The purported activity of goldenseal, Hydrastis canadensis L., against a plethora of disease states is largely attributed to the presence of the benzoisoquinoline alkaloid berberine in the plant. However, goldenseal crude extracts showed better inhibitory activity compared with pure berberine against some microorganisms such as wild type Staphylococcus aureus and azole-resistant Candida albicans isolates. Goldenseal was also more effective than berberine against the multiple drug resistance-1 (MDR1)-mediated efflux from liver cells. Using quantitative LC-MS constituent profiling, three new flavonoids, 6-desmethyl-sideroxylin, 8-desmethyl-sideroxylin and sideroxylin, were identified and isolated from the aerial portion of goldenseal. These flavonoids were found to have potential synergistic effects with berberine against wild type (NCTC 8325-4) Staphylococcus aureus strain. The flavonoids, 8-desmethyl-sideroxylin and sideroxylin, do not have intrinsic antibacterial activity but showed inhibitory activity against the multi-drug efflux pump on the NorA over-expressed S. aureus strain (K2378). All three flavonoids exhibited synergistic effects. Identifying synergists in goldenseal is a proof-of-concept of the multi-constituent efficacy of herbal plants. A new method, synergy-directed fraction, is hereby proposed as an alternative way to investigate the presence of synergists in medicinal plants.
GOLDENSEAL: A CASE STUDY ON LC-MS PROFILING
TO IDENTIFY SYNERGISTS

by

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the Faculty of The Graduate School at
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Approved by

______________________________
Committee Chair
To Mamang†, who always believed that I would go places.
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

_Hydrastis canadensis_, L. of the Ranunculaceae family is most popularly known as **goldenseal**. This name, coined by Samuel Thomson,\(^1,^2\) is attributed to the golden yellow indentation on the rhizome left by emerging petioles, which resembled old-fashioned wax letter seal. Goldenseal is a perennial herb distributed in the Northeastern United States with largest populations found in Ohio, Indiana, Kentucky, and West Virginia\(^3\). Each plant produces a single flower, which fruits into a bright red berry thus earning it another common name, **ground raspberry**. Goldenseal has also been referred to as **Indian dye** or **yellow pucon** as result of its use for dyeing weapons and fabrics owing to the yellow pigment of the rhizome.\(^1,^4,^5\) There are numerous accounts of goldenseal rhizomes having a plethora of uses by Native American communities. This plant was used by the Cherokee as an insect repellant, probably due in part to its acrid smell, and it was applied to the body as a salve, which consisted of bear fat and pounded goldenseal rhizome\(^4\). Other liniment preparations applied topically or decoctions used as a wash against local inflammation and various skin diseases were also credited to the Cherokee. The Iroquois also used infusions and decoctions from goldenseal rhizomes as drops for earaches and sore eyes, (earning it another name as **eye root**\(^1\)), and goldenseal rhizomes prepared as a tea or with whiskey were employed as a tonic for increase vigor.\(^1,^5-^6\) Goldenseal teas and
decoctions were also used for sore mouths and throats, for digestive problems such as diarrhea, ulcers, dyspepsia and stomach aches, for gall bladder, liver and heart troubles, and as an antiseptic douche for vaginal inflammation and gonorrhea. Finally, both the Iroquois and Cherokee employed goldenseal for the treatment of gastrointestinal complaints and as a soothing remedy for the internal organs. The Native Americans shared their knowledge of natural remedies with the early settlers, which allowed for the continuity of the use of herbal medicines in the new colonies. In the 19th century, goldenseal was popularized as an antiseptic by Samuel Thomson, and was used by Eclectic practitioners for other external ailments such as eczema, boils, hemorrhoids, rectal fissures and conjunctivitis. The colonists also adapted the use of goldenseal as a tonic during recuperation from major illness. In a series of advertisements in the Lewiston Morning Tribune after the Civil War on 1908, Dr. R.V. Pierce hailed the Golden Medical Discovery “for the cure of weak stomach, indigestion, or dyspepsia, torpid liver, or biliousness” as well as “for the cure of catarrhal, bronchial and throat affections accompanied by hoarseness, sore throat, lingering or hang-on-coughs and all those wasting affections”, which had goldenseal as one of its “most important and valuable ingredients” among other medical roots found growing in America. Goldenseal was dubbed as the poor man’s ginseng because its popularity as a tonic rivaled that of the imported ginseng since it was locally available thus less expensive. The plant also gained some form of infamy inspired by the novel written by John Uri Lloyd in 1901 entitled “A Stringtown on a Pike: A Tale of Northernmost Kentucky”. A passage in the book read “pure white alkaloid of Golden seal and pure morphine...when mixed in proper
proportion (one part of the alkaloid to four parts of the morphine), the reaction of strychnine presented itself…””, and was misconstrued to mean that goldenseal has the ability to mask the presence of prohibited drugs in the body. However, several studies showed that this is not true when testing for drugs using urine samples.8

Goldenseal was classified as an antiseptic and astringent in the standard drug reference of the US Pharmacopoeia and was listed in the National Formulary, the pharmacists’ reference, from 1831 to 1936 and 1936 to 1960, respectively. Its popularity was not, however, without consequence, and wild goldenseal was exploited and over-harvested to the point of being endangered, as reported in 1997 by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).9 Consequently, goldenseal was listed in Appendix II of the agreement, which “includes species not necessarily threatened with extinction, but in which trade must be controlled in order to avoid utilization incompatible with their survival”.10 Despite this restriction, goldenseal was still ranked 12th among the dietary supplements in prevalent use nationwide based on the 2002 Health & Diet Survey conducted by the Food and Drug Authority (FDA).11 This sustained demand despite of the restriction encouraged adulteration of goldenseal with other berberine-containing botanicals such as goldthread (Coptis japonica), barberry (Berberis vulgaris) and Oregon grapes (Mahonia aquifolium).12 The FDA classified goldenseal along with other herbs with undefined safety.2 Recently, the Natural Standard Herbal Pharmacotherapy13, a book published through international research collaboration, which provided evidence-based, consensus-based and peer-reviewed information on complementary and alternative therapy, gave
H. canadensis a Grade C for bacterial, parasitic, and fungal infections. This grade indicated that there are unclear or conflicting scientific evidence for goldenseal’s activity against bacterial, parasitic and fungal infections. The alkaloid berberine is considered to be the most effective component of goldenseal against both Gram-positive and Gram-negative bacteria. Berberine’s antimicrobial activity is attributed to its ability to intercalate with DNA, which inhibits the process of DNA and protein synthesis. It has also been shown to prevent bacteria from adhering to host cells either by (1) stimulating the release of lipoteichoic acid (LTA) from the cell surface resulting to the loss of LTA, the major adhesion molecule of the bacteria, or (2) breaking down the formed fibronectin-LTA complex and thereby disrupting bacterial adhesion. Although these mechanisms of actions have already been verified, little data is available to show whether the actual berberine content of goldenseal could amount to significant clinical effects against these microorganisms.
Figure 1. Goldenseal leaf with red berry.

Each leaf has a single berry. This photo was taken during the second week of July 2011 in Hendersonville, NC during a collection trip. (©Hiyas Junio)

Figure 2. Goldenseal rhizome and roots.

The golden yellow color of the exposed rhizome is due to the presence of berberine. The rhizome is the major source of berberine and is the plant part most commonly used in the preparation of herbal supplements (©Hiyas Junio).
One of the disease-causing Gram positive bacteria is *Staphylococcus aureus*, which typically can be managed with the use of commercially available antimicrobial agents. However, the emergence of virulent strain such as the infamous methicillin-resistant *S. aureus* (MRSA) has made antibiotics such as the penicillins and cephalosporins that are currently available as ineffective. This has resulted in a dramatic increase in fatal *S. aureus* infections.\textsuperscript{17} A United States population-based survey for 2001-2002 indicated that the prevalence of *S. aureus* and MRSA colonization were estimated to be 84.9 million persons (95% confidence interval) and 2.3 million persons (95% confidence interval), respectively.\textsuperscript{18} Colonization is strongly associated with bacterial infection since colonization precedes infection. An annual 8.4% and 14% increase in the incidence of *S. aureus* and MRSA infections, respectively, and annual average of \(~10,800\) (7440-13,676) and \(~5,500\) (3,809-7372) *S. aureus* and MRSA-related deaths, respectively, were estimated based on a study from 1999 to 2005 also in the United States alone.\textsuperscript{19} In a study conducted by De Marco, et al, using susceptibility data and gene expression, 72% of effluxing strains of *S. aureus* from clinical isolates are also methicillin-resistant. This indicates the relevance of efflux-mediated mechanisms in the survival of this particular virulent strain.

