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The research presented in this dissertation deals with the phenomena of bacterial efflux pump inhibition by natural products and plant extracts. Bacterial efflux pumps are active transport proteins, primarily deriving their energy source from the proton motive force, which functions to export toxic compounds outside the cell. This is a natural defense mechanism that bacteria utilize to protect themselves from toxic environments. The over-production of efflux pumps is one mechanism by which bacteria can evolve resistance to clinical antibiotics, as well as other antimicrobials. Thus, the study of efflux pump inhibitors is important because it holds the potential to reverse some forms of antibiotic resistance. In light of this importance, a series of studies were designed to improve the ability to study this phenomenon, to investigate the distribution of efflux pump inhibition in land plants, and to improve our ability to identify an important class of efflux pump inhibitors, the flavonoids.

The first aim of this research was to develop an improved method for experimentally quantifying efflux pump inhibitory activity of small molecules. Preexisting methods made this difficult due to several limitations including: the collection of indirect results, time consuming materials handling techniques, and/or matrix interference problems pertaining to the quenching of fluorescent signal. An improved method relying on mass spectrometry measurements was developed that addressed the aforementioned limitations. The importance of this

improved method lies in its ability to produce data sets useful in calculating IC_{50} values for a wider range of samples than was previously possible.

The second aim was to evaluate the presence of efflux pump inhibitor production across the land plant lineage. This is important to botanical science and the understanding of plant-microbe interactions and plant evolutionary biology. The most ancient lineages of land plants have not been previously evaluated for efflux inhibitory activity. Additionally, land plants play an important role in many traditional medicinal systems and in modern complementary and alternative medicine. Thus, understanding the distribution of efflux pump inhibitor production in this group increases our understanding of these common forms of medical treatment. In order to gain these data, a set of 14 plant species spanning the major lineages within the land plant group (bryophytes, pterophytes and lycophytes, gymnosperms, and angiosperms) were extracted and assayed to determine efflux pump inhibitory activity of the extracts. Positive results (indicating the presence of an efflux pump inhibitor) were observed for many (but not all) of the plant species tested. The observation of activity in extracts prepared from the most ancient plants tested (bryophytes—the liverworts and mosses) lends credence to the hypothesis that the production of efflux pump inhibitors is of great antiquity in land plants.

The last component of this work was the evaluation of methods for the analysis of flavonoids via mass spectrometry. This is of importance to this study due to the commonality of flavonoids in the literature pertaining to efflux pump

inhibitors, and the consistency activity of the flavonoids evaluated in Chapter II. The goal of this work was to compare two methods for the tentative identification of signals in complex data produced via high-resolution mass spectrometry that could be labeled as “possible flavonoids.” The methods evaluated were firstly the use of mass spectrometry fragmentation spectra to identify key diagnostic fragments of the flavonoid ring structure and secondly the use of mass defect filtering directly applied to high resolution data to select a short list of signals for further processing. The former method was not fruitful due to a combination of the frequent poor fragmentation and the dependency on standards for all samples. The latter proved more useful, successfully producing a list of potential flavonoids to be carried forward to other methods such as database searching and molecular formula calculation. This method was also successfully applied to a complex extract of *Hydrastis canadensis*, identifying three flavonoids known from previous work to be present. Further, the mass defect method is an intrinsic property of molecules, and therefore does not change with experimental conditions. For all of these reasons mass defect was selected as the more useful of the two methods evaluated for the identification of “possible flavonoid” signals in crude extracts.

STUDIES ON BACTERIAL EFFLUX PUMP INHIBITORS IN LAND PLANTS

by

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I dedicate this dissertation to my beloved wife and partner in all things, Renee. To her I extend my most heartfelt gratitude. Without her love and support this project would not have been possible. Thank you.

APPROVAL PAGE

This dissertation has been approved by the following committee of the Faculty of the Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION AND BACKGROUND

The advent and increased usage of modern antibiotics has resulted in a dramatic reduction in deaths due to bacterial infections. However, in more recent times, there has been an increase in the abundance of bacterial strains that are resistant to these drugs. MRSA, methicillin-resistant *Staphylococcus aureus*, is an excellent example of drug resistant bacterium that has been on the rise in both the clinical and community settings since the 1960's. One series of estimates for the damage caused by MRSA in the United States in 2005 claims 94,360 MRSA infections resulting in 18,650 deaths in the same year (Klevens et al. 2007). Antimicrobial resistance can evolve through multiple pathways, but only one is of importance to this study. This pathway is the over-expression of efflux pumps that export the antimicrobial out of the bacterial cell, preventing it from building up to toxic levels (Simões et al. 2009).

The research described in this dissertation focuses on the identification of botanical secondary metabolites that function to inhibit bacterial efflux pumps. The purpose of these studies is three fold. The first goal was to develop an improved assay for the measurement of efflux pump inhibitory activity across a diversity of sample types. The second was to elucidate the distribution of efflux pump inhibitory activity in land plants. Lastly, we sought to improve methods for

tentative identification of flavonoids (an important class of efflux pump inhibitory compounds) in complex mixtures in cases where analytical standards are not available. These three goals correspond to the research laid out in chapters two, three and four, respectively.

This research is important both for providing new insights into evolutionary biology of land plants, and also facilitating future studies aimed at the identification of efflux pump inhibitors from natural product sources. The research described in this dissertation focuses on the identification of botanical secondary metabolites that function to inhibit bacterial efflux pumps. This research is important both for providing new insights into evolutionary biology of land plants, and also facilitating future studies aimed at the identification of efflux pump inhibitors from natural product sources.

Background on Bacterial Efflux Pumps

A number of efflux pumps exist, in both prokaryotic and eukaryotic cells. One class of efflux pumps is driven by the cleavage of ATP, allowing molecules to be pumped against a diffusion gradient. Most of the remaining classes of efflux pumps use the proton motive force to drive active transport. The last group (those driven by the proton motive force) and the NorA efflux pump in particular (a member of this group) are the most prominent in *Staphylococcus aureus* (Abramson et al. 2003, Huang et al. 2003, Sabatini et al. 2008, Saidijam et al. 2006, Yin et al. 2006). The inhibition of these efflux pumps can dramatically increase the activity of a wide range of antimicrobials *in vitro* (Tegos et al. 2002).

Background on the Relevance of Efflux Pump Inhibitors to Existing Human Medicinal Plant Usage

A number of studies have shown that many individuals around the world are turning to complementary and alternative medicine (CAM), with herbal supplements being a particularly prominent part of such CAM treatments. An early study on the usage of CAM in the United States published in 1993 (Eisenberg et al. 1993) found that 34% of Americans use some form of alternative treatment. Two later studies show the specific usage of natural products are an important component of CAM—with these natural products being used by 19% (Barnes et al. 2004) in a 2004 study and by 18% in a 2008 study (Barnes and Bloom 2008).

Ethnobotanical work can inform the scientific community regarding the usage of medicinal plants in a broader context. One of the often noted observations of ethnobotanists is that plants are used in combinations (Bussmann et al. 2010, Vandebroek et al. 2010, Odhiambo et al. 2011). Even if this was not the case, the field of phytochemistry provides many illustrations pertaining to the complexity of plant extracts. It is common to begin work on an active plant extract, and then observe incremental losses in activity as the “active” compound is purified. Alternatively, it is also common for the “active” compound to be present at too low a concentration to explain the totality of the observed activity. This leads to the conclusion that some mechanism of activity is present in the extract

which is lost upon purification. This is likely due to synergistic interactions (non-additive positive interactions between mixture components) between multiple compounds (Bame et al. 2013, Berenbaum 1989, Junio et al. 2011, Stermitz et al. 2000b). The possibility of synergistic interactions complicates any study seeking to either validate the efficacy of a particular preparation or to discover its mechanism of action. In light of these observations, more studies dealing specifically with mechanisms of synergy are needed.

Significance to Public Health

This body of research is significant to public health because it relates to the problem of emerging drug resistance. The overproduction of efflux pumps is a common form of drug resistance, so identification of new efflux pump inhibitors is expected to hasten the development of effective adjuvant therapies to existing antibiotics (Lomovskaya and Bostian 2006). Toward this goal, this research is in large part aimed at improving methods for identifying efflux pump inhibitors. Secondly, a better understanding of plant derived efflux pump inhibitors is expected lead to improved application of existing plant-derived CAM therapies. Thirdly, by exploring the distribution of efflux pump inhibitors in plants; this research will increase our understanding of plant biology and evolution, as discussed in greater detail in Chapter III.

Significance to Plant Biology

This research seeks to fill two gaps in the plant biology literature. The first is the description of the distribution of efflux pump inhibitors in land plants. While efflux pump inhibitors have been identified previously, their overall distribution is unknown. The significant body of literature describing natural products that are active against bacterial efflux pumps is summarized and cited in Table 1.1. This literature demonstrates two things. The first of these is that while there are several classes of compounds that have been found to be active against bacterial efflux pumps, the flavonoids are the most common (Table 1.1). Secondly, efflux pump inhibitory activity is widely distributed in the angiosperms (flowering plants) (Table 1.1). There is one gymnosperm mentioned in the literature, and no monocots or any of the land plant groupings more ancient than the gymnosperms (Table 1.1). This makes it impossible to draw any conclusions regarding the evolutionary history of efflux pump inhibitors beyond the observation that it appears to be common in the eudicot clade. The second area of study will address the apparent commonness of efflux pump inhibitory activity in the flavonoid chemical family. Towards this end, we sought to discover whether or not efflux pump inhibitory flavonoids are common throughout the set

Table 1.1. Summarized Literature Demonstrating the Occurrence of Plants Producing Bacterial Efflux Pump Inhibitors	
Plant	Efflux Pump Inhibitory Compound(s) ^a
Gymnosperms:	
<i>Chamaecyparis lawsoniana</i>	Various diterpenes, including totarol and ferruginol (*) (#) (Smith et al. 2007a, 2007b)
Angiosperms:	
<i>Artemisia annua</i>	Flavones (*) (Gibbons 2004, 2005, Stermitz et al. 2002)
<i>Berberis spp., including B. fremontii, B. trifoliolata, B. fendleri</i>	Pheophorbide a porphyrin, 5'-methoxyhydrnocarpin a flavolignan (*) (#) (Gibbons 2004, 2005, Guz et al. 2001, Stavri et al. 2007, Stermitz et al. 2000a, 2000b, 2001)
<i>Camellia sinensis</i>	Epicatechin gallate and epigallocatechin gallate (*) (#), substituted flavonoids (Hamilton-Miller and Shah 2000, Simões et al. 2009, Stavri et al. 2007)
<i>Citrus paradisi</i>	coumarin and bergamottin derivatives (*) (Abulrob et al. 2004, Stavri et al. 2007)
<i>Dalea versicolor, D. spinosa</i>	Multiple phenolics, including flavonoids, stilbinoids, an arylbenzofuran aldehyde, a pterocarpan (*) (Belofsky et al. 2004, 2006, Stavri et al. 2007)
<i>Geranium caespitosum</i>	Polyacylated neohesperidosides (*) (Stavri et al. 2007, Stermitz et al. 2003, Gibbons 2004, 2005)
<i>Hydrastis canadensis</i>	A flavonoid (*) (#) (Ettefagh et al. 2011, Junio et al. 2011)
<i>Ipomea murucoides</i>	Various Oligosaccharides (*) (#) (Chérigo et al. 2009, Gibbons et al. 2003, Stavri et al. 2007, Stermitz et al. 2003)
<i>Jatropha elliptica</i>	A penta-substituted pyridine, propacine a coumarin lignin (*) (#) (Marquez et al. 2005, Stavri et al. 2007)
<i>Lupinus argenteus</i>	Genistein, orobol, biochanin A, isoflavones (*) (Gibbons 2004, Morel et al. 2003, Stavri et al. 2007)

<i>Lycopus europaeus</i>	Various terpenoids(*) (Gibbons et al. 2003, Gibbons 2004, 2005, Stavri et al. 2007)
<i>Mirabilis jalapa</i>	Polyphenolic amide derivatives (*) (#) (Michalet et al. 2007)
<i>Piper nigrum, P. longum</i>	Piperine, an alkaloid (*) (Khan et al. 2006, Nargotra et al. 2009, Stavri et al. 2007)
<i>Prosopis juliflora</i>	Piperidine alkaloids (#) (Gibbons 2005, Stavri et al. 2007)
<i>Rauwolfia vomitoria</i>	Reserpine, alkaloid (#) (Brenwald et al. 1998, Gibbons 2005, Simões et al. 2009, Stavri et al. 2007)
<i>Rosmarinus officinalis</i>	Carnosic acid, a diterpene (#) (Gibbons 2005, Oluwatuyi et al. 2004, Stavri et al. 2007)
<i>Silybum marianum</i>	Silybin, a flavolignan(*) (Gibbons 2004, Stavri et al. 2007 Stermitz et al. 2000a)
<i>Thymus vulgaris</i>	Baicalein, a trihydroxy flavones (*) (Stavri et al. 2007)
Plant extracts which have shown either synergy or EPI activity, for which the active compound is unknown: <i>Berberis aetnensis, Mezoneuron benthamianum, Securinega virosa, Punica granatum, Commiphora molmol, Centella asiatica, Daucus carota, Citrus aurantium, Glycyrrhiza glabra</i> [as reviewed by (Stavri et al. 2007)]	

a. Methods used to identify efflux pump inhibitory activity: (*) = tested for synergy with efflux pump substrates, (#) = tested directly for efflux pump inhibitory activity.

Knowledge pertaining to the question of flavonoid activity against bacterial efflux pumps is related to the knowledge of how this activity is distributed in land plants. This relationship exists because flavonoids are important and well-studied compounds within land plants and some algal lineages. The most ancient of all plant groups to produce flavonoids is the algal order Charales,

which is a sister group to the land plants (Becker and Marin 2009, Delwiche et al. 2002, Garbary et al. 1993, Iwashina 2000, Markham and Porter 1969b, Stafford 1991). These algae produce the simple flavonoids apigenin and luteolin, the production of which is carried on in the simplest land plants, the liverworts (Iwashina 2000, Markham and Porter 1969a, 1969b). The diversity of flavonoids produced increases in plants more derived than the liverworts, making this class of compounds in all its forms important markers of land plant evolution (Harborn 1977, Iwashina 2000, Stafford 1991). Flavonoids are also produced in some fungal lineages (Iwashina 2000), but these are not evaluated in this study.

Significance to Bioanalytical Chemistry

In addition to the significance to plant biology and human health, this research is significant to the field of bioanalytical chemistry because it advances methods for studying the phenomenon of efflux pump inhibition. The efflux inhibition assay most often used in the literature (Ettfagh et al. 2011, Kaatz et al. 2000, Lechner et al. 2008, Pereda-Miranda et al. 2006, Sabatini et al. 2008, Schindler et al. 2013, Stermitz et al. 2000, Tegos et al. 2002) is based on time series data measuring the rate of efflux of ethidium bromide (abbreviated EtBr, a well known efflux pump substrate) from cells pre-loaded with this compound. Data in this assay are collected in the form of changes in the fluorescent intensity of EtBr. The existing assays are non-robust because efflux pumps export the EtBr extremely rapidly. Preliminary experiments (Baer et al. 1982, Howitz et al. 2003, Hudson et al. 2009, Remans et al. 1999, Matsuura et al. 2002) revealed

that another problem with the use of fluorescence is the fact that many compounds can quench or otherwise interfere with a fluorescent signal, which will be discussed at greater length in Chapter II of this dissertation. To get around this problem, we sought to develop a new assay based on the use of mass spectrometry to detect changes in EtBr concentrations.

The final objective of this research effort was to evaluate methods for the identification and dereplication of flavonoids in crude plant extracts. The literature already reviewed within this chapter strongly hints at the importance of flavonoids as efflux pump inhibitors, and discusses their commonality in land plant lineages. In light of this, mass spectrometric methods for rapidly determining the flavonoid content of a mixture could be useful research tools. There are three groupings of methodologies for the determination of flavonoids via mass spectrometry in the current literature. The first and most common of these is the *a priori* choice and use of standards to quantify a specific set of flavonoids (Brolis et al. 1998, Justesen et al. 1998, Volpi and Bergonzini 2006). This method is used extensively within Chapter III. However, the ability to evaluate flavonoid content without *a priori* decisions as to which specific molecules are going to be important could also be useful. Therefore, there is need for methods which could be used to reduce a complex data set to a “short list” of signals which may be flavonoids, to facilitate further follow-up analysis. There are two such methods present in the literature. The first of these is the use of MS-MS fragmentation studies to identify potential flavonoids (Cuyckens and

Claeys 2004, Justesen 2001, March et al. 2004a, 2004b, March and Brodbelt 2008, Zhang and Brodbelt 2003, Zhou et al. 2007). The second method involves the filtering of high-resolution mass spectra based on mass defect parameters (Gu et al. 2011, Hughey et al. 2001, Sleno 2012, Wu et al. 2004, Zhang et al. 2009), which has been increasing in prominence with the proliferation of higher resolution mass spectrometers. These last two methods are compared and evaluated in Chapter IV of this dissertation.

