases cell viability in an established *in vitro* model and contributes to the positive outcome *in vivo*. The total effect of the recipe, preforming in a similar fashion as the current clinical treatment (5-ASA), depends upon the specific formulation and preparation laid down by the physicians that wrote it

Similarly, data from **Chapter 4** suggests that dietary interventions based on bitter oat bioactives may lower absorption of glucose in the gastrointestinal tract and may offer an alternative strategy to manage postprandial glucose rise in healthy and metabolically challenged individuals. This outcome gives some clout to the notion that oats, the staple grain in Scotland, have historically contributed to the health of the populace.

Taken together, these findings support historical botanical interventions as effective dietary agents to improve human health and warrant further analysis of historical herbal texts as leads for botanicals and formulations to be screened for bioactivity in a variety of preclinical experiments and clinical trials.

2. References

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