The completed sequence of *S. aureus* N315, which is similar to the prototypical *S. aureus* strain (NCTC 8325-4), is a 2.81 Mb genome and has a total of 212 transporters.\textsuperscript{20} Of the 212 transporters, at least 20 are considered to be multidrug efflux pumps, with majority classified under the major facilitator superfamily (MFS) of transport proteins. Multiple sequence analysis among the members of the cluster of MFS transporters
showed greater sequence similarities in the N-terminal halves relative to the C-terminal halves. These similarities and differences led investigators to hypothesize that the N-terminal is involved in the energization of the transport while the C-terminal is involved in determining substrate specificities of the proteins.\textsuperscript{21} Of particular interest is the NorA transporter because it is found to be most common in clinical isolates of \textit{S. aureus}, which are capable of efflux.\textsuperscript{22} The NorA confers resistance to acriflavin, fluoroquinolones, puromycin and chloramphenicol, ethidium bromide and quaternary amine compounds, which include the alkaloid berberine. It belongs to a cluster of twelve (12) transmembrane proteins, which rely on the proton motive force (PMF) to facilitate efflux, i. e., antiport of the antibiotics with protons as the dynamic force behind the efflux mechanism where the transmembrane movement of the antibiotics is opposite to that of the proton.\textsuperscript{23}

Multidrug efflux systems are transporter proteins present not only in bacteria but also in higher eukaryotes, which function as defensive machinery for cells. They export a broad range of structurally unrelated compounds thereby conferring resistance in bacterial pathogens and mammalian cancer cells. The incidence of multidrug efflux as a mechanism of resistance is either the natural role of these transporter proteins or a fortuitous function due to selective pressure imposed by toxic agents such as antibiotics and chemotherapeutic drugs.\textsuperscript{21} This was indirectly determined by examining the genetic organization, regulation and occurrence of the genes encoding the proteins, and the physiologies of the organisms. The inherent significance of multi-drug efflux pumps as potential targets in antibacterial therapy is based on the fact that they are found in many
pathogenic bacteria that displayed resistance to antibiotics.\textsuperscript{24} Regulation of these multidrug resistance (MDR) pumps is a practical strategy to overcome resistance.

Inhibition of MDR pumps to increase accumulation of antibiotics within the cells is just one type of synergy effect. An example of this is the combination of a weak antibiotic and a secondary metabolite from plants, which results in enhanced antibacterial activity.\textsuperscript{25} Other types of synergy effect are also observed for different mechanisms of resistance exhibited by virulent strains of microorganisms. Bacterial infections display resistance to antibiotics as categorized into three groups, namely, (1) modification of the receptor or active site through mutation develops resistance to rifamycin & quinolones, and penicillin, respectively; (2) degradation of the antibiotic through enzyme-cleaving strategy by way of hydrolysis, group transfer and redox reaction\textsuperscript{26}, which transform antibiotics such as cephalosporin and penicillin into their inactive forms; and (3) reduced accumulation of antibiotics within the bacterial cells via active efflux due to the presence of MDR pumps of drugs such norfloxacin and tetracycline\textsuperscript{25}. However, since virulent strains survive the assault of antibiotics with their multi-mechanistic approach, a multi-drug therapy is needed to overcome resistance. A paradigm shift from a single-drug to multi-drug therapy to combat bacterial infections could also be incorporated in the search for compounds in natural products, which are capable of working in synergy with other antibiotics.

Synergistic effects are defined as cases where the dose combination of two substances results in more than the expected total of the separate effects.\textsuperscript{27} Potentiation is a specific type of synergy whereby one compound augments the activity of another while
not possessing any activity of its own. An example of this synergy effect was observed by Stermitz, et al., (2000) with 5’-methoxyhydnocarpin-D (5’-MHC-D) (1) and pheophorbide a (2) (Figure 3) isolated from the plant of the Berberis species. As the name implied, this plant family contained berberine at high concentration with compounds 1 at 0.05-1% of dry leaf weight and 2 at least 4.2% of dry leaf weight. The combination of 5’-MHC-D and pheophorbide a at 1.2 and 0.3 µg/mL, respectively, with sub-inhibitory concentration of berberine resulted in the inhibition of the proliferation of wild type S. aureus. These compounds displayed activity against the multi-drug resistance (MDR) pump NorA as they increased the sensitivity of the wild type S. aureus strain to norfloxacin but showed no effect for the NorA- mutant. The compound 5’-MHC-D isolated by Stermitz, et al. operates against the third category of resistance by blocking the NorA pumps in S. aureus resulting in the accumulation of berberine at inhibitory concentration. Goldenseal, with berberine as one of its constituents in both roots and leaves, could have developed similar compounds that may have synergistic effect with its natural alkaloids.
Compound 1 is a flavonolignan initially reported as 5'-methoxyhydnocarpin. It was renamed to 5'-methoxyhydnocarpin-D after extensive HMBC experiments showed that this compound was a regioisomer of hydnocarpin and not the methoxy form. Compound 2 is a porphyrin leaf product formed as an intermediate in chlorophyll degradation, thus expected to be present in all green-leaved plants. However, only the extracts of berberine-containing plant species showed significant potency relative to the other plants tested by Stermitz, et al (2000).

Goldenseal’s current popularity as an herbal supplement continues to generate scientific interest, hence the current undertakings to investigate its antibacterial properties. Historical and contemporary preparations of goldenseal commonly used the rhizomes and roots as the main source of bioactive constituents. However, berberine, the most abundant alkaloid constituent in the rhizome and roots, is a natural substrate for the NorA efflux pump observed in Staphylococci resistant strains. This benzylisoquinoline
alkaloid gets easily ejected out of the cell by the NorA pumps, essentially preventing the accumulation of berberine at a toxic level inside the cell thus rendering it ineffective to arrest bacterial growth. However, recent studies showed that goldenseal is effective against bacteria, yeast, and mammalian cells that possess multi-drug resistance pumps. The results of the studies inferred that there are constituents other than berberine, which are partly responsible for the observed antibacterial\textsuperscript{32}, antifungal\textsuperscript{33} and cholesterol-lowering\textsuperscript{34} activities of goldenseal. The underlying mechanism for these activities was inferred to be synergistic in nature. Leaf extracts of goldenseal, which also contain the bioactive alkaloids albeit at lower concentrations relative to the rhizome, were shown to be effective against the efflux mechanism.\textsuperscript{35} It was reported that berberine combined with the goldenseal leaf extract showed longer intracellular retention time against MDR-1 mediated efflux from the liver cells relative to the treatment with berberine alone\textsuperscript{34}. Leaf extract activity was also reported against a strain of \textit{Candida albicans} that overexpressed multi-drug resistant (MDR) efflux pumps\textsuperscript{33}. These results suggest a multi-constituent (possibly synergistic) activity for goldenseal.

The gold standard technique for identifying and isolating bioactive compounds from natural products is bioassay-guided isolation. Bioassay-guided isolation uses bioactivity to direct the separation process through an iterative manner until a single active compound is isolated. The crude plant extract that is most bioactive is subjected to separation. Then the most bioactive of the subsequent fractions is subjected to the next stage of separation. This process is done until a single bioactive compound is purified. While this technique is outstanding for natural products drug discovery, it does have its
limitations. In bioassay-guided isolation, the particular activity of interest is directly measured, whereas miscellaneous activities due to the indirect contribution of other secondary metabolites present cannot be determined unless specifically tested or serendipitously discovered. There is a great likelihood that when bioassay-guided isolation is employed, a compound having no activity on its own and is only active in combination with other compounds may not be discovered. With this project, we sought to modify the bioassay-guided fractionation approach by incorporating combination assays and liquid chromatography-mass spectrometry (LC-MS) constituent profiling. We expected that LC-MS profiling would enable active constituents to be tracked through the isolation process, and allowed for cross referencing with databases and literature identity metabolites. Furthermore, the incorporation of combination assay as part of the isolation process would enable identification of possible synergists without intrinsic antimicrobial activity.
CHAPTER II
REVIEW OF RELATED LITERATURE