The overall purpose of this body of work has been to conduct basic research into efflux pump inhibition, improving methods for the study of this phenomenon and producing information valuable to understanding the botanical context in which it occurs. The first of these goals is of obvious importance to medical science, as has been explained previously in this introduction. The latter of these goals is of importance to understanding the role efflux pump inhibition plays in the plant kingdom. This question is one of importance because it can inform a wide array of future studies on the efflux pump inhibitors at many levels, including future pharmaceutical research, research into the ecology of plant defense mechanisms, and research into human usage of botanical medicines.

Table 1.2. Distribution in the Plant Kingdom of the 4 Example Flavonoids, Based on a Review of Existing Literature	
Flavonoid aglycone	Distribution and citations
Apigenin	<i>Chlorophyta</i> , widespread in the bryophytes including both liverworts and mosses, <i>Psiloteceae</i> , <i>Lycopodiaceae</i> , <i>Selaginellaceae</i> , <i>Isoetaceae</i> , <i>Equisetum</i> , <i>Psilotaceae</i> , <i>Pteridopsida</i> ferns, widespread in flowering plants (Basile et al.1999, Hiraoka 1978, Iwashina 2000, Iwashina et al. 2006,Markham et al. 1998, Markham and Porter 1973, Miean and Mohamed 2001, Richardson 1989, Salatino and Pradao 1998, Veit et al. 1995, Wallac 1989, Wollenweber 1989)
Luteolin	Liverworts, Mosses, <i>Equisetum</i> , <i>Lycopodiaceae</i> , <i>Pteridopsida</i> ferns, widespread in flowering plants (Basile et al. 1999, Hiraoka 1978, Iwashina 2000, Markham et al. 1998, Mian and Mohamed 2001, Richardson 1989, Salatino et al. 2000, Salatino and Pradao 1998, Veit et al. 1995)
Quercetin	Liverworts, mosses, <i>Pteridopsida</i> ferns, <i>Equisetum</i> , <i>Psilotaceae</i> , <i>Pinaceae</i> , widespread in flowering plants (Chen and Xiao 2005, Hertog et al. 1992, Hiraoka 1978, Hollman et al. 1997, Kaundun et al. 1998a, 1998b, Iwashina 2000, Iwashina et al. 1995, 2006, Mian and Mohamed 2001, Richardson 1989, Salatino et al. 2000, Salatino and Pradao 1998, Veit et al. 1995, Wallace 1989)
Kaempferol	Mosses, <i>Pteridopsida</i> ferns, <i>Equisetum</i> , <i>Psilotaceae</i> , <i>Pinaceae</i> , widespread in flowering plants (Hertog et al. 1992, Hiraoka 1978, Kaudun et al. 1998a, 1998b, 2000, Iwashina 2000, Iwashina et al. 1995, 2006, Mian and Mohamed 2001, Niemann and Genderen 1980, Richardson 1989, Salatino et al., 2000, Salatino and Pradao 1998, Veit et al. 1995, Wallace 1989, Wollenweber 1989)

CHAPTER II

A MASS SPECTROMETRY-BASED ASSAY FOR IMPROVED QUANTITATIVE MEASUREMENTS OF EFFLUX PUMP INHIBITION

Note: This chapter of the dissertation is a paper currently in press with the journal Plos One.

Abstract

Bacterial efflux pumps are active transport proteins responsible for resistance to selected biocides and antibiotics. It has been shown that production of efflux pumps is up-regulated in a number of highly pathogenic bacteria, including methicillin resistant *Staphylococcus aureus*. Thus, the identification of new bacterial efflux pump inhibitors is a topic of great interest. Existing assays to evaluate efflux pump inhibitory activity rely on fluorescence by an efflux pump substrate. When employing these assays to evaluate efflux pump inhibitory activity of plant extracts and some purified compounds, we observed severe optical interference that gave rise to false negative results. To circumvent this problem, a new mass spectrometry-based method was developed for the quantitative measurement of bacterial efflux pump inhibition. The assay was employed to evaluate efflux pump inhibitory activity of a crude extract of the botanical *Hydrastis canadensis*, and to compare the efflux pump inhibitory activity of several pure flavonoids. The flavonoid quercetin, which

appeared to be completely inactive with a fluorescence-based method, showed an IC₅₀ value of 75 µg/mL with the new method. The other flavonoids evaluated (apigenin, kaempferol, rhamnetin, luteolin, myricetin), were also active, with IC₅₀ values ranging from 19 µg/mL to 75 µg/mL. The assay described herein could be useful in future screening efforts to identify efflux pump inhibitors, particularly in situations where optical interference precludes the application of methods that rely on fluorescence.

Introduction

Bacterial efflux pumps are active transport proteins that function to extrude toxic compounds, including antimicrobial drugs, from the cell. These pumps serve to protect bacteria from damage by toxins, and can play a role in the development of resistance to antimicrobials (Bambeke et al. 2000, Brenwald et al. 1998, Saidijam et al. 2006, Tegos et al. 2002, Yin et al. 2006). For example, it has been shown that production of efflux pumps is up-regulated in drug resistant strains of many bacteria, including methicillin resistant *Staphylococcus aureus* (Junio et al. 2011, Piddoc 2006a, 2006b, Stermitzet al. 2000b, Walsh 2000). Compounds that inhibit bacterial efflux pumps are of interest because of their potential to increase antimicrobial effectiveness (Simões et al. 2009). Thus, our laboratory has been engaged in experiments to find new efflux pump inhibitors (EPIs) from natural product sources.

Current methods for evaluating efflux pump inhibitory activity rely on an efflux pump substrate that fluoresces only when it is located inside a cell (due to

intercalation with DNA) (Olmsted and Kearns 1977). The majority of existing protocols operate by pre-loading cells with the efflux pump substrate ethidium bromide, which gives them a high initial fluorescent intensity. The extent of efflux pump inhibition is then measured by comparing the rate of decrease in fluorescence intensity over time in the presence of varying amounts of the putative EPI (Ettefagh et al. 2011, Kaatz et al. 2000, Lechner et al. 2008, Pereda-Miranda et al. 2006, Sabatini et al. 2008, Schindler et al. 2013, Stermitz et al. 2000, Tegos et al. 2002). Related experiments utilizing measurements based on the intracellular accumulation of fluorescent substrates have also been reported (Mahamoud et al. 2006, Stermitz et al. 2000b). For accumulation experiments, fluorescence increases over time as the substrate diffuses into cells.

Ethidium bromide is attractive as a substrate for assaying efflux pump inhibition because of extensive literature precedent of its application in efflux inhibition assays (Ettefagh et al. 2011, Kaatz et al. 2000, Lechner et al. 2008, Pereda-Miranda et al. 2006, Sabatini et al. 2008, Schindler et al. 2013, Stermitz et al. 2000, Tegos et al. 2002). This compound has also been established to be active via intracellular action, with literature precedent stretching back to the 1950's (Newton 1957, Olmsted and Kearns 1977, Waring 1965). However, the existing methods for testing efflux pump inhibition with ethidium bromide gave false results in our study due to matrix quenching effects (the suppression of fluorescence by various components of the mixture) in crude extracts and even with some pure compounds. We endeavored to circumvent

these quenching effects by developing a new mass spectrometry-based efflux pump inhibition assay. There is extensive literature support for the efflux pump inhibitory activity of flavonoids and related compounds (Ettefagh et al. 2011, Gibbons 2004, 2005, Guz et al. 2001, Hamilton-Miller and Shah 2000, Junio et al. 2011, Simões et al. 2009, Stavri et al. 2007, Stermitz et al. 2000a, 2000b, Stermitz et al. 2001, Stermitz et al. 2002). Thus, we sought to validate the new assay by comparing efflux pump inhibitory activity of a series of pure flavonoids. In addition, to test the validity of the new assay in a more crude sample matrix, we compared the efflux pump inhibitory activity of an extract from the botanical goldenseal (*Hydrastis canadensis*), which is known to contain EPIs (Ettefagh et al. 2011, Junio et al. 2011), using both fluorescence and mass spectrometry-based approaches for data collection.

Materials and Methods

Preparation of plant material

The goldenseal leaf and petiole material used was cultivated in a woodland setting in Hendersonville, North Carolina, (N 35°24.2770', W 082°20.9930', 702.4 m elevation) and was made available by William Burch; this population has been utilized in previously published work (Ettefagh et al. 2011, Junio et al. 2011) and is represented by a voucher (NCU583414) curated in the University of North Carolina at Chapel Hill herbarium. The goldenseal extract was prepared using previously described methods (Gu et al. 2004, Junio et al. 2011). Dried plant material was macerated for at least 24 hr, and the methanol

extract was subsequently separated from the plant material. This extract was dried in a rotary evaporator to reduce the volume of methanol, and partitioned against an equal volume of hexane. The resulting mixture was stirred for at least one hr and the layers were collected separately using a separatory funnel. The methanol partition was added to water and chloroform in a ratio of 1:5:4, stirred for at least 1 hr, then separated. The chloroform partition from this step was evaporated and used as the starting material for all experiments described herein, and will be referred to as “goldenseal extract” from this point forward.

96 well plate ethidium bromide accumulation assay

This assay is an adaptation of published ethidium bromide efflux-based assays (Ettefagh et al. 2011, Kaatz et al. 2000, Schindler et al. 2013) and previously published reports of measurements on intracellular accumulation of berberine and chloramphenicol (Mahamoud et al. 2006, Stermitz et al. 2000b). All experiments presented here were performed in the 96 well plate format. Activity was tested using *Staphylococcus aureus* strain NCTC 8325-4 (Novick 1967). The final assay composition was 10% DMSO, 50% Muller-Hinton broth, 40% water (by volume), an estimated $1.6-1.8 \times 10^8$ CFU/mL *S. aureus*, 1.25 $\mu\text{g/mL}$ ethidium bromide, and a range of analyte concentrations. Data collection, and hence bacterial exposure to these conditions, was limited to 30 min. The alkaloid piperine was used as a positive control, as it is well established in the literature to be an EPI (Khan et al. 2006, Nargotra et al. 2009, Stavri et al. 2007). Each analyte concentration was tested in triplicate, and the positive control (a piperine

dilution series ranging from 4.7 µg/mL to 300 µg/mL prepared via 2-fold dilution) was included on each plate. Fluorescence was measured using a BioTek SynergyH1 microplate reader (BioTek, Winooski, VT) with an excitation wavelength of 520 nm and emission wavelength of 600 nm at 1 min intervals for a total of 30 min. All experiments were performed in triplicate and vertical bars reported as standard deviation.

Use of mass spectrometry to measure ethidium bromide accumulation

To enable mass spectrometric measurements of ethidium bromide accumulation, the experimental parameters were identical to those described in the previous section, except that the method of data collection was modified. The prepared samples were incubated at room temperature in a EMD Millipore MultiScreen™ fritted-bottom 96-well filter plate (pore size 0.22 µm, EMD Millipore, Darmstadt, Germany). At the conclusion of the 30 min incubation period, these were filtered simultaneously under vacuum into a receiving 96 well plate. All solutions were stored at 4 °C prior to analysis.

Ethidium bromide in the bacterial supernatant was analyzed using high performance liquid chromatography (HPLC) electrospray ionization-mass spectrometry (ESI-MS). Liquid chromatography separations were achieved using a ThermoFinnigan Surveyor HPLC system (Thermo Finnigan, San Jose, CA). The autosampler was temperature controlled at 8 °C, and the column (Agilent Prevail C₁₈, 3 µm packing, 50 x 2.1 mm) was heated to 40 °C. Sample injection volume was 5 µL and a flow rate of 0.2 mL/min was employed.

Samples were eluted using binary gradients consisting of acetonitrile acidified with 1% acetic acid and deionized water (CH₃CN:H₂O) as follows:— 0 min, 0:100; 1.5 min, 0:100; 2 min, 95:5; 10 min, 95:5; 10.5 min, 0:100; 18 min, 0:100. Mass spectrometry analyses were conducted with an LCQ DECA XP Plus ion trap mass spectrometer with electrospray ionization source (Thermo Fisher Scientific) using the following conditions: capillary temperature, 250 °C; sheath gas flow, 10 (arbitrary units); no auxiliary gas; source voltage 4.5 kV; capillary voltage, 42 V; tube lens offset, -25 V. The instrument was operated in the positive ion mode with two scan events. The first was full scan, followed by the data-dependent CID fragmentation (50% collision energy) of *m/z* 314.20 (the [M]⁺ ion of ethidium). The selected ion chromatogram was plotted for the main product ion *m/z* 286, and its peak area was determined. All experiments were performed in triplicate and vertical bars set to standard deviation.

Mass spectrometry data were analyzed to determine an IC₅₀ value for each test compound. The IC₅₀ of piperine was defined as the midpoint between the peak area for vehicle control and that of the 300ppm piperine sample, similar to an approach employed previously [42]. Once determined for piperine, the same peak area was used as a set point for determining IC₅₀ values of the test compounds on the same plate.

Bacterial growth inhibition

Minimum inhibitory concentrations (MICs) were determined according to Clinical Laboratory Standards Institute guidelines (Wikler et al. 2006). Solutions were prepared in 96 well plates with a final well volume of 250 μ L, 2% DMSO in Mueller-Hinton broth, and variable concentrations of test compound or extract ranging from 4.7 to 150 μ g/mL, prepared in triplicate. Duplicate plates of each experiment were employed, one inoculated with a bacterial concentration of 5×10^5 CFU/mL, the other containing only analyte and vehicle. All plates were incubated for 18 hr at 37 °C, after which turbidity at 600nm (OD_{600}) was measured with a BioTek SynergyH1 microplate reader. To correct for background due to absorbance of the analyte compounds, the mean OD_{600} for each treatment without addition of bacteria was subtracted from the mean OD_{600} of treated wells. MIC was determined as the concentration where there was no statistically significant difference between the mean absorbance of the treated wells and that of the negative control (vehicle in broth).

Results and Discussion

Assay development and comparison of efflux pump inhibition assay methods

The first goal of our experiments was to determine the applicability of a fluorescence-based accumulation assay to measure the efflux pump inhibitory activity of various flavonoids. Towards this goal, we first validated the assay using a known efflux pump substrate (ethidium bromide) and a known EPI (piperine). As expected, when *S. aureus* is exposed to ethidium bromide,

fluorescence increases over time (Figure 2.1). This increase is due to intracellular accumulation of ethidium, which fluoresces at 600 nm when it is intercalated with DNA (Ettfagh et al. 2011). Ethidium bromide is a substrate of NorA, a major chromosomally-encoded *Staphylococcus aureus* efflux pump (Bambeke et al. 2000, Saidijam et al. 2006). Thus, the intracellular accumulation of ethidium bromide by *S. aureus* is counteracted by the action of NorA (and other efflux pumps). As evidence of this, the addition of piperine, a known NorA inhibitor, caused a more pronounced increase in fluorescence over time than was observed for the cells in the absence of the inhibitor (Figure 2.1).