1. Isolated constituents of goldenseal

Most studies conducted to assess the bioactivity of goldenseal have used the rhizome as the primary source of plant materials due to the abundance of the berberine in this part of the plant. Based on literature, berberine (the yellow alkaloid) and hydrastine (the white alkaloid) were the most abundant constituents of goldenseal or Hydrastis. This was documented as early as the 19th century. In a comprehensive account done by John Uri Lloyd and Curtis Gates Lloyd (1884-1887), which they compiled in the book Drugs and medicines of North America, it was Constantine Samuel Rafinesque who they credited for documenting the presence of berberine in goldenseal. According to Lloyd and Lloyd, Rafinesque, in 1828, in his publication entitled Medical Flora of the United States, wrote of a constituent of Hydrastis as “a peculiar principle hydrastine, of a yellow color”. Meanwhile, the name berberine was given by Buchner and Herberger in 1830 to an extract they isolated instead from *Berberis vulgaris*. In the late 19th century, the yellow alkaloid was more popularly called hydrastine in the Americas due to Eclectic doctors. It was more popular as berberine in Europe. Hydrastine, the white alkaloid, was credited to be discovered, and first reported by Alfred B. Durant on 1851 in the American Journal of
Pharmacy where he wrote, “...I shall therefore call the substance hydrastine...after repeating my experiment in large scale, in fully establishing its rank among the alkaloids.”

1.1. Alkaloids in goldenseal

There are twelve (12) known alkaloids present at varying concentration isolated from goldenseal, which are shown in Figure 4. These alkaloids are berberine (3), canadaline (4), canadine (5), canadinc acid (6), β-hydrastine (7), hydrastidine (8), isohydrastidine (9), hydastinine (10) corypalmine (11), isocorypalmine (12), (S)-tetrahydropalmatine (13), and 8-oxotetrahydrothalifendine (14). Of these alkaloids, the most abundant are berberine (1-6% w/w), β-hydrastine (2-5% w/w) and canadine (0.1-1% w/w). The others are present in trace amounts relative to the most abundant alkaloid berberine, and have not been quantified because of lack of available standards. Among these, berberine is the most investigated for its pharmacological effects both in in vivo and in vitro studies. In a compilation of evidenced-based reviews on goldenseal by Braun and Cohen (2010), the activity attributed to berberine included antimicrobial, anti-diarrhea, hypocholesterolaemic/anti-atherogenic, anti-diabetic, anti-inflammatory, immune activity, anticancer, neuroprotective and antidepressant. Berberine was also used in clinical trials for diarrhea, eye infection, hypercholesterolaemia, chronic congestive heart failure, diabetes and radiation-induced lung injury. β-Hydrastine (7) was used formerly as astringent, vasoconstrictor and uterine stimulant and canadine (also called
tetrahydroberberine, 6) was reported to have antagonistic activity to a specific dopamine receptor\textsuperscript{40, 41}.
Figure 2. Alkaloids isolated from *H. canadensis*.

Canadine (5) and β-hydrastine (7) are reported to be unique to goldenseal and used for the detection of adulteration of commercial goldenseal root powder with other berberine-containing plants.\textsuperscript{12,42} However, it was also reported that canadine and β-hydrastine were detected from plants of the *Corydalis* species\textsuperscript{43,-44} as well. Compounds 8 and 9 can be converted to 7 through methylation with diazomethane while compounds 11 and 12 are intermediates in the biosynthesis of 5 through oxidation of the ortho-methoxy phenol.\textsuperscript{36}
1.2. Flavonoids and other compounds in goldenseal

The flavonoids 6, 8-di-C-methylluteolin-7-methyl-ether (C_{18}H_{16}O_{6}, 13) and 6-C-methylluteolin-7-methyl ether (C_{17}H_{14}O_{6}, 16) were isolated by Hwang, et al, from powdered goldenseal rhizomes. Survey of the literature using SciFinder® and the Dictionary of Natural Products® indicated that these compounds were new to goldenseal and had not been reported to be present in the aerial portion of the plant nor have they been reported to be present in other plants. Both compounds showed weaker activity relative to berberine against a cariogenic oral pathogen *Streptococcus mutans* (ATCC 10449), and *Fusobacterium nucleatum* (ATCC 25586), a periodontopathic oral pathogen. The MIC values of these compounds against *S. mutans* and *F. nucleatum* were >500 and 375 µg/mL (15) and 250 and 375 µg/mL (16), respectively. Compound 15 also exhibited additive effect with berberine against *S. mutans*, which lowered the MIC of berberine from 125 µg/mL to 62.5 µg/mL and had an FIC of 0.5-1.037.

The alkaloid 8-oxotetrahydrothalifendine (C_{19}H_{17}NO_{5}, 14) and two quinic acid feruloyl esters (C_{21}H_{28}O_{9}, 21 & 22) were reported by Gentry, et al, and were isolated from goldenseal root powder. Compound 14 was initially reported by Messana, et al., to be present but the structure was not reported due to insufficient physical data to locate the hydroxyl groups. The group reported its molecular formula as C_{19}H_{21}NO_{4}. All three compounds were new to goldenseal and only compound 14 had been previously isolated from another plant, *Coscinium fenestratum*, which had berberine as its most abundant alkaloid. All three compounds were inactive against *Staphylococcus aureus* (ATCC...
13709), Klebsiella pneumonia (ATCC 10031), Mycobacterium smegmatis (ATCC 607), and Candida albicans (ATCC 10231) when isolated from the most active extract.

Compound 5-O-(4’-[β-D-glucopyranosyl]-trans-feruloyl) quinic acid, GFPQ, (C_{23}H_{30}O_{14}, 20) was isolated by McNamara, et al, from ground rhizomes of goldenseal plant grown in New Zealand but sourced from North America. Both the above ground and below ground parts of the plant were analyzed in search of biomarkers for the rhizome, which can be used as indicators for the integrity of goldenseal herbal supplements sold. Compound 20 is a new constituent found in goldenseal and considered to be a major component in the roots and rhizomes being apparently more abundant than canadine (5). This compound was not detected in the aerial portion of the plant, which makes it a potential biomarker for adulteration. Compound 20 showed no activity against methicillin-resistant Staphylococcus aureus with an MIC > 1.0 mg/mL. Another constituent reported by McNamara, et al newly identified in goldenseal was neochlorogenic acid (C_{16}H_{18}O_{9}, 19).{42} Based on previous studies, compound 19 was almost always detected with chlorogenic acid (C_{16}H_{18}O_{9}, 18) but has never been reported before in goldenseal. Compound 18 is a known constituent of goldenseal and is found to be common in green plants. It was reported that 18 isolated from artichoke (Cynara scolymus, L) displayed bacteriostatic activity with relatively high antimicrobial activity against fungi than bacteria.{46}
Figure 5. Other compounds isolated from goldenseal.

Most of the compounds listed above were recently discovered as constituents of goldenseal with the exception of compounds 17 and 18, which are found abundantly in plants. Compound 17 is bound to the fibers of plants\textsuperscript{47,48,49} while 18 is a common constituent of green plants discovered in 1909 from green coffee beans\textsuperscript{50}. 
2. Literature survey of antibacterial activity of goldenseal against efflux pumps

2.1. Activity of goldenseal against microorganisms

Scazzocchio, et al., investigated the antibacterial activity of goldenseal and its alkaloids, berberine, β-hydrastine, canadine and canadaline, based on their bactericidal and bacteriostatic activity against Gram-positive bacteria *Staphylococcus aureus* (ATCC 2953, ATCC 6538P), *Streptococcus sanguis* (ATCC 10556), and Gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853)\(^2\). Bactericidal activity is determined by the absence of growth of the microorganism in the sub-cultures of disks exposed at predetermined time in the test solutions after being loaded with low density inoculums \((2.0\times10^3 \text{ CFU/mL})\). Hydrogen peroxide \((3\% \text{ w/w})\) was used as reference for bactericidal activity because of its effectiveness as a topical disinfectant. Meanwhile, bacteriostatic activity (the ability to inhibit bacterial growth) was determined using broth microdilution assay with the minimum inhibitory concentration (MIC) as the measure of efficacy for the samples tested at concentration range of \(0.0020\) to \(1.0 \text{ mg/mL}\). Cefetamet pivoxil® (La Roche LTD CH-4002 Basel Switzerland), an oral cephalosporin, was used as the positive control drug. The goldenseal extract was tested at standardized berberine concentration. The results indicated that the extract \((2.5, 5 \text{ and } 10 \text{ mg/mL})\) and all the alkaloids \((1.5 \text{ and } 3.0 \text{ mg/mL})\) except for β-hydrastine exhibited bactericidal activity against at least three of the microorganisms. Berberine and canadeline were ineffective against *P.aeruginosa* and *E. coli*, respectively. For the bacteriostatic activity, all test samples where ineffective.
against the Gram-negative bacteria with MIC values of >1.0 mg/mL versus the Cefetamet at 0.002 and 0.5 mg/mL for the *E. coli* and *P. aeruginosa*, respectively. Canadaline had the same MIC at 0.25 mg/mL against all the Gram-positive bacteria whereas canadine had an MIC range of 0.5 to >1.0 mg/mL. The extract and berberine had the same MIC against both Gram-positive and Gram-negative except for one strain, *S. aureus* (ATCC 25923) with MIC at 0.12 and 0.25 mg/mL, respectively. Although this is only a two-fold difference, the investigators asserted that this result was significant in that the activity of the canadine and canadaline could not account for the difference in the MIC values since canadine had low activity against this strain (>1mg/mL) and both alkaloids were present in the tested extract at trace amounts. The presence of other compounds other than alkaloids was further suggested as possibly being responsible for the observed activity.