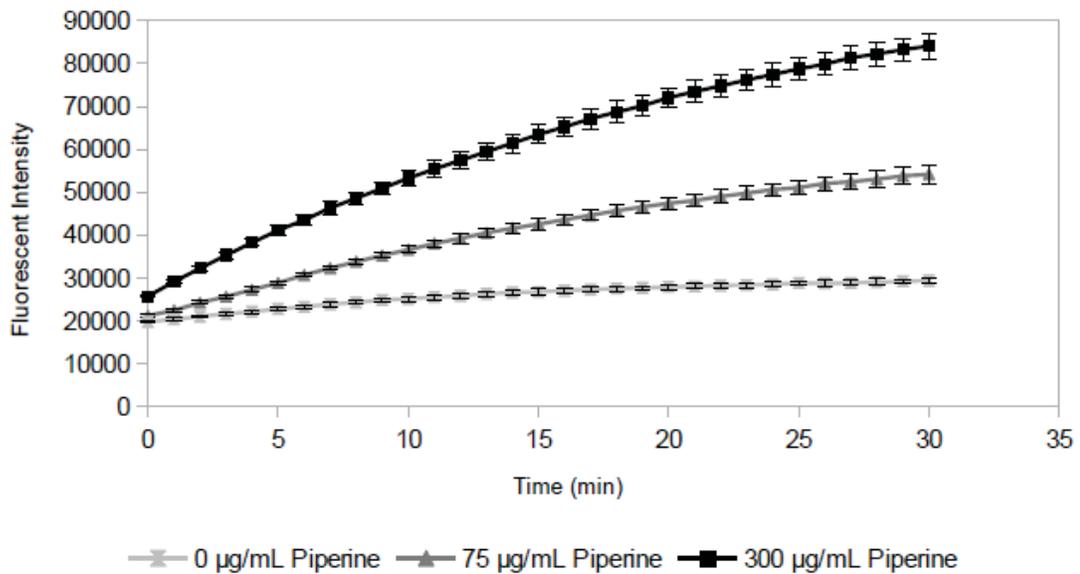


Figure 2.1. Change in Absolute Fluorescent Intensity Over Time for *Staphylococcus aureus* Exposed to Ethidium Bromide and the Efflux Pump Inhibitor Piperine.

Fluorescence increases over time due to intracellular accumulation of ethidium bromide. The increase is more pronounced in the presence of piperine, which enhances intracellular accumulation of ethidium bromide by blocking efflux. Data points represent the mean of 3 samples, vertical bars represent standard deviation.

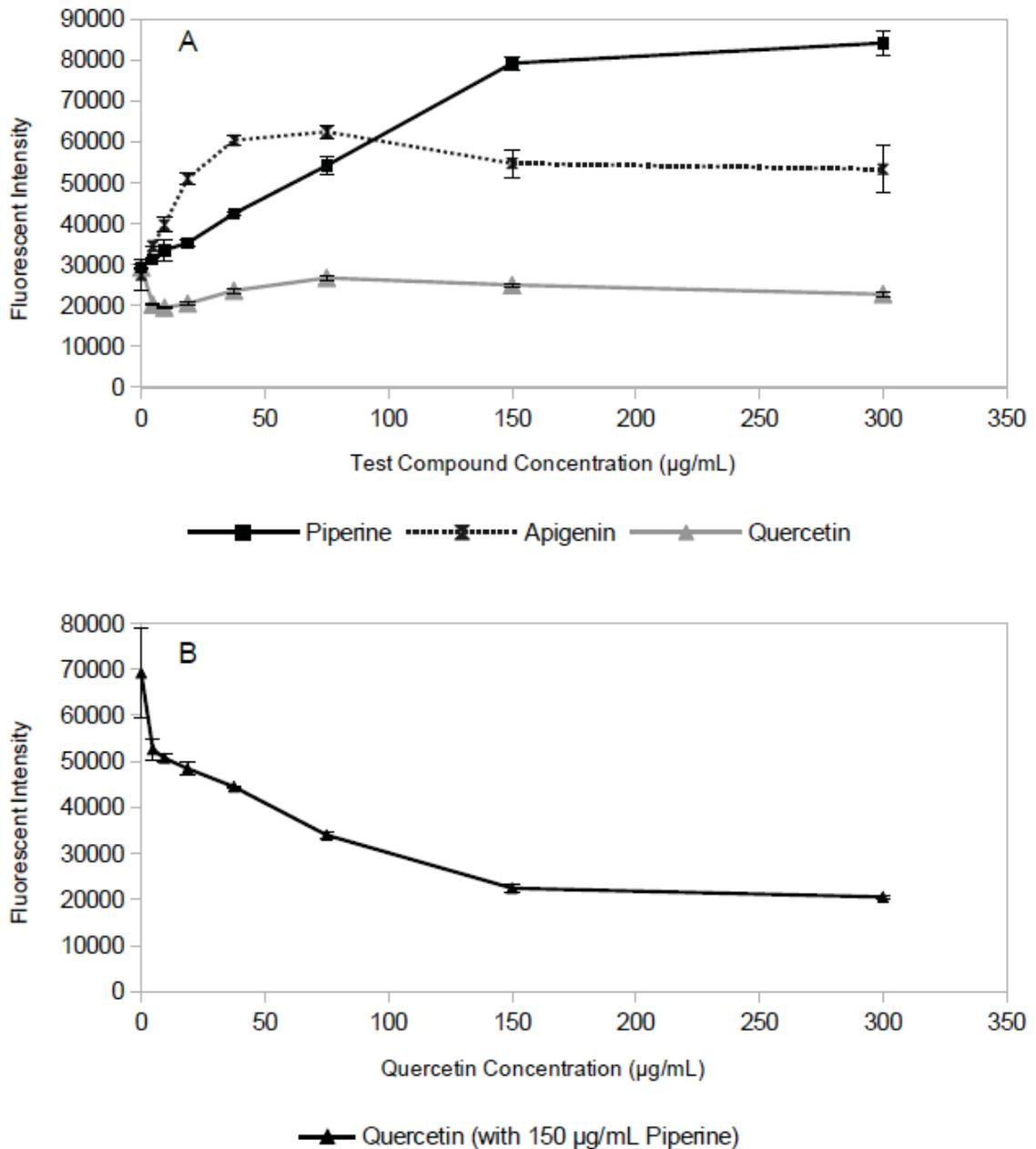


Figure 2.2. Change in Fluorescence Due to Ethidium Accumulation by *S. aureus* in the Presence of Putative Inhibitors. (A) Dose-response curves for the flavonoids apigenin and quercetin. (B) Fluorescence observed for 150 µg/mL piperine in the presence of increasing concentrations of quercetin. Decrease in fluorescence with increasing quercetin concentration in (B) can be attributed to fluorescence quenching by the flavonoid. Data points represent the mean of triplicate measurements (biological replicates), vertical bars represent standard deviation.

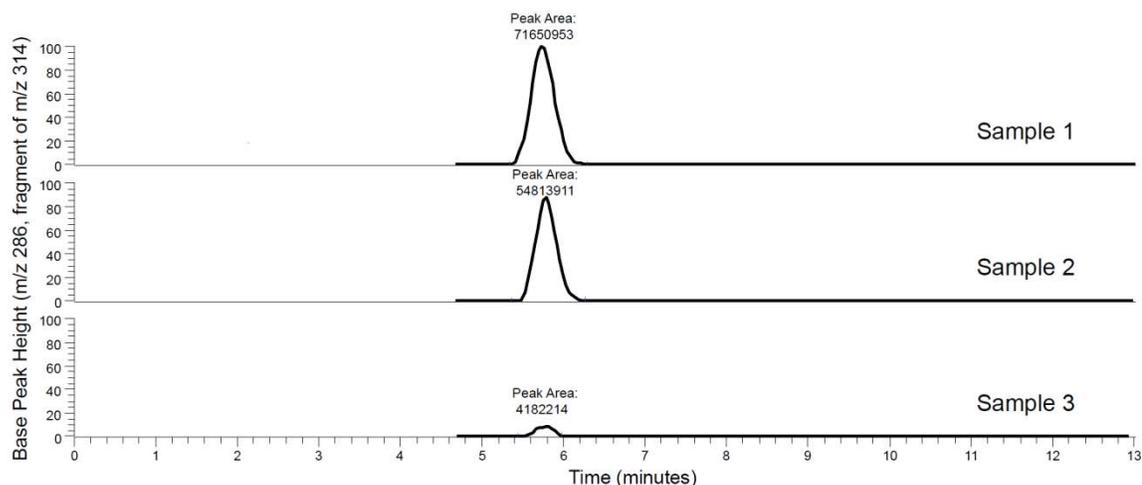


Figure 2.3. Representative Selected Chromatograms of Filtered, Spent Broth Showing a Peak for the Ethidium Ion (MS-MS Transition of m/z 314 to 286).

Sample 1 is the negative control (*S. aureus* cultured for 30 min in Mueller Hinton broth with 1.25 $\mu\text{g}/\text{mL}$ ethidium bromide and 10% DMSO). Samples 2 and 3 were cultured under the same conditions as sample 1 with the addition of 75 $\mu\text{g}/\text{mL}$ and 300 $\mu\text{g}/\text{mL}$ piperine, respectively. All three peaks are normalized to a signal intensity of 3.45×10^6 . As piperine is added, efflux pumps are blocked, trapping the ethidium inside the cells and decreasing the quantity of ethidium (as indicated by the area of the ethidium peak) in the spent broth.

To circumvent the problem with optical matrix interference, a method was developed using liquid chromatography - mass spectrometry (LC-MS) to measure ethidium bromide concentrations in the spent broth filtrate. The concentration-response relationship for the data generated by the LC-MS method was expected to be the inverse of that generated by the measurement of fluorescence—as the concentration of an inhibitor increases, it traps the ethidium inside the bacterial cells, and the concentration of ethidium in the spent broth filtrate (as measured by LC-MS) should decrease.

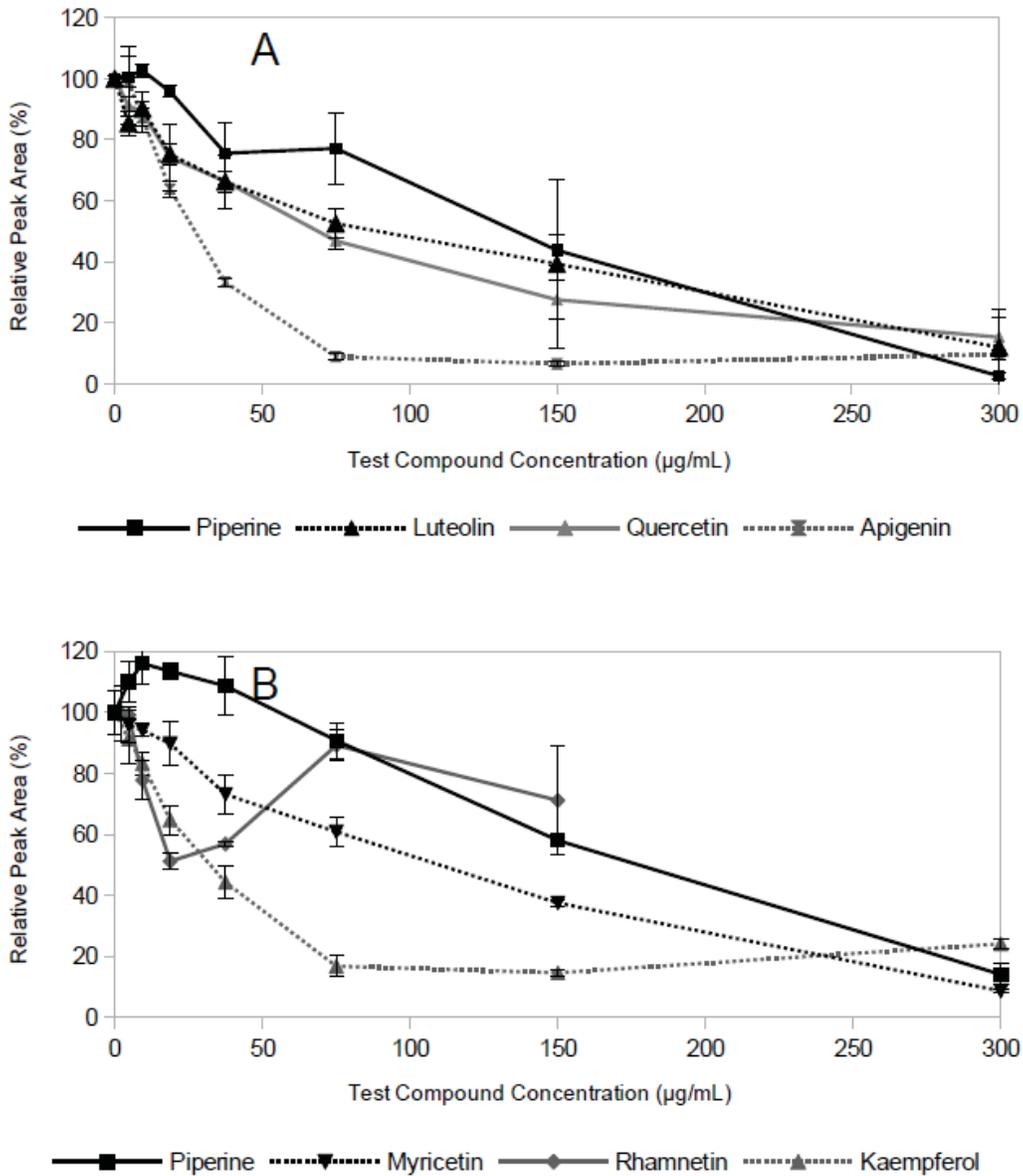


Figure 2.4. Efflux Pump Inhibitory Activity of 6 Flavonoids (Luteolin, Quercetin, Apigenin, Myricetin, Rhamnetin and Kaempferol) Compared to that of Piperine, as Indicated by LC-MS Measurement of Residual Ethidium Bromide in Spent Broth After a 30 Min Incubation.

Relative peak area (expressed as a percentage) for ethidium is plotted as a function of concentration of the putative inhibitor. Data points represent the mean of 3 measurements (biological replicates), with vertical bars representing standard deviation. Rhamnetin was only prepared at concentrations up to 150 µg/mL due to solubility problems. Panels A and B represent data taken from two different 96 well plates, generated via the same method.

The known EPI piperine was again used for assay validation and it was observed that, as expected, the concentration of ethidium in the filtrate decreased with increasing concentration of piperine (Figure 2.3). These results were plotted in a dose-response curve, with peak area displayed on the y-axis and concentration on the x-axis (Figure 2.4).

With the LC-MS assay, it is possible to quantify the extracellular levels of ethidium *without relying on fluorescence*. Thus, if quercetin is in fact an active EPI and the negative results observed in the fluorescence based assay (Figure 2.2) are due to quenching, quercetin should have demonstrable activity in the new LC-MS based assay. Consistent with this expectation, the LC-MS based method showed quercetin to be active as an EPI (Figure 2.4). Once the confounding effect of quenching is removed, it is apparent that quercetin is similar in its EPI activity to piperine, and apigenin is the most active EPI of the three. Furthermore, the LC-MS method showed a more typical dose-response relationship for apigenin (Figure 2.4) compared to the fluorescence-based assay (Figure 2.2A), suggesting that apigenin quenches ethidium fluorescence at concentrations $\geq 38 \mu\text{g/mL}$.

To further evaluate the applicability of the LC-MS based efflux pump inhibition assay, we measured IC_{50} values (Table 2.1) for a series of six structurally diverse flavonoids and the aforementioned compound piperine. All six flavonoids were active (sample data shown in Figure 2.4), with IC_{50} values ranging from $19 \mu\text{g/mL}$ (kaempferol and rhamnetin) to $75 \mu\text{g/mL}$ (quercetin,

luteolin, and myricetin.) (Table 2.1). Piperine demonstrated an IC₅₀ value in this assay between 75 µg/mL and 150 µg/mL.

Finally, it was of interest to evaluate whether the mass spectrometry-based efflux assay would be applicable to samples with more complex matrices. Toward this goal, a botanical extract prepared from goldenseal (*Hydrastis canadensis*) was evaluated for EPI activity using both the fluorescence-based and mass spectrometry-based ethidium bromide accumulation assays. When assayed by the fluorescence-based method, the extract appeared inactive (no apparent IC₅₀, Figure 2.5A), contradicting literature that indicates *H. canadensis* extracts contain EPIs (Ettfagh et al. 2011, Junio et al. 2011). However, when the activity of the extract was evaluated with the mass spectrometry-based assay, an IC₅₀ value of 75 µg/mL was observed (Figure 2.5B). These results demonstrate that quenching can hamper the measurement of efflux pump inhibition for complex extracts using fluorescence, and that the interference can be overcome using the method developed herein. This finding is particularly important given that it may be useful in drug discovery efforts to screen complex extracts for the presence of EPIs.