2.2. Activity of goldenseal against azole-resistant yeast

Kolaczkowski, et al, investigated seven (7) medicinal plants, *Mahonia* (Berberis) *fortunei* (Chinese mahonia51), *Berberis fendleri* (Colorado barberry52), *Pinus edulis* (two-needle pinyon52), *Zanthoxylum zanthoxyloide* (candlewood tree53), *Hydrastis canadensis* (goldenseal52), *Dalea formosa* (featherplume52) and *Artemisia annua* (sweet sagewort52) for their anti-fungal activity against different strains and clinical isolates of *C. albicans* and *C. glabrata*. Plant samples extracted from the whole above-ground material were also tested against *Escherichia coli* K12, *Staphylococcus aureus* F137 and isogenic *Saccharomyces cerevisiae* strains. Goldenseal leaves extracted with methanol showed better inhibitory effects than extracts prepared in ethyl acetate against *C. glabrata* and *C.*
*albicans* with MIC values at 40 and 160 µg/mL, respectively. The methanol extracts also showed comparable MIC values with an azole-sensitive clinical isolate of *C. glabrata* BPY112 and the resistant BPY126, which overexpresses multidrug efflux pumps CgCDR1, CgCDR2, and CgSNQ2. Similar activity was also displayed against two isolates of azole-resistant *C. albicans*, with one isolate overexpressing CDR1 and CDR2 and the other overexpressing CDR2. An MIC of 80 µg/mL for the methanol extract was also observed for *S. aureus* but it was found to be inactive against *E. coli*. *Saccharomyces cerevisiae* was also used to further characterize the extracts. *S. cerevisiae* has a well-characterized network of multi-drug resistance genes, which could be manipulated easily, compared to its homolog *C. glabrata*. The use of *S. cerevisiae* enabled better characterization of the growth inhibitory and efflux modulatory activity of the extracts tested. An MIC of 180 µg/mL was displayed by goldenseal against both the *S. cerevisiae* strain with the wild-type PDR1 allele and the strain with the double disruption of the regulators PDR1 and PDR3. The goldenseal methanol extract also displayed an MIC value of 90 µg/mL against the triple knockouts of YOR1, SNQ2 and PDR5, which are multidrug transporter genes. The above results suggested that the goldenseal methanol leaf extract contained secondary metabolites, which exhibit possible synergistic effects via pump-inhibitory activity against the azole-resistant *Candida* isolates. However, there were no quantitative data collected on the plant samples to implicate berberine or other constituent in this activity.
2.3. **Activity against *Helicobacter pylori* of a crude goldenseal extract**

*Helicobacter pylori* is the main organism implicated in chronic gastritis, peptic ulcer disease and gastric cancer. There are reported cases of this bacterium exhibiting resistance to the current drug treatment, which is a triple drug therapy consisting of two antibiotics with a proton pump inhibitor or bismuth salts. The susceptibility of *H. pylori* berberine, β-hydrastine, and the methanol extract of *H. canadensis* rhizome was investigated by Mahady, et al, (2003) using 14 clinical isolates and one ATCC strain (43504) of the microorganism. The alkaloids and the crude extract were tested at a final concentration range of 0.78 to 100 µg/mL using the agar dilution procedure. Results showed that β-hydrastine was not as effective, with MIC range of 25 to > 100 µg/mL, relative to berberine and the crude *H. canadensis* methanol extract, which both showed comparable activity against the bacterium. Berberine had an MIC range of 0.78 to 25 µg/mL while the goldenseal extract had an MIC range of 0.78 to 50 µg/mL. In 8 of the 14 clinical isolates, berberine and *H. canadensis* exhibited the same MIC value against each of the isolates. The MIC value of the compounds differed from one isolate to the other. With the remaining 6 isolates, including the ATCC 43504, the MIC of berberine was better by two-fold compared with the MIC of *H. canadensis* against each isolate. Again, the MIC values differed from one isolate to the other. It was communicated by the principal investigator to this author that the *H. canadensis* extract concentrations used for the susceptibility testing were not standardized to berberine. This indicated that the extract was tested at a lower berberine concentration relative to the pure berberine since
both were tested at the same final test concentration range of 0.78 to 100 µg/mL. The improved activity of the extract compared with berberine against H. pylori indicates either that there are compounds in the extract with more potent antibacterial activity than berberine, or that there are auxiliary compounds that act with berberine to increase its antimicrobial activity.

2.4. Activity of goldenseal against MDR1-mediated efflux from liver cells

High level of low density lipoprotein cholesterol (LDL-c) in the blood plasma is postulated to be the primary risk factor involved in the development of coronary heart disease and atherosclerosis. Berberine is known to have a cholesterol-lowering effect and its mechanism of action is considered novel relative to those of the commercially successful statin drugs, which include atorvastatin (Lipitor®) and lovastatin (Mevacor®, Altocor®, Altoprev®). The statins work by inhibiting HMG-CoA reductase, an enzyme involved in the critical step in the biosynthesis of cholesterol in the liver. In a study by Kong, et al, berberine isolated from the Chinese herb huanglian was found to upregulate hepatic LDL receptor (LDLR) by increasing the LDLR mRNA and protein expression. LDLR mediates the uptake of plasma cholesterol from circulation to the liver where it is oxidized into different bile acids. On the basis of its berberine content, goldenseal was investigated by Abidi, et al, for its cholesterol-lowering activity. Goldenseal root extract with equivalent berberine concentration as the pure berberine tested showed better activity in increasing the expression of LDLR in HepG2 cells, which led the investigators to probe the mechanism associated with the observed activity. With comprehensive
quantitative analysis showing lower berberine concentration in the extracts, the
goldenseal extracts prepared from four different lots were able to increase the LDLR
mRNA expression better than the pure berberine. This was independently confirmed by
Northern blot analyses and real-time quantitative RT-PCRs of the total RNA harvested
from HepG2 cells. The observed upregulation of LDLR expression was proposed to be
via stabilization of the mRNA since the LDLR promoter (oncostatin M) activity was not
affected by the extracts both in the sterol-dependent and sterol-independent pathway of
LDLR transcription. In the presence of the transcription suppressor actinomycin D, the
LDLR mRNA level in the cells was not diminished but instead was increased 3.4-fold by
the goldenseal root extract compared with controls. Both canadine and berberine were
also able to increase the level of LDLR mRNA by 2.5-fold under the same restriction.
However, the concentrations of berberine and canadine present in the root extract tested
could not account for the observed activity. In a time-dependent experiment, the cellular
level of LDLR mRNA was increased with a faster kinetics by goldenseal compared with
berberine. Applied at standardized berberine concentration, the goldenseal extract caused
the rapid accumulation of berberine in the cells exhibited by a ~13-fold increase in the
fluorescence intensity relative to only ~2-fold increase for the pure berberine within a 5-
minute period. This observation led Abidi and his co-workers to postulate that since
berberine is actively effluxed from bacterial cells by multidrug resistance pumps, the
multi-drug transporter 1 (MDR1) pgp-170 in HepG2 cells could be responsible for the
low intracellular accumulation of berberine. In an experiment to verify MDR1 inhibitory
activity of goldenseal, the efflux of DiOC2, a fluorescent small molecule, which is a
known substrate of the MDR1 transporter, was investigated with verapamil as a positive control. Experimental results showed that goldenseal was able to increase the uptake of DiOC₂ compared with the untreated cells, and the cellular retention of the fluorescent molecule was comparable with that of verapamil. Based on these results, the investigators further asserted that there are minor components in goldenseal that act as natural antagonists of the MDR1 transporter, which enhances the berberine uptake in HepG2 cells resulting in increased LDLR mRNA expression.

Abidi, et al, also investigated the effect of goldenseal in vivo using Golden Syrian male hamsters fed with a high-cholesterol (HC) diet. The pooled-serum from the goldenseal-treated animals exhibited lower plasma total cholesterol by 31.3%, LDL-c by 25.1%, triglyceride by 32.6% and FFA by 33.8% relative to the control. The reduction in the total cholesterol and LDL-c was directly correlated with the increased liver LDLR mRNA expression from sacrificed animals whereas the increased in the triglyceride and FFA were attributed to the activation of the ERK signaling pathway through acetyl CoA-carboxylase. Overall, the study provided substantial evidence for the presence of natural antagonist of the MDR1 transporters in goldenseal, which improved the uptake of berberine in liver cells resulting in the stimulation of LDLR mRNA expression resulting in the subsequent attenuation of LDL-c in the blood plasma.
2.5 Synergy in antimicrobial activity

Synergy in an antimicrobial combination is characterized by an effect larger than the expected dose-combination effect of two drugs when evaluated for their single-dose mixture.\textsuperscript{27} In a simplistic representation, it would mean that if the activity of each drug in the combination is assigned as 1, then the overall effect would be 1+1 >2. If one of the drugs in the combination has zero activity but resulted in an overall effect such that 1+0 >2, then the combined effect is considered potentiation. Potentiation is a specific type of synergy. Quantification of synergistic interactions was proposed by Berenbaum (1977) using algebraic and geometric methods based on approaches used in immunosuppressive therapy. The algebraic method uses Equation 1,

\[
\frac{\text{dose of } A_{e}}{A_{e}} + \frac{\text{dose of } B_{e}}{B_{e}} = 1 \quad (\text{Equation 1})
\]

where \(A_{e}\) and \(B_{e}\) are equi-effective doses, i.e., doses of drugs A and B that produce an equal effect.\textsuperscript{56} If the dose-combination is additive then the result of equation 1 is 1; if the dose-combination is synergistic, the result of equation 1 is less than 1, and if it is antagonistic, the result of equation 1 is greater than 1. For antimicrobial combination experiments, the \textbf{fractional inhibitory concentration (FIC) index} is often used to quantify combination interactions using the same concept proposed by Berenbaum. The FIC index is calculated using Equation 2\textsuperscript{57},
where A and B are the minimum doses of drugs A and B in the combination that resulted in complete inhibition of the growth of the microorganism. MIC is the minimum inhibitory concentration of each of the drugs alone against the microorganism in question. The checkerboard technique is done to measure the FIC index of dose combinations. The checkerboard experiment is a two-dimensional experiment where paired combinations of an array of serial concentrations of the test compounds are evaluated. The overall effects of the paired combinations are classified as synergy if FIC<0.5, antagonism if FIC >4.0 and no interaction if FIC>0.5-4.0. For example, it was determined that the minimum inhibitory concentration (MIC) for drug A or B is 5.0 µg/mL against *S. aureus*, i.e., 5.0 µg/mL is the lowest concentration of drug A or B that inhibits the proliferation of *S. aureus*. It was also determined that growth of the bacteria was arrested by the combination of 1.0 µg/mL A + 4.0 µg/mL B, 2.0 µg/mL A + 2.0µg/mL B or 2.5 µg/mL A + 2.5 µg/mL B, etc. If we use the MIC values of drugs A and B, and the combination doses, then we can calculate Equation 2 as

\[
\frac{(A)}{(MIC_A)} + \frac{(B)}{(MIC_B)} = FIC_A + FIC_B = FIC \text{ Index (Equation 2)}
\]

which indicates that the drug interaction effect is additive against *S. aureus*. The graphic demonstration of the FIC index is represented by an isobole, a termed coined by Loewe.
& Muischnek.\textsuperscript{56} Each point on the isobole is a dose-combination of the MIC of the drug plotted at the $y$-coordinate in combination with a concentration of the drug plotted on the $x$-axis\textsuperscript{57}. Typical isoboles are shown in Figure 6, which can be used to classify the combination effect as antagonism, additivism, or synergy depending on the shape of the plot.
**Figure 6.** Isoboles for identifying interactions effects.

The geometric method for identifying dose-combinations as additive (no interaction, straight isobole), synergistic (enhanced activity, concave isobole) or antagonistic (diminished activity, convex isobole) as proposed by Berenbaum\(^{28}\).
3. Synopsis

The compilation of experimental studies on goldenseal clearly identified berberine as an important active constituent against a plethora of disease states. Some evidence-based studies showed that complex goldenseal extracts are also effective to arrest both bacterial and fungal growth and the crude goldenseal extract is able to increase berberine concentration inside mammalian cells. The bacterial, fungal and mammalian cells used in these studies possessed multi-drug resistance pumps. However, given that berberine is a substrate of MDR pumps, it is unlikely that this compound alone is responsible for all of the observed activities of crude H. canadensis extracts. Berberine accumulates only at low concentration in bacterial and mammalian cells because it is effectively effluxed out of the cells. Goldenseal extracts standardized to their berberine content were more effective than berberine against MDR pumps in bacterial and mammalian cells. Non-standardized extracts tested at the same concentration as pure berberine also showed comparable activity against both wild type and clinical isolates of bacteria and yeast. The displayed activity of goldenseal against MDR pump-expressing microorganisms is believed to be a result of synergistic interaction of berberine with other constituents present in the plant. However, due to the lack of methods correlating activity with concentration of constituents in goldenseal extracts, it is difficult to correlate the observed activities with the presence of specific chemical compounds. In addition, reliable methods to identify synergists that do not have activity on their own but are able to potentiate the activity of other compounds are lacking in the existing literature. It is, therefore, the aim of this research to identify and quantify
goldenseal constituents that exhibit possible synergistic effects with berberine through a technique that will be developed as part of this study. With this technique, we aim to correlate quantitative data with biological activity and ultimately to identify constituents from *H. canadensis* that synergistically enhance the antimicrobial activity of berberine.
CHAPTER III
MATERIALS AND METHODS

1. Plant Material

The plant samples collected were grown on a private lot owned by a local grower, Mr. Bill Burch, in Hendersonville, North Carolina. The GPS coordinates for the collection site are N 35° 24.277’, W 082° 20.993’, 702.4 altitude. Aerial portions of the plant, which consisted of the mature leaf and the petiole, were harvested and transported in paper bags back to Greensboro, NC. The samples were air-dried at ~37°C before processing. Sample collections were done in the summer, late July to mid-August. A total of three collections were performed throughout the course of this research. Each batch of leaves was processed accordingly within two weeks of collection. A voucher specimen of the plant was submitted to UNC Chapel Hill Herbarium (CB# 3280, Coker Hall, University of North Carolina, Chapel Hill, NC 27599-3280, accession #NCU583414) and identified by Dr. Alan S. Weakly (PhD, Botany, Duke University, 1984).
Figure 7. Cultivated goldenseal grown in the plant’s natural habitat.