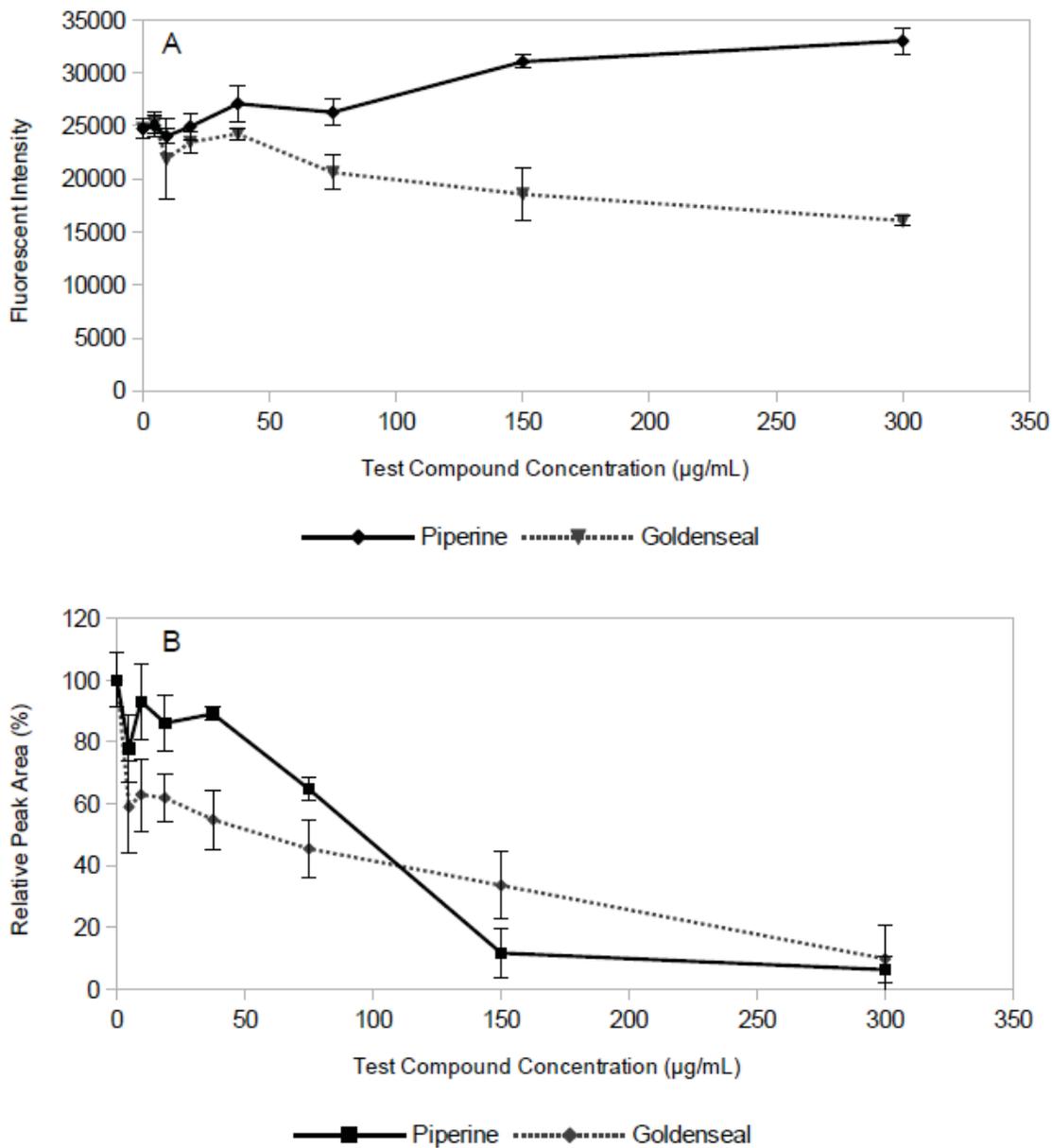


Figure 2.5. Efflux Pump Inhibition in *S. aureus* by a Goldenseal (*Hydrastis canadensis*) Extract.

Data collected using the fluorescence-based ethidium accumulation assay for a range of *H. canadensis* extract concentrations. (B) Data collected using the mass spectrometry-based ethidium accumulation assay. Incubation time was 30 min for both A and B, data represents mean of 3 samples, vertical bars represent standard deviation. Note, bacterial growth was less vigorous in this assay (Figure 2.5) as compared to Figures 2.1 and 2.2, which is reflected in the lower overall fluorescence values.

Flavonoid	IC₅₀ for efflux inhibition^a	MIC^b
Apigenin	38 µg/mL (140 µM)	>150 µg/mL (>560 µM)
Kaempferol	19 µg/mL (66.0 µM)	>150 µg/mL (>520 µM)
Rhamnetin	19 µg/mL (60.0 µM)	>150 µg/mL (>470 µM)
Quercetin	75 µg/mL (250 µM)	>150 µg/mL (>500 µM)
Luteolin	75 µg/mL (260 µM)	75 µg/mL (260 µM)
Myricetin	75 µg/mL (240 µM)	150µg/mL (470 µM)

- a. Efflux pump inhibition was measured via LC-MS analysis of ethidium in spent, filtered culture supernatant after a 30 min incubation in triplicate wells.
- b. Growth inhibition was measured by optical density at 600nm (in triplicate) after an 18 hr incubation.

Potential interference by growth effects

Many botanical compounds possess antimicrobial activity. Thus, it was important to evaluate whether growth effects might confound the measurements of efflux pump inhibition. Towards this goal, the flavonoids and the goldenseal extract were screened for inhibition of bacterial growth across a range of concentrations, from 4.7 µg/mL to 150 µg/mL. Only two samples (the flavonoids luteolin and myricetin, Table 2.1) demonstrated measureable MICs under these conditions. To determine whether growth inhibition by these compounds was likely to confound data interpretation, the two inhibitory flavonoids (luteolin and

myricetin) were incubated with test strains under the experimental conditions at twice their IC₅₀ values (Table 2.1). *S. aureus* cells were tested for loss of viability in the presence of these flavonoids (as well as in the presence of the flavonoid apigenin which does not inhibit bacterial growth) by replicating the experimental conditions of bacterial, ethidium bromide, broth and DMSO content, and plating aliquots of the resulting culture onto supplemented Mueller-Hinton agar at 0, 15 and 30 min time points. No loss of viability was observed after a 30 min exposure to the experimental conditions, as determined by colony count enumeration (Figure 2.6). Additionally, to further evaluate the potential for simple toxicity to confound evaluation of data in this assay, the commercial antibiotics gentamicin and nafcillin were subjected to the mass spectrometry-based efflux pump inhibition assays, and no IC₅₀ was observed to the maximum concentration tested of 100 µg/mL (Figure 2.7). The highest tested concentration for the antibiotics was well above their reported MICs against *Staphylococcus aureus* (~0.5 µg/mL (LaPlante and Rybak 2004, Mulazimoglu et al. 1996)). Collectively, these results suggest that growth inhibition does not confound the measurements of efflux pump inhibitory activity reported in Table 2.1.

In light of the health risks posed by the increased occurrence of antibiotic resistant bacterial strains, the need for reliable methods for their study is of high importance. The mass spectrometry-based method to quantitatively investigate efflux pump inhibition is just such a tool, and as such is expected to be of high value to the scientific community. Our study shows that misleading results (false

negative in the case of assays that rely on ethidium bromide accumulation) can be obtained when screening crude extracts and even pure compounds with fluorescence-based efflux pump inhibition assays. The new method presented here circumvents these problems. Additionally, the ubiquity of activity in the flavonoids tested in the validation process of this assay reinforces the importance of this class of natural products in the reversal of efflux-pump mediated drug resistance.

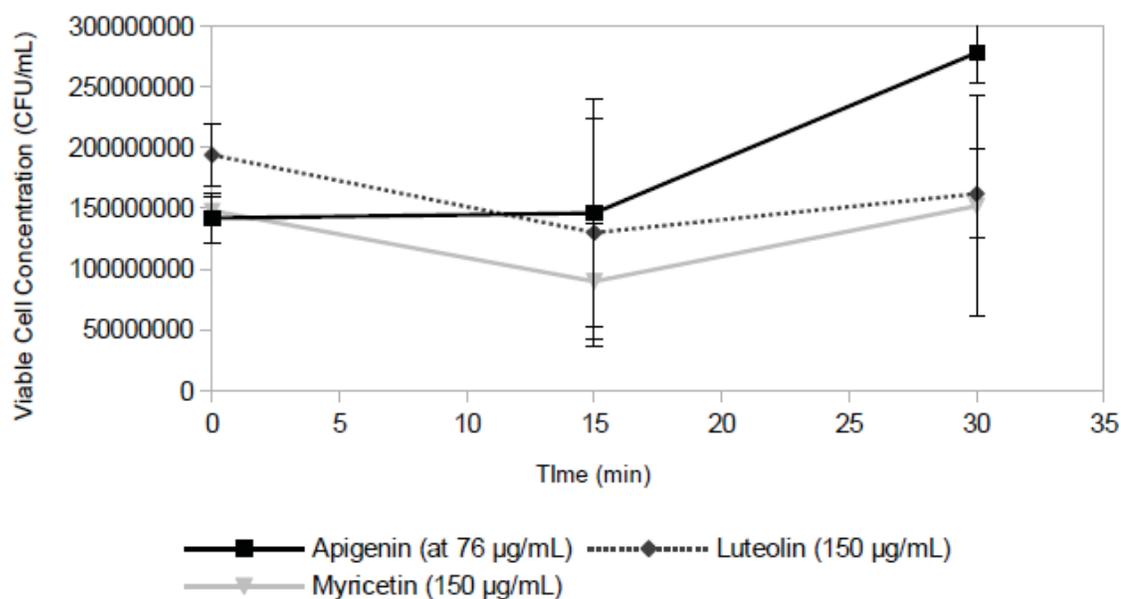


Figure 2.6. Time Series Data Evaluating the Viability of *Staphylococcus aureus* Cultures Exposed to Experimental Conditions.

Conditions are as follows: 10% DMSO, 50% Muller-Hinton broth, 40% water (by volume), with 1.25 µg/mL ethidium bromide, for a maximum of 30 min. Test compounds include two flavonoids that inhibit the growth of this strain (luteolin and myricetin), and one that does not (apigenin) (Table 2.1). Two-tail T-test values comparing the 0 and 30 minute time points are as follows: apigenin, $p= 0.15$; luteolin, $p= 0.16$; myricetin, $p= 0.95$. Thus, no statistically significant changes at the 95% confidence interval were observed in cell viability.

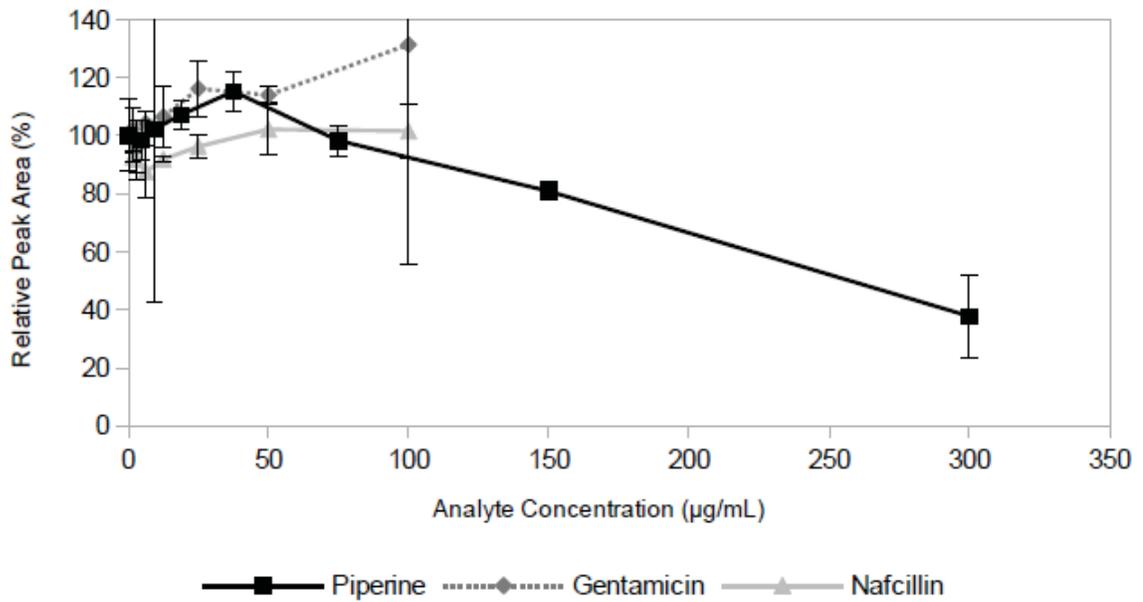


Figure 2.7. Efflux Pump Inhibitory Data Generated Via the Mass Spectrometry-Based Assay Performed on the Known Antibiotic Compounds Gentamicin and Nafcillin.

Also shown is a control dose-response curve performed on the positive control piperine.

CHAPTER III
DISTRIBUTION OF BACTERIAL EFFLUX PUMP INHIBITORS IN LAND
PLANTS

Manuscript in preparation for submission for publication

Abstract

Secondary metabolites play a critical role in plant defense against disease. One important example of this is the interaction between plant antibiotic secondary metabolites and other metabolites that inhibit bacterial efflux pumps. Bacterial efflux pumps are active transport proteins that bacterial cells use to protect themselves against multiple toxic compounds, including many antimicrobials. Plant efflux pump inhibitors can block these efflux pumps, rendering antimicrobial compounds more effective as defense molecules than they otherwise would be. The goal of this research was to employ a recently developed mass spectrometry-based assay to evaluate the distribution of efflux pump inhibitors in fourteen plant extracts spanning the important land plant lineages (two bryophytes, five pteridophytes, three gymnosperms, and four angiosperms). For these studies, *Staphylococcus aureus* was used as a test bacterium. Efflux pump inhibitory activity was observed in extracts from all but four species (*Nephrolepis exaltata*, *Ginkgo biloba*, *Cycas circinalis*, and *Glaucidium palmatum*). The most active extracts were those prepared from

Osmunda claytoniana and *Pinus strobus*, which both demonstrated IC₅₀ values for efflux inhibition of 19 ppm. Our results indicate that efflux pump inhibitors are common in land plants and that they appeared early in the evolutionary history of land plants.

Introduction

Understanding plant defense against bacterial infection is a vital component to the study of plant evolution and ecology. One very interesting plant defense mechanism is the production by plants of bacterial efflux pump inhibitors (Stermitz et al. 2000b). Efflux pumps are active transport proteins located in bacterial cell membranes. Bacteria use the pumps to export, or expel, toxins that enter from the bacterial environment. Efflux pump inhibitors block the function of these pumps, causing intracellular accumulation of toxic compounds. It has been proposed that the presence of bacterial efflux pump inhibitors may partially explain the efficacy of botanical medicines traditionally used to treat infection (Tegos et al. 2002). Efflux pump inhibitors may also play an important role in plant evolution (Pieterse and Dicke 2007, Treutter 2005). For example, certain plants have been shown to produce a mixture of antimicrobial compounds (substrates for efflux pumps) and efflux pump inhibitors that synergistically enhance the efficacy of the antimicrobials (Junio et al. 2011, Stermitz et al. 2000b). Plants produce a large number of secondary metabolites with little or no known activity or usefulness. The reason for this continues to be something of an evolutionary quandary, and has been for some time (Jones and Fern 1991).

The presence of efflux pump inhibitors could shed light on at least one aspect of this issue, and on the evolution and ecology of plant defense against infection.

Plant extracts and secondary metabolites that inhibit bacterial efflux pumps are also of interest because of their potential to increase human relevant antimicrobial effectiveness (Simões et al. 2009). Over-production of efflux pumps is one mechanism by which bacteria have evolved antibiotic resistance (Brenwald et al. 1998, Saidijam et al. 2006, Yin et al. 2006). Thus, efflux pump inhibitors are being studied as potential therapeutics against drug-resistant bacterial infections (Lomovskaya and Bostain 2006), and investigations aimed at identifying new efflux pump inhibitors have potential to be of relevance not only to ecology and plant evolution, but also to medical research.