GPS coordinates of N 35° 24.277’, W 082° 20.993’, 702.4 altitude where collection of plant samples were done for the duration of this research work. The area is surrounded by trees in a private lot. Ginseng plants were originally planted in the same area. (©Hiyas Junio)
2. **Solvent Extraction**

Pre-weighed air-dried leaves of goldenseal were homogenized using a commercial coffee grinder (Kitchen Aid). The ground leaves were percolated with methanol for at least 24 hr., at which time the solvent was removed and replaced with an equivalent volume of methanol. The residue-free methanol extract was collected and concentrated in vacuo using a rotary evaporator (Heidolph Laborota 4000 Efficient). The methanol extract was re-suspended in 9:1 methanol:water and then partitioned with hexane in a separatory funnel. The aqueous methanol layer was separated from the non-polar hexane layer and subsequently partitioned with 4:1 ratio of chloroform to methanol in water in a separatory funnel. The hexane layer was collected, dried in vacuo and was set aside for testing. The chloroform-methanol layer was washed with 1% saline solution to remove water-soluble tannins, which were known to interfere during cell-based bioassay by giving false positive results in enzymes of proteins. The aqueous layer was also collected, dried in vacuo and set aside. The schematic diagram for solvent extraction is as shown in Figure 8.
Goldenseal leaves were initially air-dried for 2 to 3 days and then in the oven at 37°C overnight. Ground leaves were percolated with methanol multiple times using recycled methanol collected from the rotary evaporator (Heidolph). Hexane was also recycled for use for the fractionation procedure.
3. **Chromatographic Separation and Isolation**

The crude tannin-free organic extract was subjected to four stages of separation to obtain the purified flavonoids of interest. After each stage, collected eluates were pooled according to similarity of thin layer chromatography (TLC) and LC-MS profiles, and each was characterized qualitatively and quantitatively for its alkaloid and flavonoid content using LC-MS (Materials and Methods, Section 4). Subsequently, the pooled eluates were tested for their antibacterial activity using the 96-well plate microdilution assay (Materials and Methods, Section 7.2). After each round of biological assay, the most active fraction was advanced to the next stage of separation.

First and second stage separations were carried out using normal phase chromatography with the Combi Rf flash chromatograph (Teledyne-Isco; Lincoln, NE, USA) using silica gel columns (330 g and 120 g, respectively). The gradient for both stages of separation was run based on column volume (CV) instead of time. The use of column volume is preferred because it is more efficient and practical to assign the volume for each gradient, which is based on the column dimension, than it is to assign the duration for each gradient. First stage separation was run at a flow rate of 60 mL per minute for a total run length of 14.5 CV (121.5 min). The gradient initiated at 40:60 hexane/chloroform for 0.5 CV (3.68 min) then was increased to 0:100 hexane/chloroform over 3.0 CV (18.4 min); an isocratic condition of 0:100 was maintained over 2.0 CV (14.72 min), after which the solvent gradient was initiated at 2:98 methanol/chloroform over 2.3 CV (16.3 min) then followed by a series of methanol/chloroform gradients, 5:95 over 2.3 CV (16.3 min), 10:90 over 1.7 CV (12.5 min), 20:80 over 1.7 CV (12.5 min)
then at 100:0 methanol/chloroform over 1.1 CV (8.10 min) then held isocratic at the same condition for 2.5 CV (18.4 min) to wash the column.

The second stage separation utilized increasing concentration of ethyl acetate (EtOAC) in hexane followed by increasing methanol in ethyl acetate up to 100% methanol. The flow rate was at 85 mL per minute with total run length of 22.3 CV (50.3 min). The initial solvent composition of 100:0 hexane/EtOAc was maintained for 0.9 CV (2.03 min) and was then followed by a steep gradient change to 0:100 Hexane/EtOAc over 12 CV (27.1 min). The solvent composition was held isocratic for 3.10 CV (7.00 min) after which the solvent system was switched to MeOH/EtOAc at 3:97 for 1.8 CV (4.06 min) followed by 10:90 over 1.3 CV (2.93 min), 20:80 over 1.0 CV (2.56 min), and the final gradient at 100:0 MeOH/EtOAc over 1.0 CV (2.56 min), then held isocratic at the same condition for 1.2 CV (2.71 min) to wash the column.

Third stage of separation was accomplished with preparative HPLC using a C-18 YMC ODS-a (5µ, 120A; 250x20 mm) column with 8.0 mL/min flow rate. A solvent system of methanol (MeOH) in water was used with gradient starting at 70:30 MeOH/H₂O and increased to 85:15 over 60 min; isocratic condition of 85:15 is maintained until 84.5 min then decreased to 70:30 over 0.50 min then held at isocratic condition until 95 min. UV trace at maximum wavelengths (λ_max) of 270 and 330 nm were monitored during the run. The flavonoid sideroxylin was collected at this stage with 98.8% purity.

Fourth stage of separation was achieved with preparative HPLC using a Luna-PFP® (pentafluorophenylpropyl; 5µ, 100A; 250 × 21.20 mm) column at flow rate 21.24
mL per minute with a total run time of 25.0 min. The solvents system was acetonitrile (CH$_3$CN) and water. The initial solvent composition of 45:55 CH$_3$CN/H$_2$O was changed to 58:42 over 12.0 min followed by 58:42 to 60:40 at 12.1 to 15.0 min; then isocratic at 60:40 at 15.1 to 20.0 min with final gradient change from 60:40 to 65:35 at 20.1 to 25.0 min. UV absorbance at maximum wavelengths ($\lambda_{\text{max}}$) of 270 and 330 nm was monitored during the run. The major isomer 8-desmethyl-sideroxylin was collected with 98.9% purity and the minor isomer 6-desmethyl-sideroxylin was collected with 99.7% purity.

Analyses of the purity of the pooled eluates were carried out using analytical HPLC with PDA after the third and fourth stage using Luna PFP® (5 $\mu$, 100A; 250x4.6 mm) columns using the same solvent system as that of the preparative scale for each corresponding stage of separation but at a flow rate of 1.0 mL per minute.

4. **Quantification using HPLC-ESI Mass Spectrometry**

Separation of the complex plant mixture and subsequent detection of constituents was accomplished using HPLC coupled to electrospray ionization mass spectrometry with ion trap mass analysis. The HPLC (Agilent, 110 series) separation was carried out with a Prevail C-18 column (3$\mu$; 50x2.1 mm) with solvent of increasing concentration of water in acetonitrile. The water was prepared with 1% acetic acid to aid in the ionization process. The gradient elution was done starting at 100:0 CH$_3$CN/H$_2$O to 68:32 over 5 min and decreased to 0:100 until 20 min. An isocratic condition of 0:100 was maintained until 40.0 min.
The alkaloids berberine, canadine and β-hydrastine were quantified in each of the pooled eluates after each stage of separation. A calibration curve was constructed from standard mixture of the three alkaloids, berberine (Sigma), canadine (Sequoia) and β-hydrastine (Sigma), at $1.56 \times 10^{-5}$ M up to $1.22 \times 10^{-2}$ M concentration prepared via two-fold serial dilution. The alkaloid standard solutions, fractions, and sub-fractions were analyzed in the positive mode. The flavonoid constituents were also quantified using a calibration curve constructed from standard mixture of the three compounds previously isolated at a concentration range of $2.50 \times 10^{-7}$ M to $5.00 \times 10^{-5}$ M prepared via a two-fold serial dilution. The flavonoid standard solution, fractions and sub-fractions were analyzed in the negative mode.

The mass spectrometer was set for two scan events for both the positive and negative mode. The first scan event for both ionization modes was a full scan at mass range 50 to 2000. The second scan event was a data dependent scan. The precursor masses for the positive mode were 336.12 (berberine), 340.00 (canadine) and 384.14 (hydrastine). The precursor masses for the negative mode were 297.1 and 311.1. Collisionally induced dissociation (CID) was performed on the most abundant ion from scan event 1 if the specified precursor ions were not detected. The normalized collision energy (%) was at 35 and a default charge state of one (1) was set. The acquisition time was set for 40.80 mins. The capillary temperature was 275 °C, the sheath gas pressure was 20 (arbitrary units), spray voltage was 4.5 kV, capillary voltage was 3 V and tube lens voltage was 60 V.
5. Accurate mass determination and fragmentation pattern study

The accurate masses of the purified crystals 6-desmethyl-sideroxylin, 8-desmethyl-sideroxylin and sideroxylin were determined using an Acquity® ultrahigh performance liquid chromatograph (UPLC) coupled to a Thermo linear trapping quadrupole – orbitrap high resolution mass spectrometer (HRMS) with electrospray ionization (ESI) source. The UPLC method gradient elution was performed over 10.0 min. at a flow rate of 3.0 mL/min with acetonitrile (CH$_3$CN) and 0.1 % aqueous formic acid as solvents. The gradient was initiated at 10:90 CH$_3$CN/H$_2$O then increased to 50:50 CH$_3$CN/H$_2$O over 2.5 min. It was increased from 50:50 to 52:48 followed by 52:48 to 55:45 then 55:45 to 60:40 CH$_3$CN/H$_2$O over 0.5 min. for each gradient change. This was followed by a steep gradient increase from 60:40 to 100:00 CH$_3$CN/H$_2$O over 0.5 min. The column was equilibrated back to the initial gradient condition from 100:00 to 10:90 over 0.5 mins.

The mass spectrometer was set for two scan events. The first scan was a full scan at mass range 150 to 2000 with FTMS analyzer (Orbitrap) for accurate mass determination. The second scan event was a data dependent scan with the ion trap mass analyzer. The precursor masses for the negative mode were 297.1 and 311.1, and the instrument was set to fragment the most abundant ion from scan event 1 if these ions were not detected. The normalized collision energy (%) was at 35 and a default charge state of one (1) was set. The acquisition time was set for 10.0 mins. The capillary temperature was 300 °C, the sheath gas pressure was 25 (arbitrary units), spray voltage was 4.25 kV, capillary voltage was -41 V and tube lens voltage was -103.27 V.
6. Disc diffusion assay

The hexane, organic (4:1 chloroform/methanol), tannin and aqueous extracts collected from solvent partitioning (Materials and Methods, Section 2) were tested using disc diffusion assay. In this assay, discs preloaded with the test samples (extract, extract+berberine, solvent control, and berberine control) are incubated for 24 hrs with the bacteria on an agar plate. The plate is divided into imaginary quadrants and each quadrant is assigned to a disc. The agar plate is loaded with four discs corresponding to extract (1.0 mg/mL); extract (1.0 mg/mL) plus berberine (5.0 mg/mL), solvent control and berberine control (5.0 mg/mL). Each plate is prepared in duplicate. Another plate is prepared for the positive control, the antibiotic kanamycin (BD BBL™ Sensi Disc).

A single bacterial colony bacterium is incubated in 2.0 mL Müeller-Hinton broth for a 24-hr period prior to testing. After incubation, 100 µL of the bacterial stock solution is mixed with 2.0 mL top agar and then vortexed to ensure homogenous mixing. The top agar is a bacterial solution that allows the plating of the bacteria. It is made of 0.70 % agar (BD Bacto®) in Müeller-Hinton broth. The top agar is then poured in the middle of the agar plate and allowed to spread evenly. Before the top agar fully solidifies, the discs are laid into the plates at the assigned quadrant. The ring of inhibition, the region around the disc where there is no visible growth of bacteria, is measured after 24 hrs of incubation. The percent increase in the inhibition is calculated for each of the extracts in combination with berberine and compared with the inhibition due to berberine by itself. The extract producing the highest percent increase in inhibition was moved to the first stage of separation (Materials and Methods, Section 3).
7. Determining the minimum inhibitory concentration

7.1. Constructing the growth curve for *Staphylococcus aureus*

A single colony of the bacteria was incubated in 5.00 mL of Müeller-Hinton broth for a 24-hr period. After incubation, it was added to 50.0 mL müeller-Hinton broth in a 150 mL Erlenmeyer flask to make a stock solution. The optical density at 600 nm (OD600) of the stock solution was measured using a Spectrometer 20 and was designated as the OD600 at time T = 0. To measure colony forming units (CFU) of this original bacterial solution, a 100 µL aliquot of the original stock solution was diluted with 900 µL PBS in an Eppendorf tube, which resulted in a $1.00 \times 10^{-1}$ dilution (A). After preparing solution A, the original stock solution was incubated at 37°C while shaking at 200 rpm. Then from solution A, a series of 10-fold dilutions were performed, giving $1.0 \times 10^{-2}$ (B), $1.0 \times 10^{-3}$ (C), $1.0 \times 10^{-4}$ (D), $1.0 \times 10^{-5}$ (E), and $1.0 \times 10^{-6}$ (F) dilutions of the stock solution. Each dilution of the bacteria was grown in agar plates, starting from the most diluted (F) to the most concentrated (A). A 100 µL solution was smeared unto the agar plate using a miniature glass “hockey stick” while spinning the Petri dish with a small rotating circular platform. The growth plate was prepared in duplicate for each dilution and incubated for 24 hr at 37°C.

To monitor bacterial growth based on optical density, the original stock bacterial solution was incubated at 37°C while shaking at 200 rpm. The OD600 of the stock solution was measured at a 30-minute time interval for a 5-hr period. In addition, the growth plates were prepared concomitant with each OD600 measurement at every time
interval for the entire period. The colony forming units (CFU) in each plate were counted after 24 hrs. The average CFU was calculated from the duplicate plates prepared for each dilution at each time interval. All plates with 30 to 300 CFUs were considered measureable, and the CFU per mL for each plate was calculated by the following formula:

\[
\text{CFU/mL} = (\text{CFU/100 µL}) \times \text{dilution factor} \times 1000,
\]

where the average CFU (between 30 to 300 colonies) for every 100 µL of the solution smeared unto the agar plate is the CFU/100 µL; the dilution factor is the total number of unit volume used to make the final solution volume, i.e. Solution A is 10 times more dilute than the original stock solution while solution B is 10 times more dilute that solution A therefore the solution B should be multiplied by 100; and 1000 is the conversion factor from µL to mL. A growth curve was constructed by plotting the OD600 (primary y-axis) and CFU/mL (secondary y-axis) versus time as shown in Figure 9. The growth curve was collected each time a new bacteria stock was prepared and cryopreserved. Cryopreservation was accomplished by incubating a single colony of bacteria in 2.00 mL of Müller-Hinton broth for a 24-hr period. After incubation, it was added to 20.0 mL Müller-Hinton broth in a 150 mL Erlenmeyer flask to make a stock solution and was shaken for 2 hr at 200 rpm at 37°C. After 2 hrs, the bacterial solution is dispensed into sterile cryovials with glycerol. The cryovials were incubated for another 24-hr period to allow the bacteria to grow. Then the vials were stored at -80°C.
Figure 9. Growth curve for the wild type *S. aureus* (NCTC 8325-4).

Based on the growth curve, the bacteria start the log phase growth after 2.5 hrs of shaking at 200 rpm at 37°C. At the log phase, the bacteria grow exponentially at which point a $5.0 \times 10^5$ CFU/mL stock is made and added to the 96-well plate with the test samples combined with berberine. The growth of the bacteria in the plate after 18-hr incubation period is assessed using OD600 measurements.
7.2. **Broth Microdilution Assay**

The antibacterial activity and potential synergistic activity of the test samples were investigated against wild type *Staphylococcus aureus* (NCTC 8325-4)\(^{60}\), and a NorA overexpressed strain (K2378)\(^{61}\) using the broth microdilution assay with a 96-well microtiter plate (Costar). The test samples were combined with different concentrations of berberine and then incubated with the bacteria for 18 hrs\(^{29,62}\). All the test samples (fractions, sub-fractions and pure flavonoids) were added to the wells at a volume of 50 µL, with the fractions and sub-fractions at 75 µg/mL, and the pure flavonoids at 60 µg/mL final well concentration. All test samples were in 2% DMSO at the final well volume. For the positive control reserpine, a 1.0 mg/mL reserpine dissolved in 100% DMSO was dispensed at 5.0 µL per well to give a 20 µg/mL reserpine in 2% DMSO solution at the final well volume of 250 µL.

The direct antibacterial activity of the fractions, sub-fractions and pure flavonoids were also determined from the microdilution assay. The wells with test samples in 2% DMSO at the final well volume, incubated with the bacteria without berberine (usually the wells at Row A), showed the direct antibacterial activities of the fractions and sub-fractions (at 75 µg/mL) and the pure flavonoids (at 60 µg/mL). The direct antibacterial activities of both 8-desmethyl-sideroxylin and sideroxylin against the wild type *S. aureus* were further investigated by constructing minimum inhibitory concentration (MIC) curves for each flavonoid. The curves were constructed using 4.69, 9.38, 18.8, 37.5, 75.0, 150 and 300 µg/mL final well concentration of each flavonoid. MIC is the lowest
concentration of the test sample at which growth of the bacteria is completely inhibited. In our experiments, MIC was defined as the point at which there was no statistically significant difference between the treatment and the negative control (broth with 2% DMSO). The lower the MIC value, the more effective the test sample is in arresting bacterial growth. Meanwhile, the synergistic activity of the test samples was assessed in combination with different concentrations of berberine and the activity was quantified based on the MIC of berberine. If the MIC of berberine was significantly lowered (FIC < 0.5), the test sample was considered to have synergistic activity.