A number of studies have focused on identifying bacterial efflux pump inhibitors from natural sources (Gibbons 2005, Hemaiswarya et al. 2008, Simões et al. 2009, Tegos et al. 2002). Literature shows that efflux pump inhibitors are widely distributed in angiosperms, occurring in at least 25 genera. Among the reported efflux pump inhibitors from botanicals, the flavonoid chemical class is particularly prominent (Abulrob et al. 2004, Belofsky et al. 2004, 2006, Brenwald et al. 1998, Chérigo et al. 2009, Gibbons et al. 2003, Gibbons 2004, 2005, Guz et al. 2001, Ettefagh et al. 2011, Hamilton-Miller and Shah 2000, Junio et al. 2011, Khan et al. 2006, Marquez et al. 2005, Michalet et al. 2007, Morel et al. 2003, Nargotra et al. 2009, Oluwatuyi et al. 2004, Simões et al. 2009, Stavri et al. 2007, Stavri et al. 2007, Stermitz et al. 2000a, 2000b, 2001, 2002, 2003). Additionally,

there are two reports which mention the efflux inhibitory activity of the gymnosperm *Chamaecyparis lawsoniana* and its essential oils (Smith et al. 2007a, 2007b). However, mention of efflux pumps in plants more primitive than the gymnosperms is lacking in the literature. The flavonoid chemical family (beginning with the most primitive aglycones apigenin and luteolin) is both shared between and localized to the land plant lineage (Buer et al. 2010, Crawford 1978, Harborn 1977, Lwashina 2000, Kaundun et al. 1998, Markham and Porter 1969a, Richardson 1989, Wiedenfeld et al. 2000) and its sister group of green algae, order Charales (Becker and Marin 2009, Delwiche et al. 2002, Garbary et al. 1993, Lwashina 2000, Markham and Porter 1969b, Stafford 1991). Additionally, the flavonoids apigenin and luteolin, which are produced by many species, are efflux pump inhibitors (Brown et al. 2015). In light of this, and the observation of efflux pump inhibitory activity in *C. lawsoniana* (Smith et al. 2007a, 2007b), it stands to reason that bacterial efflux pump inhibitor production is likely to be more widely distributed than just the flowering plants that dominate the literature. Thus, we hypothesized for this study that the ability to produce efflux pump inhibitors developed early in the evolution of land plants. To test this hypothesis, we measured the efflux pump inhibitory activity of extracts from a series of plants from various branches of this lineage, and we quantified known efflux pump inhibitory flavonoids in these plant extracts.

Materials and Methods

Collection and preparation of plant material

The species of land plants used for the study sampled all major groups of land plants (Fig 1). Plant material was acquired via wild collections, cultivation, or purchase. Wild-collected material was obtained from private property (Pennsylvania, United States of America) with permission of the land owner and from the campus of the University of North Carolina Greensboro. No protected species were collected. A voucher specimen representing each collection was preserved and deposited at the UNC herbarium (see voucher numbers in Table 1). The plant material was cleaned of debris and air-dried until crisp. Only the aerial portions of each plant were used except in the case of *Marchantia sp.*, for which the whole plant was used because of its small size.

Plant extracts were prepared according to previously described extraction methods (Gu et al. 2004, Junio et al. 2011). For each plant species, dried plant material was macerated in methanol for at least 24 hr, and the liquid extract was subsequently separated from the solid material via vacuum filtration. This procedure was repeated three times, and all three extracts from one batch of plant material were combined and partitioned using an equal volume of hexane. This mixture was then stirred for at least one hr, and the methane/hexane layers were collected using a separatory funnel. The methanol partition was added to water and chloroform in a ratio of 1:5:4, stirred for at least 1 hr, and then separated. The chloroform partition was evaporated and used as the starting

material for all experiments described herein, and will be referred to as “plant extract” from this point forward.

Determination of efflux pump inhibitory activity

Standard methods for measuring efflux inhibition rely on ethidium bromide-based fluorescence assays (Ettefagh et al. 2011, Kaatz et al. 2000, Lechner et al. 2008, Pereda-Miranda 2006, Sabatini et al. 2008, Schindler et al. 2013, Stermitz et al. 2000B, Tegos et al. 2002). Consistent with a recent report from our laboratory (Brown et al. 2015), we observed severe quenching effects with fluorescence based methods of evaluating efflux pump inhibition for the plant extracts studied herein (data not shown). To circumvent interference due to fluorescence quenching, we employed a recently developed liquid chromatography-mass spectrometry (LC-MS) based assay as an alternative approach for measuring efflux pump inhibitory activity. Rather than attempting to measure activity by fluorescence, the quantity of ethidium ion remaining in the filtered spent broth from *S. aureus* cultures was measured using LC-MS. The presence of an efflux pump inhibitor causes ethidium to accumulate inside the bacterial cells, with the result that the concentration of ethidium measured in the spent broth decreases as inhibitor concentration increases. Thus, efflux pump inhibition was evaluated via a mass spectrometry-based assay as described previously (Brown et al. 2015).

Plant extracts were tested by micro dilution in 96 well plates, prepared via 2-fold serial dilution at a range of concentrations from 300 µg/mL to 4.7 µg/mL.

The vehicle composition was 50% Mueller-Hinton broth [containing a culture of *Staphylococcus aureus* strain number NCTC 8325-4 (Novick 1967) grown to an OD₆₀₀ of 0.7-0.9], 40% deionized water, and 10% DMSO (by volume) with the addition of 1.25 µg/mL ethidium bromide. The approximate final reaction concentration of bacteria was $1.6-1.8 \times 10^8$ CFU/mL. Bacteria were incubated at the above conditions for 30 min, and then removed via filtration with an EMD Millipore MultiScreen™ fritted-bottom 96-well filter plate (pore size 0.22 µm, EMD Millipore, Darmstadt, Germany). Residual ethidium bromide was analyzed in the spent broth via an LC-MS method as described in (Brown et al. 2015), and the data were plotted as dose-response curves of ethidium peak area versus inhibitor concentration. IC₅₀ values were calculated from the dose-response curves as described previously (Brown et al. 2015).

Evaluation of bacterial growth inhibition

The minimum inhibitory concentration (MIC) was evaluated for all plant extracts against *Staphylococcus aureus* used in this study in accordance with Clinical Laboratory Standards Institute (CLSI) methods (Wikler et al. 2006). Briefly, a 24 hr single-colony seed culture of *Staphylococcus aureus* NTCC 8325-4 was grown to log phase and diluted such that the final starting reaction concentration of bacteria was 5×10^5 CFU/mL. The final reaction mixture was 250 µL composed of 2% DMSO, 98% Mueller-Hinton broth, and varying concentrations of a given test extract (ranging from 150 µg/mL to 4.7 µg/mL, prepared via serial dilution) in 96-well plates. A duplicate plate without bacteria

was also prepared to establish the background absorbance of the samples. All samples were incubated for 18 hr at 37 °C, and absorbance was measured with a BioTek SynergyH1 microplate reader (Winooski, VT, USA) at 600nm. The optical density from the background plate was subtracted from that of the test plate and the data plotted graphically to determine minimum inhibitory concentrations (MICs).

Identification and quantitation of flavonoids in plant extracts

An Acquity UPLC (Waters) coupled to a Q Exactive Plus mass spectrometer (Thermo Scientific) was employed for quantitative analyses of six flavonoids found in our previous studies to be active efflux pump inhibitors (Brown et al. 2015). The instrument conditions were as follows: capillary temperature, 310 °C; sheath gas flow, 25 (arbitrary units); auxiliary gas, 5 (arbitrary units); source voltage 3.7 kV; HESI probe temperature, 50°C; S lens, 50; scan range 250 m/z —325 m/z in the positive mode. Separation was achieved with a 2.1 × 50 mm Acquity UPLC BEH shield C₁₈ column with 1.7 μM particle size operated at a column temperature of 40 °C. The solvent was comprised of acetonitrile with 0.1% formic acid and deionized water with 0.1% formic acid at a flow rate of 0.3 mL/min. The 10 min gradient employed the following ratios (CH₃CN:H₂O):— 0 min., 10:90; 5 min., 50:50; 6 min, 50:50; 7 min., 60:40; 7.5 min., 60:40; 8 min., 70:30; 8.5 min., 100:0; 9 min., 100:0; 9.5 min., 10:90; 10 min., 10:90 (Junio 2011). Flavonoids were identified by accurate mass and comparison of retention time with standards, and quantified using

linear regression by comparing peak area of flavonoids in plant extracts to external calibration curves for the relevant standards.

Results and Discussion

The hypothesis tested in this study is that the ability to produce efflux pump inhibitors developed early in the evolution of land plants. This hypothesis is supported by the finding that many extracts tested here are active efflux pump inhibitors, including extracts from one gymnosperm (*Pinus strobus*) four pteridophytes (*Diphasiastrum digitatum*, *Osmunda claytoniana*, *Equisetum hyemale*, *Psilotum nudum*) as well as both bryophytes *Marchantia spp.* and *Sphagnum spp* (Table 3.1). Sample data showing this effect are shown in Fig 3.2A and 3.2B for the positive control piperine, a known efflux pump inhibitor (Khan et al. 2006, Nargotra et al. 2009, Stavri et al. 2007). Also as indicated in Fig 3.2A and 3.2B, a clear dose-response curve was obtained for the botanical extracts of *P. strobus* and *D. digitatum* using the mass spectrometry-based assay. Thus, bacterial efflux pump inhibitory activity is a common phenomenon, and it is more parsimonious to assume it to be of great antiquity than it would be to assume it evolved multiple times in recent geological history. This conclusion is congruent with the previously reported findings that the flavonoids, apigenin and luteolin, are efflux pump inhibitors (Brown et al. 2015) that evolved prior to land plants (Lwashina 2000). The majority of the plant extracts investigated demonstrated efflux pump inhibitory activity (Table 3.1), with the most active being the *Osmunda claytoniana* and *Pinus strobus* extracts (IC₅₀ values of 19

ppm). Extracts from four species, *Nephrolepis exaltata*, *Ginkgo biloba*, *Cycas circinalis* and *Glaucidium palmatum*, showed no activity. Only one sample, the extract of *Marchantia spp.* (Table 3.2) had a measurable MIC under the study conditions. Notably, it has been previously shown (Brown et al. 2015) that the occurrence of a growth inhibition is not likely to introduce an artifact into data generated via this method, and thus the growth inhibition caused by the *Marchantia spp.* Extract is not likely to be problematic in data interpretation.

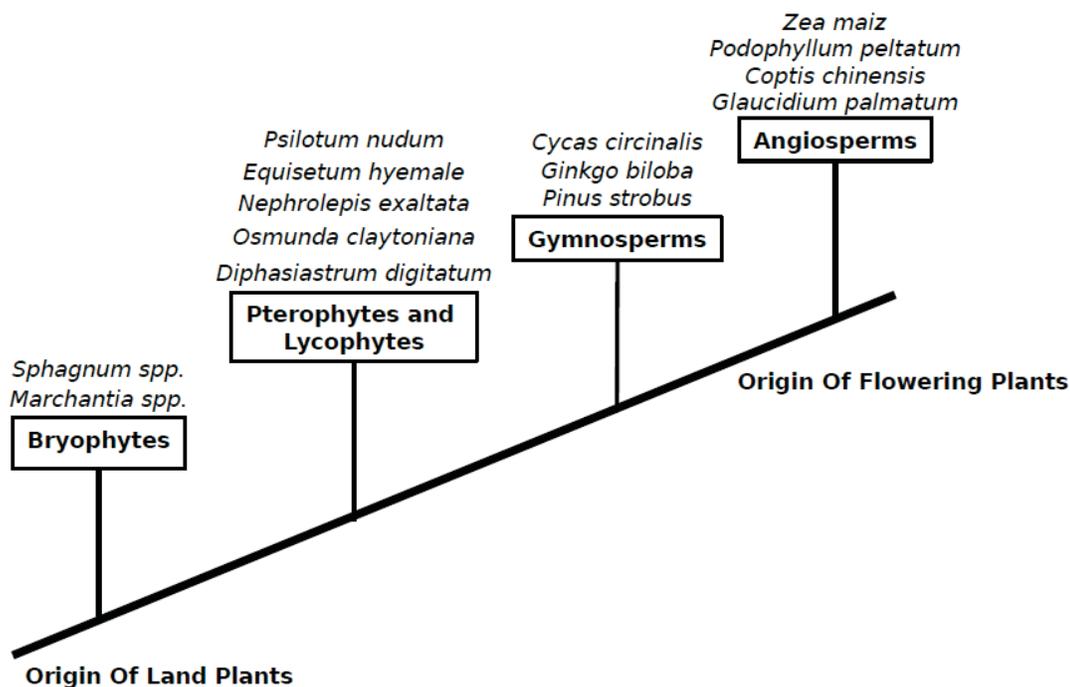


Figure 3.1. Dendrogram Depicting the Major Lineages of Land Plants and which Species Extracted in this Study Belong to Each.

These assignments are supported by the following sources: Aagaard et al. 2009, Bhattacharya and Medlin 1998, Wang and Chen 2007, Wang et al. 2007, Qiu et al. 2007, Wikstrom and Kenrick 2000.

High resolution mass spectrometry analysis indicated the presence of flavonoids in all but one (*Glaucidium palmatum*) of the fourteen plant extracts tested. In all extracts, the flavonoids were present at levels below their individual IC₅₀ values for efflux pump inhibition of *S. aureus* (Table 3.1). As an example, at the IC₅₀ concentration of *Podophyllum peltatum* (75 µg/mL), the assay concentration of kaempferol was 2.4 × 10⁻¹ µg/mL (Table 3.1). This well below the IC₅₀ value for pure kaempferol, which was reported to be 19 µg/mL (Brown et al. 2015). The before mentioned concentration of kaempferol was the highest

observed concentration of a flavonoid in this study. Also, kaempferol along with rhamnetin (which also showed an IC_{50} value of 19 $\mu\text{g/mL}$ [Brown et al. 2015]) were the most active flavonoids in this study, all others have activities ranging from 38 $\mu\text{g/mL}$ to 75 $\mu\text{g/mL}$ (Brown et al. 2015). Thus, even in extracts with multiple detectable flavonoids, the combined activity of these compounds is not sufficient to appreciably contribute to the activity of the extract. These data demonstrate that the individual flavonoids quantified herein are only partially (if at all) responsible for the overall efflux pump inhibitory activity of the complex botanical extracts, and that it is not possible to definitively link their presence to the activity of the extracts. When considering these data, it is worth noting that numerous possible classes and derivatives of flavonoids have been shown to be present in botanical extracts, including flavones, isoflavones and flavonols (Gibbons 2004, 2005, Stermitz et al. 2002), gallic acid substituted flavonoids (Guz et al. 2001, Hamilton-Miller and Shah 2000, Simões et al. 2009, Stavri et al. 2007), flavolignans (Gibbons 2004, 2005, Guz et al. 2001, Stavri et al. 2007, Stermitz et al. 2000a, 2000b, 2001), and other similar phenolic compounds (Belofsky et al. 2004, 2006, Stavri et al. 2007). It is possible that these flavonoid derivatives (alone or in combination with other chemical classes) are at least partially responsible for the combined efflux pump inhibitory activity of the extracts. Ultimately, more data are needed to clearly elucidate the specific role flavonoids and their derivatives play in the efflux pump inhibitory activity of modern plants.

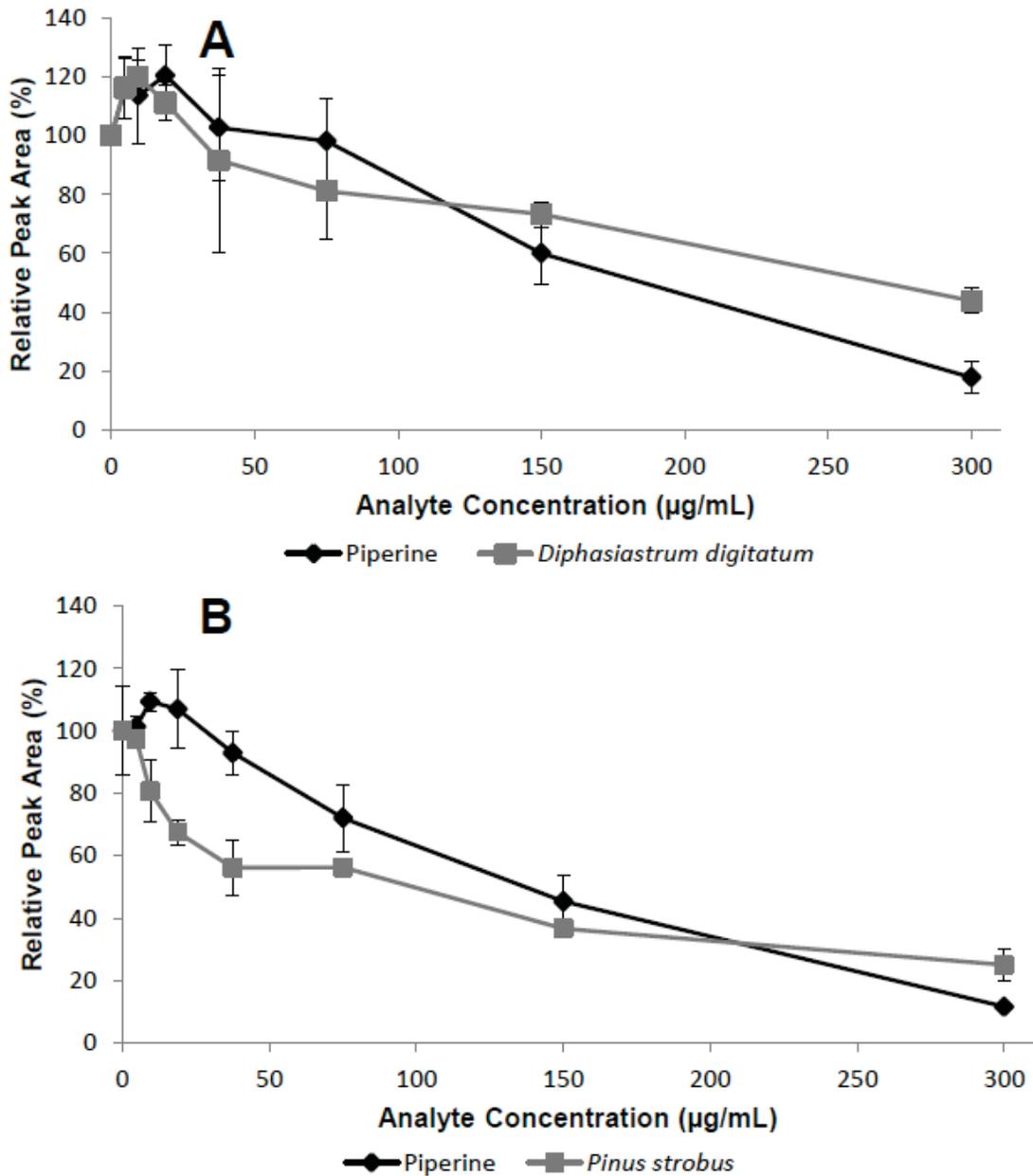


Figure 3.2. Sample Data Showing Dose Response Curves for Efflux Pump Inhibition by the Botanical Extracts *Pinus strobus* (A) and *Diphasiastrum digitatum* (B) Collected with Mass Spectrometry-Based Methods.

A positive response is indicated by a decrease in extracellular residual concentration of ethidium, as indicated by decreased peak area with increasing concentration of the putative inhibitor. The known efflux pump inhibitor piperine is used as a positive control in all experiments. Shown are the means of triplicate measurements and standard deviations.

The data presented herein raise several interesting possibilities. The commonality of efflux pump inhibitory activity demonstrates that land plants are rich sources of bacterial efflux pump inhibitors. Thus, land plants might serve as research tools or as therapeutic agents. It appears that many botanical extracts contain efflux pump inhibitors other than the active flavonoids investigated herein, as inferred from the fact that their activity cannot be explained by the presence of known active flavonoids. Future studies to identify these compounds would be worthwhile. Finally, more in-depth studies to explore the role of efflux pump inhibitors in plant defense would be extremely interesting.

Table 3.1. Comparison of the Efflux Pump Inhibitory Activity, Antimicrobial Activity, and Flavonoid Concentration of Botanical Extracts Spanning the Land Plant Lineage.			
Species and family^a (mode of acquisition, herbarium number)	IC₅₀^b	MIC^c	Active Flavonoids Present at Quantifiable Concentrations^d (w/w percent of extract; Reaction concentration at the IC₅₀, in µg/mL)
<i>Marchantia spp.</i> (Marchantiaceae) (purchased ^g , NCU633652)	150 µg/mL	150 µg/mL	Apigenin (3.1x10 ⁻³ %, 4.7x10 ⁻³ µg/mL) Luteolin (6.5x10 ⁻³ %, 9.7x10 ⁻³ µg/mL)
<i>Sphagnum spp.</i> (Sphagnaceae) (purchased ^h , NCU633524)	150 µg/mL	>150 µg/mL	Myricetin (2.5x10 ⁻² %, 3.8x10 ⁻² µg/mL)
<i>Diphasiastrum digitatum</i> (Dill.) Holub (Lycopodiaceae) (collected ^e , NCU602026)	150 µg/mL	>150 µg/mL	Myricetin (2.7x10 ⁻² %, 4.0x10 ⁻² µg/mL)
<i>Osmunda claytoniana</i> L. (Osmundaceae) (collected ^e , NCU602025)	19 µg/mL	>150 µg/mL	Myricetin (2.4x10 ⁻² %, 4.5x10 ⁻³ µg/mL)

<i>Nephrolepis exaltata</i> (L.) Schott (Nephrolepidaceae) (Cultivated ⁱ , NCU633530)	>300 µg/mL	>150 µg/mL	Myricetin (3.1×10 ⁻² %, 9.5×10 ⁻² µg/mL)
<i>Equisetum hyemale</i> L. (Equisetaceae) (collected ^f , NCU633528)	75 µg/mL	>150 µg/mL	Myricetin (3.9×10 ⁻² %, 2.9×10 ⁻² µg/mL)
<i>Psilotum nudum</i> (L.) P. Beauv. (Psilotaceae) (cultivated ⁱ , NCU633529)	75 µg/mL	>150 µg/mL	Apigenin (8.9× 10 ⁻³ %, 6.7× 10 ⁻³ µg/mL) Luteolin (3.0× 10 ⁻⁴ %, 2.3× 10 ⁻⁴ µg/mL) Rhamnetin (3.9× 10 ⁻³ %, 3.0× 10 ⁻³ µg/mL)
<i>Pinus strobus</i> L. (Pinaceae) (collected ^e , NCU602028)	19 µg/mL	>150 µg/mL	Apigenin (1.1× 10 ⁻³ %, 2.2× 10 ⁻⁴ µg/mL) Kaempferol (1.2× 10 ⁻³ %, 2.2× 10 ⁻⁴ µg/mL) Myricetin (2.3× 10 ⁻² %, 4.4× 10 ⁻³ µg/mL) Rhamnetin (2.8× 10 ⁻³ %, 5.4× 10 ⁻⁴ µg/mL)
<i>Ginkgo biloba</i> L. (Ginkgoaceae) (cultivated ⁱ , NCU633527)	>300 µg/mL	>150 µg/mL	Apigenin (1.8× 10 ⁻³ %, 5.6× 10 ⁻³ µg/mL) Kaempferol (5.8× 10 ⁻⁴ %, 1.7× 10 ⁻³ µg/mL) Luteolin (7.2× 10 ⁻⁴ %, 2.2× 10 ⁻³ µg/mL) Myricetin (1.5× 10 ⁻² %, 4.4× 10 ⁻² µg/mL) Quercetin (7.5× 10 ⁻³ %, 2.2× 10 ⁻² µg/mL) Rhamnetin (3.6× 10 ⁻³ %, 1.1× 10 ⁻² µg/mL)
<i>Cycas cir cinalis</i> L. (Cycadaceae) (cultivated ⁱ , NCU633526 and NCU633525)	>300 µg/mL	>150 µg/mL	Apigenin (4.7× 10 ⁻³ %, 1.4× 10 ⁻² µg/mL) Luteolin (4.9× 10 ⁻⁴ %, 1.5× 10 ⁻³ µg/mL) Myricetin (3.2× 10 ⁻² %, 9.5× 10 ⁻² µg/mL) Rhamnetin (3.3× 10 ⁻³ %, 9.9× 10 ⁻³ µg/mL)
<i>Glaucidium palmatum</i> Siebold&Zucc. (Ranunculaceae) (cultivated ^j , NCU602023)	>300 µg/mL	>150 µg/mL	None

<i>Coptis chinensis</i> Franch. (Ranunculaceae) (cultivated ^l , NCU602015)	75 µg/mL	>150 µg/mL	Myricetin (3.5×10^{-2} %, 2.6×10^{-2} µg/mL)
<i>Podophyllum peltatum</i> L. (Berberidaceae) (collected ^e , NCU602021)	75 µg/mL	>150 µg/mL	Apigenin (4.2×10^{-3} %, 3.2×10^{-3} µg/mL) Kaempferol (3.2×10^{-1} %, 2.4×10^{-1} µg/mL) Luteolin (1.5×10^{-4} %, 1.1×10^{-4} µg/mL) Myricetin (5.1×10^{-2} %, 3.8×10^{-2} µg/mL) Quercetin (4.7×10^{-2} %, 3.5×10^{-2} µg/mL) Rhamnetin (4.5×10^{-2} %, 3.4×10^{-2} µg/mL)
<i>Zea mays</i> L. (Poaceae) (purchased ^k , NCU633531 and NCU633532)	38 µg/mL	>150 µg/mL	Apigenin (1.5×10^{-3} %, 5.9×10^{-4} µg/mL) Kaempferol (2.2×10^{-3} %, 1.1×10^{-3} µg/mL) Luteolin (1.5×10^{-4} %, 5.5×10^{-5} µg/mL) Myricetin (5.7×10^{-2} %, 2.2×10^{-2} µg/mL)

- a. Authorities and validity of botanical names were verified using The Plant List (<http://www.theplantlist.org/>).
- b. Efflux pump inhibition was measured via analysis of ethidium bromide in spent, filtered bacterial supernatant after a 30 min incubation, as measured by mass spectrometry.
- c. Growth inhibition was measured by optical density at 600nm after an 18 hr incubation.
- d. Flavonoid concentration was estimated based upon a calibration series of pure compounds, shown in Table 3.2. Flavonoid IC₅₀ values from previous work (Brown et al. 2015) are as follows: kaempferol and rhamnetin—19 µg/mL; apigenin—38 µg/mL; quercetin, luteolin and myricetin—75 µg/ml. Analytical parameters are shown in supplemental Table 3.2.
- e. Collection location: Near Drake Road, Dushore, Pennsylvania, United States of America.
- f. Collection location: The campus of the University of North Carolina Greensboro, Greensboro, North Carolina, United States of America. Near 36.44.331 latitude, -79.48.506 longitude.
- g. Purchased as a living specimen from Wards Natural Science, 5100 West Henrietta Road, PO Box 92912, Rochester, NY 14692-9012
- h. Purchased. Original supplier: Mosser Lee Company, Millston, Wisconsin 54643, United States of America. Purchased retail in Greensboro, North Carolina, United States of America

i. Cultivated (original source unknown) at the University of North Carolina at Greensboro, North Carolina, United States of America. Near 36.44.331 latitude, - 79.48.506 longitude. The sample of *Ginkgo biloba* was obtained from a campus planting at the University of North Carolina, Greensboro. The sample of *Cycas circinalis* was obtained from the Department of Biology plant collection, University of North Carolina, Greensboro.

J. Garden origin, plants supplied by Seneca Hill Perennials Fall, 2011, stored in a refrigerator until spring, 2012 and grown out in original pot to produce the specimen.

k. Purchased at a farmers market, Hwy 24, Biscoe, North Carolina, United States of America.

l. Origin was Horizon Herbs, LLC, Post Office Box 69, Williams, Oregon 97544, United States of America.

Table 3.2. Data from the Calibration Curves Used for Flavonoid Quantifications Presented in Table 3.1. Calibration curves were plotted as log-area of the relevant selected ion trace versus log-concentration. All samples were tested in combination at seven concentrations prepared via serial dilution from a starting concentration of 10 µg/mL.					
Compound	Slope (± standard deviation)	Intercept (± standard deviation)	Standard Deviation for logY	Concentration Range (µg/mL)^a	R² value
Apigenin	8.81× 10 ⁻¹ (± 7.75× 10 ⁻²)	8.47 (± 1.06× 10 ⁻¹)	1.73× 10 ⁻¹	5× 10 ⁻³ —5	0.985
Kaempferol	1.05 (± 6.84× 10 ⁻²)	8.25 (± 9.41× 10 ⁻²)	1.53× 10 ⁻¹	5× 10 ⁻³ —5	0.992
Rhamnetin	1.50 (± 1.48× 10 ⁻¹)	7.97 (± 1.29× 10 ⁻¹)	2.10× 10 ⁻¹	5× 10 ⁻³ —5	0.990
Quercetin	1.55 (± 1.45× 10 ⁻¹)	7.89 (± 1.26× 10 ⁻¹)	2.05× 10 ⁻¹	5× 10 ⁻² —5	0.991
Luteolin	9.09× 10 ⁻¹ (± 5.16× 10 ⁻²)	8.16 (± 9.92× 10 ⁻²)	1.63× 10 ⁻¹	5× 10 ⁻⁴ —5	0.990
Myricetin	3.15 (± 6.64× 10 ⁻¹)	5.72 (± 4.82× 10 ⁻¹)	6.40× 10 ⁻¹	5× 10 ⁻¹ —10	0.957

CHAPTER IV
IDENTIFICATION OF FLAVONOIDS IN COMPLEX MIXTURES

Manuscript in preparation for submission for publication

Abstract

The previous two chapters have established that flavonoids act as efflux pump inhibitors, and that they are common in land plants. In light of these observations, it stands to reason that improved methods for the study and identification of flavonoids would be a benefit to the field. The present chapter compares two methods described in the literature for the identification of flavonoids in extracts. The first of these is the analysis of fragmentation data generated via mass spectrometry. The second is the usage of mass defect filtering data to process high resolution mass spectrometry data into a “short list” of potential flavonoids. A series of studies involving pure flavonoids and fractions generated from an extract of goldenseal (*Hydrastis canadensis*) were used to demonstrate the potential usefulness of mass defect filtered data as a complement to fragmentation data.

Introduction

As has been discussed previously (Chapter I), the most common class of natural products that has been shown to act as efflux pump inhibitors is the flavonoids (Table 1.1). Flavonoids also have a diversity of bioactivities in eukaryotic cells, as well as antibacterial and antiviral activity (Buer et al. 2010, Cushnie and Lamb 2005). Therefore, a method for the identification of these compounds in crude mixtures would be very useful. While there is a large body of literature pertaining to identifying and quantifying specific flavonoids, a general method for the identification of compounds belonging to the flavonoid chemical family has not yet been developed. Zhou et al. (2007) published a paper identifying polymethoxylated flavones from a plant extract using tandem mass spectroscopy. However, their method was primarily based on observing losses related to the fragmentation of the O-methyl groups. Many compounds can possess these substitutions; thus, their fragmentation is not sufficient evidence to identify a compound as a flavonoid.

A second approach attempting general flavonoid analysis relies most heavily on the usage of specialized HPLC columns, which interact well with flavonoids. As a second stage of confirmation, the method identifies flavonoids as compounds with a peak UV absorbance of 254 nm (Huck et al. 2002). This method is limited by the fact that UV detectors produce less data than mass spectroscopy. While the Huck et al. (2002) report did use mass spectrometry for quantitation, the spectra and fragmentation patterns were not fully interpreted.

There are a plethora of articles that publish methods for identification of a specific set of flavonoids through comparison to standards, as well as articles describing the structural analysis of flavonoids (Abad-García et al. 2009, Cuyckens and Claeys 2004, Justesen 2001, March et al. 2004a, 2004b, March and Brodbelt 2008, Xu et al. 2012, Zhang and Brodbelt 2003), but these fall short of comprehensive flavonoid identification methods for complex mixtures. To address this lack, we propose to evaluate two mass spectrometry approaches for the identification of flavonoids in complex mixtures without the *a priori* selection and usage of external standards, specifically fragmentation analysis and mass defect filtering.

The evaluation of flavonoid fragmentation and its utility performed in this study was based on three published cleavage patterns for flavonoids that have the potential to produce a manageable set of diagnostic fragments (March et al. 2004a, 2004b). Our studies were designed to identify key diagnostic fragments in a MS² spectrum using flavonoid standards, which would allow the classification of unknown chemical species in a complex mixture as “probable flavonoids.” These three hypothesized fragmentation patterns and their likely fragments, supported by the literature (March et al. 2004a, 2004b), are depicted in Tables 4.1, 4.2 and 4.3. In the 1,3 and 1,4 fragmentation patterns (the flavonoid numbering is shown in Figure 4.1) depicted in Tables 4.1 and 4.2, the C ring is opened. In the 1,3 cleavage pattern, there is a subsequent rearrangement which preserves the A ring. The 1,4 cleavage pattern does not produce this

rearrangement. The cleavage pattern shown in Table 4.3 follows the loss of C_2H_2O from the C ring, which is followed by linkage of the B and C rings. What these patterns all have in common is the fact that the A ring is relatively unaffected (Justesen 2001, March et al. 2004a, 2004b, March and Brodbelt 2008), and because of this, these were selected as the most promising sources of diagnostic fragments. The complete tables (Table 4.1-4.3) were composed using the preceding observations in the literature in combination with commonly observed methylations—which most often occur at carbon 6 and carbon 8. Additionally, secondary methylation can occur on any hydroxyl group present (Dewick2008). These patterns were selected as the most likely to be diagnostic of the flavonoid structure because the effect the A/C fused ring system directly and this is more likely to be distinctive than fragments effecting the B ring only.

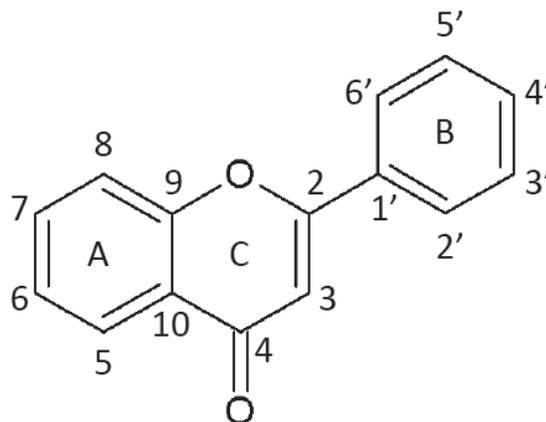


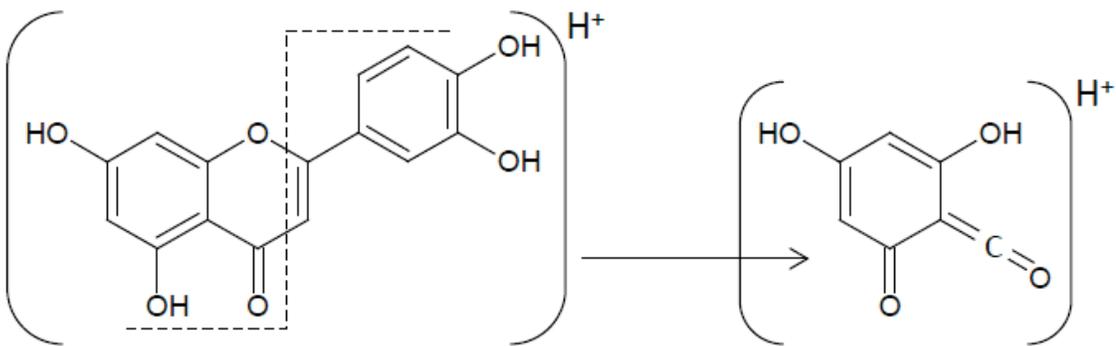
Figure 4.1. Numbering of the Flavonoid Ring System

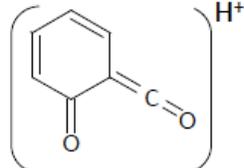
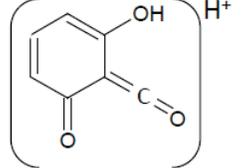
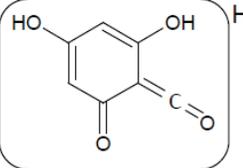
The flavonoid numbering and lettering system is documented in the following sources: (Dewick 2008, Justesen 2001, March and Brodbelt 2008)

Since flavonoids are derived from polyketide synthesis (Dewick 2008), a limited number of flavonoid substitution patterns with predictable masses are likely to occur. Flavonoids are commonly methylated, which most often occur at carbon 6 and carbon 8. Additionally, secondary methylation can occur on any hydroxy group present (Dewick 2008). In accordance with these patterns, Tables 4.1-3 also include the masses of all potential methylated fragments (from one to seven methyl groups, as appropriate).

Table 4.1. Probable Fragments Formed Following 2,3 Cleavage of the Flavonoid C Ring and Subsequent Rearrangement

a



Potential fragment structure^b			
Mass of fragment^c	121.02841	137.02332	153.01824
Fragment plus one methyl^d	136.05134	152.04625	168.04117
Fragment plus two methyls^d	151.07427	167.06918	183.06410
Fragment plus three methyls^d	N/A	N/A	198.08703

a. The fragment formed following 2,3 cleavage of the C ring followed by rearrangement from the precursor molecule quercetin (March et al. 2004a, 2004b).

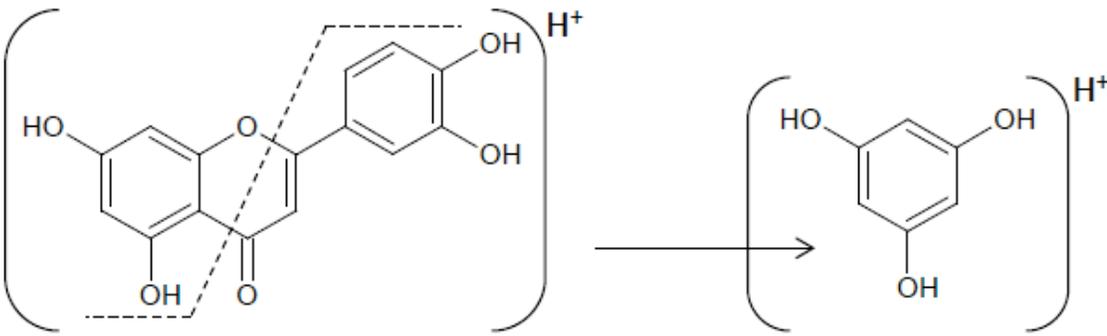
b. Probable fragments formed from similar rearrangements of flavonoids of flavonoid aglycones, based on the known biosyntheses of flavonoids (Dewick 2008).

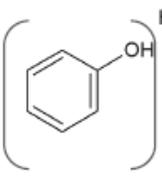
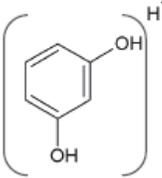
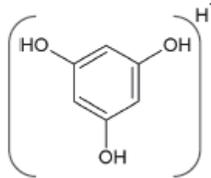
c. Monoisotopic masses of the above flavonoid fragments.

d. Monoisotopic masses of methylated flavonoid fragments, based on probable methylation patterns (Dewick 2008).

Table 4.2. Probable Fragments Formed Following 2,4 Cleavage of the Flavonoid C Ring

a



Potential fragment structure^b			
Mass of fragment^c	95.04914	111.04406	127.03897
Fragment plus one methyl^d	110.07207	126.06699	142.06190
Fragment plus two methyls^d	125.09500	141.08992	157.08483
Fragment plus three methyls^d	N/A	156.11285	172.10776
Fragment plus four methyls^d	N/A	N/A	187.13069

a. The fragment formed following 2,4 cleavage of the C ring followed by rearrangement from the precursor molecule quercetin (March et al. 2004a, 2004b).

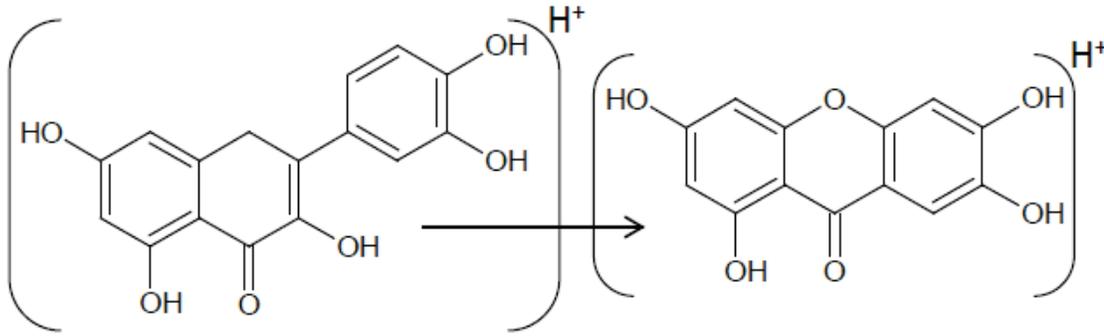
b. Probable fragments formed from similar rearrangements of flavonoids of flavonoid aglycones, based on the known biosynthesis of flavonoids (Dewick 2008).

c. Monoisotopic masses of the above flavonoid fragments.

d. Monoisotopic masses of methylated flavonoid fragments based on probable methylation patterns (Dewick2008).

Table 4.3. Probable Flavonoid Fragments and Their Masses, Following Loss of C₂H₂O and Ring Rearrangement

a.



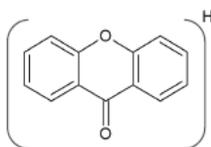
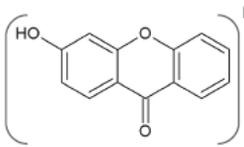
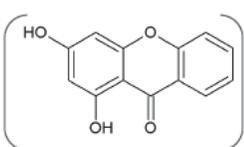
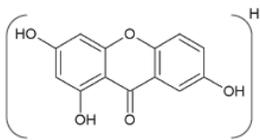
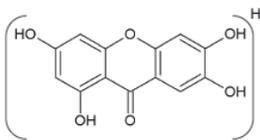
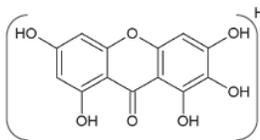
Potential fragment structure^b			
Mass of fragment^c	197.05971	213.05462	229.04953
Fragment plus one methyl^d	212.08264	228.07755	244.07246
Fragment plus two methyls^d	227.10557	243.10048	259.09539
Fragment plus three methyls^d	N/A	258.12341	274.11832
Fragment plus four methyls^d	N/A	N/A	289.14125

Table 4.3 Continued. Probable Flavonoid Fragments and Their Masses, Following Loss of C₂H₂O and Ring Rearrangement

Potential fragment structure ^b			
Mass of fragment ^c	245.04445	261.03936	277.03428
Fragment plus one methyl ^d	260.06738	276.06229	292.05721
Fragment plus two methyls ^d	275.09031	291.08522	307.08014
Fragment plus three methyls ^d	290.11324	306.10815	322.10307
Fragment plus four methyls ^d	305.13617	321.13108	337.12600
Fragment plus five methyls ^d	320.15910	336.15401	352.14893
Fragment plus six methyls ^d	N/A	351.17694	367.17186
Fragment plus seven methyls ^d	N/A	N/A	382.19479

a. The fragment formed after the loss of C₂H₂O from the parent molecule quercetin and fusion of the B and C ring (March et al. 2004a, 2004b).

b. Probable fragments formed from similar rearrangements of flavonoids of flavonoid aglycones, based on the known biosyntheses of flavonoids (Dewick 2008).

c. Monoisotopic masses of the above flavonoid fragments.

d. Monoisotopic masses of methylated flavonoid fragments, based on probable methylation patterns (Dewick 2008).

The tool evaluated for the identification of possible flavonoids in plant extracts is the usage of mass defect filtering. The mass defect of an ion is the difference between its nominal and accurate mass, and the usage of high-

resolution instrumentation allows for the direct measurement of this intrinsic molecular property (Gu et al. 2011, Hughey et al. 2001, Sleno 2012, Wu et al. 2004, Zhang et al. 2009). Mass defect values for the four most important elements in organic compounds are as follows: ^{12}C , 0.00000 Da; ^1H , 0.00783 Da; ^{16}O , -0.00509 Da; and ^{14}N , 0.00309 Da (Gu et al. 2011, Sleno 2012). As can be seen from this, the number of carbons has no effect on the mass defect, oxygen has a negative effect and hydrogen has the strongest positive effect. Because of this, mass defect has been shown to be a useful tool for distinguishing between molecules with different core carbon/hydrogen core structures (Gu et al. 2011, Hughey et al. 2001, Sleno 2012, Wu et al. 2004, Zhang et al. 2009).

There currently exist software tools capable of calculating mass defect from raw spectra and using it as a data filter parameter. This allows compounds with related mass defect values but unrelated nominal masses to be grouped together and separated from the rest of the mass spectrum (Sleno 2012). A review published by Zhang et al. (2009) evaluated mass defect filtering data produced to evaluate drug metabolites and found it to be an effective method for reducing background and identifying a large suite of metabolic byproducts in a wide range of matrices. Further, they concluded that when compared to the more traditional MS^2 procedure of neutral loss filtering, mass defect filtering was frequently more robust due to the often unpredictable nature of fragmentation analysis. The study produced by (Gu et al. 2011) goes further and evaluates the feasibility of using mass defect filtering to successfully identify a variety of natural

products including flavonoids, coumarins and steroidal alkaloids in plant extracts. They were successful in identifying components of the mixtures belonging to all three groups of compounds, and suggest that the usage of more than one mass defect filter could be a useful technique for fingerprinting plant extracts (Gu et al. 2011). One shortcoming of the approach used is the fact that the mass defect windows of coumarins and flavonoid aglycones have a very wide region of overlap. Another shortcoming that is readily identifiable from the flavonoid literature is the fact that the mass defect window does not include highly methylated aglycones. Two examples of such aglycones include sideroxylin (monoisotopic mass 312.09977 Da) and tangeretin (monoisotopic mass 372.12090 Da). Both of these possess very high ratios of methyl groups relative to the number of oxygens in the molecule (Nelson 1934, Junio et al. 2011), and therefore have mass defects higher than the highest flavonoid mass defect mentioned in Gu et al. (2011), that of pachypodol (mass defect 89.6 mDa). Nevertheless, the above was an excellent study and was used as the model for the present work with flavonoid mass defects, in combination with other techniques. The goal of this study was to compare and contrast the ability of mass defect filtering and fragmentation analysis to distinguish compounds as “possible flavonoids” in complex mixtures. Since this study did not involve isolation, definitive structural identifications have not been proposed.

Materials and Methods

Mass spectrometry and fragmentation analysis

Flavonoid standards were dissolved in methanol and analyzed in combination at a concentration of 0.1 mg/mL. Separation of flavonoid mixtures was accomplished using an Aquity ultraperformance liquid chromatograph (UPLC) (Waters). The chromatographic program was performed at 0.3 mL/min (all ratios are CH₃CN:H₂O): 0 min, 10:90; 5 min, 50:50; 6 min, 50:50; 7 min, 60:40; 7.5 min, 60:40; 8 min, 70:30; 8.5 min, 100:0; 9 min, 100:0; 9.5 min, 10:90; 10 min, 10:90 (Brown et al. 2015). Following separation, mass analysis was executed using a Q Exactive Plus mass spectrometer (Thermo Scientific) operated in the positive ion mode under the following conditions: capillary temperature, 310 °C; sheath gas flow, 25 (arbitrary units); auxiliary gas, 5 (arbitrary units); source voltage 3.7 kV; HESI probe temperature, 50 °C; S lens, 50 kV; scan range 250 *m/z*—325 *m/z* (Brown et al. 2015). MS³ fragmentation was conducted by data-dependent CID at 35% collision energy, followed by a second data-dependent event using the HCD collision cell at 80% collision energy.

Extraction and fractionation of goldenseal

Goldenseal plant aerial portions were cultivated and provided by Mr. Bill Burch, and are represented by a vouchered specimen in the University of North Carolina Chapel Hill Herbarium (accession number NCU583414). This species was selected due to existing experience and publications pertaining several of

the flavonoids it contains (Junio et al. 2011). Plant material was air dried until crisp, then macerated in methanol for a minimum of 24 hrs, followed by filtration. The extract was dried via rotary evaporation, then re-dissolved in equal portions of methanol and hexanes. These were shaken in a separatory funnel and allowed to separate. The methanol partition was then added to water and chloroform at a ratio of 1:5:4 (methanol:water:chloroform) and allowed to separate again. The chloroform partition was dried and carried on to normal phase liquid chromatography. Liquid chromatography was accomplished using a Teledyne Isco (Lincoln, Nebraska) flash chromatography apparatus, and a 1200 mL "Gold" silica gel column. The chromatographic gradient was performed at 200 mL/min using the following solvent ratios and times (all ratios are chloroform:methanol): 0 min, 100:0; 6 min, 100:0; 18 min, 97:3; 30 min, 90:10; 36 min, 80:20; 42 min, 0:100; 54 min, 0:100. Fractions were collected with a total volume of 250 mL, and all fractions were evaporated prior to experimental usage.

Analysis of goldenseal extract by high resolution mass spectrometry

The fractions produced from the chromatographic separation of goldenseal were analyzed via high resolution mass spectrometry using a Q Exactive Plus mass spectrometer (Thermo Scientific) in combination with reverse phase liquid chromatography using an Aquity UPLC (Waters). The chromatographic gradient was (all ratios are CH₃CN:H₂O): 0 min, 10:90; 5 min, 50:50; 6 min, 50:50; 7 min, 60:40; 7.5 min, 60:40; 8 min, 70:30; 8.5 min, 100:0; 9 min, 100:0; 9.5 min, 10:90; 10 min, 10:90. The flow rate was 0.3 mL/min (Junio

2011). Instrumental parameters were as follows: Sheath gas flow, 25 arbitrary units; capillary temperature, 300 °C; source voltage, 3.5 kV; source current, 100 μ A; capillary voltage, 42 V; tube lens voltage, 42 V.

The chromatograms generated for all 44 fractions and the crude chloroform partition were initially processed using MetWorks (Thermo Scientific). The filter parameters limited output to a mass range of 250-400 Da, and the mass defect range to 37.6-121 mDa. The mass defect range was based upon previously published range (Gu et al., 2011), but expanded to include the mass defect range for highly methylated flavonoids, as discussed in the introduction to this chapter. Peaks were extracted from the mass defect filtered chromatograms using IntelliXtract software (ACDLabs), with the data being reduced to include only those peaks identified as $[M+H]^+$ with a peak area 10^7 instrument response units or greater. This alone is inadequate for the identification of these compounds as flavonoids, so the data were further processed in two ways.

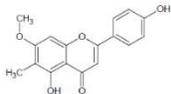
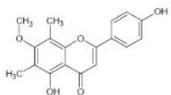
Accurate mass data was further processed to determine probable molecular formulas using the MIDAS formula calculator, with the objective of determining whether or not its formula had the potential to be a flavonoid. Parameters provided for formula calculation were: 5 ppm or less mass accuracy, an unlimited number of carbon and hydrogen atoms, up to 5 nitrogen atoms, and up to 10 oxygen atoms. A formula was considered a "potential flavonoid" if its calculated degree of hydrogen deficiency ranged from 9-12 and it possessed no nitrogen and more than two oxygens. These parameters were selected based on

known patterns in the biosynthesis and diversity of flavonoids in plants (Dewick 2008, Lwashina 2000)

Results and Discussion

The botanical extract *Hydrastis canadensis* was chosen as a test case in the study because it has previously been shown to contain flavonoids (Junio et al. 2011) that could serve as internal controls for the identification of “possible flavonoids”.

Chromatographic separation of the *H. canadensis* extract produced a total of 44 fractions. These were analyzed via high-resolution mass spectrometry followed by mass defect filtering along with the crude extract (all at a concentration of 1 mg/mL). Initial processing of these data using the mass defect filter returned a total of 45 unique chemical entities in all fractions of the *Hydrastis canadensis* extract (data not shown). A signal was considered a unique chemical entity if it had a unique pairing of accurate mass and retention time data. Of these, only 8 were also detected by the same analytical methodology in an unfractionated sample of the same *H. canadensis* extract (data not shown), which indicates the importance of the fractionation process for facilitating identification of relevant chemical signals. These signals were further processed by calculating molecular formulas, yielding 12 compounds with the potential to be flavonoids. The masses and retention times of these 12 signals are shown in Table 4.4.

Table 4.4. “Potential Flavonoids” in <i>Hydrastis canadensis</i> Fractions. A total of 45 unique accurate mass/retention time entities were present in the mass defect filter results. Of those, 12, represented here, produced a potential flavonoid molecular formula.			
Measured accurate mass (m/z)	Retention time (min)	Potential diagnostic fragments (m/z) [error, using masses in tables 4.1, 4.2 and 4.3]^a	Flavonoid structures known to exist in <i>Hydrastis canadensis</i> with corresponding monoisotopic mass
299.091	4.77	197.060 [1.5 ppm] ^d	
299.091	6.24	121.028 [3.4 ppm] ^b 153.018 [1.6 ppm] ^b 213.055 [1.8 ppm] ^d 229.049 [2.3 ppm] ^d	 <p>8-desmethyl-sideroxylin, monoisotopic mass 298.08412^e</p>
301.071	4.65	229.050 [2.1 ppm] ^d	
301.108	6.22	NF ^f	
313.107	6.64	137.023 [2.3 ppm] ^b	 <p>Sideroxylin, monoisotopic mass 312.09978^e</p>
315.086	4.20	NF ^f	
315.086	5.51	153.018 [1.6 ppm] ^b 136.051 [2.5 ppm] ^b 245.044 [1.8 ppm] ^d	
329.102	4.41	NDF ^g	
329.102	5.73	95.049 [1.5 ppm] ^c 137.023 [2.3 ppm] ^b	
329.102	6.76	136.052 [4.8 ppm] ^b 197.061 [6.5 ppm] ^d	
359.111	6.88	121.028 [3.4 ppm] ^b 151.074 [1.8 ppm] ^b	
385.075	4.56	NF ^f	

- Only masses matching to within 10 ppm mass accuracy were accepted
- Masses compared to a fragment in table 4.1
- Masses compared to a fragment in table 4.2
- Masses compared to a fragment in table 4.3

- e. Compounds previously isolated from a *Hydrastis canadensis* extract (Junio et al. 2011). The tentative identifications for 8-desmethyl-sideroxylin and sideroxylin were tentatively determined by comparing the elution order to previous work using the same UPLC instrument and solvent gradient (Junio 2011).
- f. Samples labeled NF failed to produce a fragmentation spectrum with sufficient signal intensity to analyze
- g. Samples labeled NDF produce a spectrum with strong signal but no diagnostic fragments.

Experiments pertaining to the usage of mass spectrometry MS² fragmentation spectra showed that it was common to discover fragments from table 4.1, but that fragments from table 4.2 and 4.3 were lacking (data not shown). Further exploration indicated that MS³ data, consisting of a low-energy initial fragmentation event (35% collision energy, CID) followed by a data-dependent second higher-energy (80% collision energy, HCD) fragmentation event yielded fragments predicted by all three tables, data which is congruent with expectations set forth in the literature. A sample fragmentation spectrum produced by this method is shown in figure 4.2. The sample spectrum 4.2B shows masses from tables 4.1 and 4.2, as well as masses with the correct nominal mass but incorrect accurate mass, which leads to the conclusion that accurate mass measurements of fragments is critical for the correct interpretation of these data.

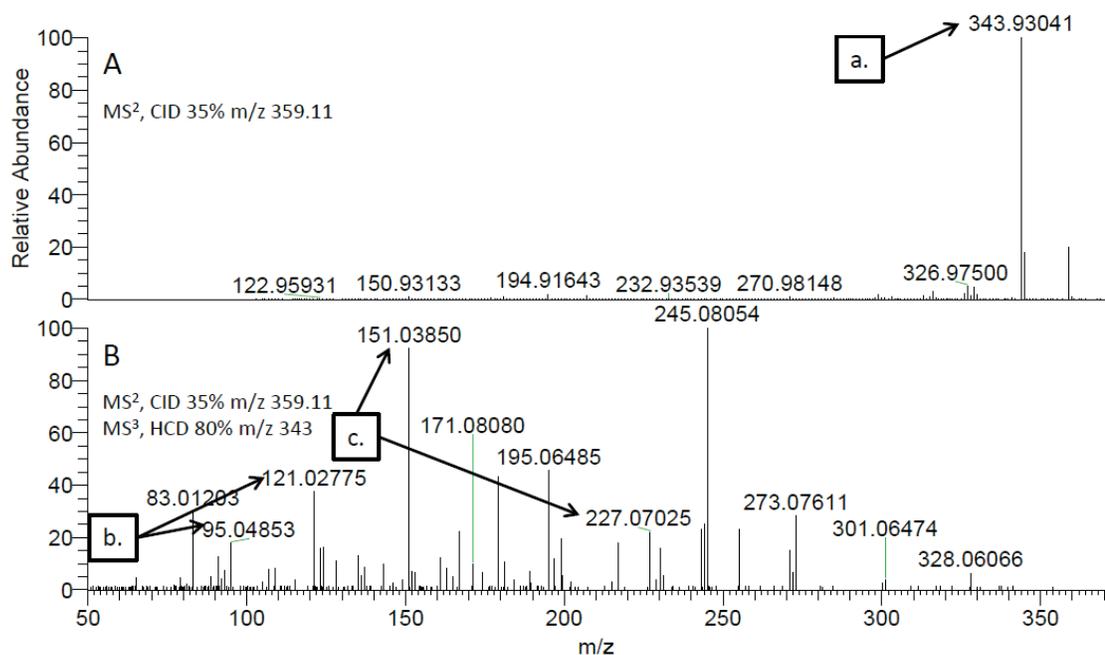


Figure 4.2. Sample MS² Data (4.2A) and MS³ Data (4.2B) Derived from the Precursor Ion m/z 359.11.

- The first low-energy fragmentation (35% collision energy, CID) event resulted in the base peak being m/z 343.9, representing the loss of one methyl group. This is a low resolution spectrum produced in the ion trap mass analyzer in the LCQ-Orbitrap.
- High energy collision-induced dissociation (HCD) of the precursor mass 343.9 revealed two masses considered to be potentially diagnostic of a flavonoid structure (error less than 10 ppm, values listed in Table 4.4).
- The indicated masses are of the correct nominal mass to be indicative of a flavonoid core structure, but the error is too great to have confidence in such an assignment (151.03850, 15 ppm mass error; 227.07025, 240 ppm mass error) when compared to the masses in Tables 4.1 and 4.3.

The MS³ fragmentation spectra for each of the 12 masses which returned a potential flavonoid formula were further examined and of these 8 presented diagnostic fragments (Table 4.4). Additionally, 4 compounds failed to produce a fragmentation spectrum which could be analyzed, and only one compound produced a quality spectrum that lacked any diagnostic fragments (Table 4.4).

The masses for these compounds were compared the masses for three flavonoids previously isolated from goldenseal leaf material extracted by the same methods used here (Junio et al. 2011, Junio 2011) include sideroxylin (monoisotopic mass 312.09977 Da), 6-desmethyl-sideroxylin, and 8- desmethyl-sideroxylin (isomers, monoisotopic mass 298.08412 Da). Two of these were tentatively identified in the present data (Table 4.4) and thus providing an internal control suggesting the usefulness of these methods.

These data build upon, and are highly congruent with, the findings of Gu et al. (2011). Both the published study and the data presented here demonstrate that mass defect filtering has a high potential for utility for the tentative identification of natural products in complex mixtures; particularly in cases where analytical standards are not available. Both studies also show that mass defect filtering alone is inadequate for the identification of flavonoids, since other compounds have similar mass defect windows. Further, these data indicate that the use of formula calculation is a viable method for the secondary processing of mass defect filtered raw data, and greatly refines the determinations made.

Finally, it is important to remember when interpreting these data that all results are to be treated as tentative potential identifications, and cannot be treated as firm identifications in the absence of further study involving comparison to standards and/or isolation and structure elucidation. These data also underscore the importance of utilizing high resolution mass spectrometry in any such study. These results are suggestive that the flavonoid chemical

diversity in *H. canadensis* exceeds that indicated by the literature. However, since the data presented here only allows the identification of a chemical signal as a “potential flavonoids” and cannot fully distinguish false positive results, further research involving the isolation and structure elucidation of these compounds is indicated.

CHAPTER V

CONCLUDING REMARKS

The work presented in this dissertation began with a purpose of exploring the distribution of efflux pump inhibitors in land plants; exploring the commonality of efflux pump inhibitory activity within the flavonoid chemical family; and lastly evaluating techniques for the detection and examination of flavonoids in complex mixtures. A body of preliminary experimentation rapidly indicated that new methods were necessary to produce the type of quantitative data needed for true comparisons between the level of activity present in plant extracts, as well as in many pure compounds. The method that was developed made use of tandem mass spectrometry to observe changes in the level of ethidium bromide residual in filtered extracellular matrix following a 30 min exposure of bacterial cells to the ethidium and test compound or plant extract. This proved useful in determining relative efflux pump activity, because ethidium is an efflux pump substrate that accumulates in cells, and is readily detectable via mass spectrometry. The use of mass spectrometry allowed the ethidium signal to be readily separated from the complex matrix formed when bacterial growth medium and plant extracts are combined. This is something not possible with the conventional optical instruments used in the previous literature pertaining to bacterial efflux pump inhibition.

Once developed, this assay was successfully employed to determine the relative levels of efflux pump inhibitory activity present in extracts prepared from 15 plants representing all of the main extant branches of the land plant lineage. All tests were carried out using the bacterium *Staphylococcus aureus*. The data for one of these plants was presented in Chapter II, the remainder was presented in Chapter III of this dissertation. In summary, all extracts except four showed activity against ethidium efflux in *S. aureus*, and there was no taxonomic pattern to the distribution of the plants whose extracts failed to show activity in this assay. This led to the conclusion that the production of bacterial efflux pump inhibition is widespread (though not ubiquitous) in land plants. Further, activity against bacterial efflux pumps was observed in the most ancient plants examined (the bryophytes). Since it is more parsimonious to assume one evolutionary origin of efflux pump inhibitor production than multiple origins over evolutionary time, it was also concluded that the production of efflux pump inhibitors is likely to have occurred early in the history of land plants. This conclusion is further strengthened by the results indicating that flavonoids commonly possess the ability to inhibit ethidium efflux; because their ubiquity and ancient origin in land plants is well established in the literature.

The above conclusion was then complicated by measuring the concentration of known active flavonoids in the active plant extracts. These measurements indicated that while present, the flavonoids examined were not sufficiently abundant to fully explain the activity levels present in the plant

extracts. This finding lead to a further conclusion that there must be additional, unknown chemical diversity in these extracts to explain their activity, and that additional experimentation is needed to fully elucidate these unknown details.

In light of the apparent chemical complexity underlying the ability of plant extracts to inhibit bacterial efflux pumps, improved methods are needed to aid in preliminary description of these mixtures. In order to accomplish this goal, a series of techniques present in the literature were combined into a set of sequential steps designed to accomplish preliminary identification of a population of chemically related signals. Flavonoids and an extract prepared from the plant *Hydrastis canadensis* were used as sample cases. The crude *H. canadensis* extract was separated into 44 fractions via normal phase chromatography. This proved necessary in order to concentrate uncommon chemical entities and lift them above the detection threshold set for the method. Each fraction was subjected to UPLC-MS³ analysis using a high-resolution mass spectrometer. The first exclusion criteria applied was mass defect filtering, which narrowed the list of “possible flavonoids” down to 45 chemical entities with distinct accurate mass and retention times. Formula calculation was then applied to this list, narrowing it down to 12 entities which yielded at least one “possible flavonoid” formula. The MS³ fragmentation spectra of these 12 entities were then examined to determine if they contained fragments which could have been produced by published fragmentation patterns indicative of the flavonoid core A/C ring structure. This was deemed the set of fragments most likely to be diagnostic of

the flavonoid chemical family in general. This series of exclusion criteria produced a list of 8 “possible flavonoids”. Of these, three can be attributed to compounds previously published as flavonoids produced by *H. canadensis*. There are two conclusions pertaining to this last body of research. Firstly, the usage of sequential exclusion criteria beginning with intrinsic molecular properties does hold promise for making preliminary determinations about components of complex chemical mixtures. Secondly, these data suggest that the flavonoid complexity of *H. canadensis* is richer than what the present literature indicates and that there is a need for additional research describing this richness.

In the final analysis, the most important component of this body of research may well prove to be the development of the mass spectrometry based assay for bacterial efflux pump inhibition. This is because it opens doors for the exploration of this phenomenon that are closed without it. While there are many potential uses for this method, three are specifically suggested as a continuation of the research presented herein. The first of these is the exploration of efflux pump inhibitors outside the flavonoid chemical family, as has been discussed previously in this conclusion. The second is the evaluation of efflux pump inhibitors in plant extracts active against plant pathogens. The data collected here suggests that efflux pump inhibition may have played a role in the evolution of land plant disease ecology. However, testing more bacteria in the mass spectrometry based efflux pump inhibition assay would provide stronger

evidence supporting or refuting this possibility. Lastly, the presence of this assay and the quantitative phenotypic comparisons it is capable of producing are expected to be an asset in drug discovery research efforts aimed at curing antimicrobial resistant infections.

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