The broth microdilution assay required four (4) consecutive working days with the first two days allotted for growing and incubating the bacteria, the third day for actual plating of test samples into the microtiter plate, and the fourth day for reading the OD600 with the plate reader (Optima). The microorganisms used for all tests were grown directly from a vial of the bacteria cryopreserved in glycerol at -80°C. The cryopreserved bacteria were grown initially from a single colony in order to ensure that the microorganisms used were genetically the same. On the first day, the thawed bacteria in glycerol were streaked unto an agar plate using a sterile stick, and allowed to grow for a 12-hr period in the incubator set at 37°C. This initial step was done to ensure that the bacteria were still viable after the inactive state in the -80°C. On the second day, a single growth colony was taken from the agar plate using a sterile loop, and was incubated in 2.00 mL Müeller-Hinton broth for 24 hrs.hr For the NorA overexpressed strain of *S. aureus*, a 20 μL of 1.0 mg/mL chloramphenicol (Sigma) was added into the broth to give a final concentration of 0.010 mg/mL in order to maintain the plasmid of the mutant
strain, which confers chloramphenicol resistance. Any bacteria, which did not maintain the plasmid would not survive. After the 24-hr incubation period, the bacterial stock solution was diluted with 18.0 mL broth in a 125 mL Erlenmeyer flask, covered with tin foil and was shaken at 200 rpm at 37° C for 2.5 hrs to reach log phase, as determined from the growth curve. Prior to the end of this 2.5-hr period, the microtiter plate was ready to be loaded with the bacteria with all the wells filled accordingly.

The OD600 of the solution was adjusted to fall within the range of 0.08 to 0.132, as recommended by the Clinical Laboratory Standards Institute (CLSI).63 This solution was then diluted further to a final OD600 corresponding to $5.0 \times 10^5$ CFU/mL. The relationship between OD600 and CFU/mL was established by the data collected for the growth curve (Figure 9) and a calculation to determine the desired OD600 to correspond with $5.0 \times 10^5$ CFU/mL was performed using the best fit line for the plot of OD600 vs. CFU/mL (Figure 10). This final diluted stock solution at $5.0 \times 10^5$ CFU/mL was then dispensed at 50 µL per well to give $1.0 \times 10^5$ CFU/mL at final well volume, consistent with CLSI guidelines63.
Figure 10. Calibration curve for determining the colony forming units per milliliter of solution (CFU/mL) at an optical density at 600 nm.

A more quantitative approach was taken to calculate for the CFU/mL of the solution at the onset of the log phase of the bacteria after 2.5 hrs. The solution was diluted to fall within the range recommended by the CLSI. The absorbance corresponding to the desired CFU/mL was calculated using the equation $x \text{ (in CFU/mL)} = \frac{(y + 2.09 \times 10^{-2})}{2.60 \times 10^{-9}}$ where $y$ is the OD600 of the stock solution prepared to fall in the range of 0.08 to 0.132. From this stock solution, a $5.0 \times 10^5$ CFU/mL solution was prepared using the dilution equation.
The timing for the end of the 24-incubation period for the bacteria on the second day plus the 2.5 hrs growth to the log phase on the third day was synchronized with the completion of the loading of the wells of the microtiter plate, also on the third day. Depending on the number of samples and concentration to be tested, at least 3 hrs prior to the end of the 24-hr incubation time was allotted for the preparation of solutions for plating. Berberine stock solution was prepared by dissolving commercially available berberine chloride (Sigma) in warm Müeller-Hinton broth at an initial concentration of 1.0 mg/mL. The commercially available berberine chloride (Sigma) was somewhat difficult to dissolve in Müeller-Hinton broth at 1.0 mg/mL. The solubility was improved by dissolving berberine in warm broth and using the sonicator (Fisher Scientific) to help solubilize the alkaloid. The solution was sterile-filtered using a tuberculin syringe fitted with a 0.22 μm pore nylon cartridge filter (Fisher) and collected in a wide-mouthed 20-mL sterile vial. A series of seven two-fold dilutions in broth was performed, which resulted in berberine solutions of varying concentration. The seven stock berberine concentrations from lowest to highest were 7.81, 15.6, 31.33, 62.5, 125, 250, 500 µg/mL. The berberine solutions and the test samples (fractions 1, 2, 3, etc…) were diluted into the plate according to the schemed shown in the photo below:
Berberine was dispensed into the well in combination with all test solutions (fraction 1 (F1), fraction 2 (F2), etc.) including reserpine (75 µg/mL at the final well volume), a known efflux pump inhibitor. The concentration of berberine remained the same across the plate on the same row and the concentration increased down each row. The berberine concentration down the plate, starting at the topmost row, was at (A) 0, (B) 4.6969, (C) 9.3838, (D) 18.88, (E) 37.5, (F) 75.0, (G) 150 and (H) 300 µg/mL after dilution to final well volume of 250 µL. Each well contained 150 µL of the corresponding stock berberine concentration, i.e., wells at Row B were loaded with 150 µL of 7.81 µg/mL stock berberine to give a 4.69 µg/mL berberine at the final well volume of 250 µL.
The concentration of berberine dispensed into the plate increases from zero to 500 µg/mL going down the plate but the concentration dispensed was the same across the plate for each corresponding row. A single concentration of the test sample (fraction, sub-fraction, reserpine or flavonoids) was allotted three columns (e.g. Columns 1 to 3, Rows A to H, 8x3 wells), which allowed for a total of four test samples loaded per plate. Duplicate plates are prepared for each round of testing with one plate was without bacteria (blank) and the other plate loaded with bacteria (treated). Using the schematics as shown in Figure 11, the “berberine” only wells at Row A, Columns 1 to 3 were at zero berberine concentration, which contained 200 µL broth and 50 µL of 10% DMSO (blank) or 150 µL broth, 50 µL of 10% DMSO and 50 µL bacteria stock solution at 5.0 × 10^5 CFU/mL (treated). For the wells at Rows B to H of the same “berberine” column, there was 150 µL of the alkaloid, 50 µL of 10% DMSO with 50 µL broth (untreated) or with 50 µL bacteria (treated). The other wells across Row A, Columns 4 to 12 loaded with the fractions (F1, F2, F3…etc.) were also at zero berberine concentration hence contained 50 µL of fraction (or sub-fraction) at 10% DMSO stock concentration (2% DMSO assay concentration) with 200 µL broth (untreated) or with 150 µL broth, and 50 µL bacteria (treated). For wells, which contained a combination of berberine and each fraction, the blank plate (Rows B to H, Columns 1 to 12) contained in each well 150 µL berberine, 50 µL of the fraction (or sub-fraction) in 10% DMSO with 50 µL of broth (blank) or with 50 µL bacterial solution (treated). Each well had a final volume of 250 µL and a final concentration of 2% DMSO.
CHAPTER IV

RESULTS AND DISCUSSION

1. A known flavonoid, 8-desmethyl-sideroxylin, isolated from goldenseal leaves enhances the activity of the alkaloid berberine

1.1. Quantitative LC-MS profiling correlates the activity of fraction 4 (F4) and sub-fraction 2 (sF2) with the presence of three (3) flavonoids, 6-desmethyl-sideroxylin, 8-desmethyl-sideroxylin and sideroxylin

It has already been established that goldenseal (*Hydrastis canadensis*, L.) has antibacterial activity against several strains of microorganisms due to the presence of alkaloids. Of all the alkaloids isolated from goldenseal, the three most abundant are berberine, canadine and β-hydrastine. Of these alkaloids, it is berberine that is generally considered responsible for all the observed bioactivity of goldenseal, which ranges from antibacterial to anticancer. However, in the emergence of resistant bacterial strains, some of which express the multi-drug resistance (MDR) pumps, berberine cannot account for all of the observed bioactivity. This is because berberine, being a quaternary ammonium compound, is a substrate of these MDR pumps, i.e., berberine only accumulates inside the bacterial cells at low levels because it is effectively effluxed by the MDR pumps. The published results on the activity of goldenseal against MDR pump-expressing cells of bacterial, fungal and mammalian origin suggested the presence of yet to be identified constituents that play a partial role in the activity of this plant. These yet to be identified
constituents could possibly interact with the efflux pumps, thereby rendering berberine effective against efflux-pump expressing bacteria strains. It is therefore the goal of this research to identify and isolate these constituents in goldenseal and to develop a method that would allow the discovery of such compounds given the constraint that berberine is a substrate of MDR pumps.

Goldenseal leaves were collected and used as plant samples instead of the rhizomes for this study. Most of the published work on goldenseal used either commercially available powdered rhizomes or rhizomes obtained from a particular supplier for content analysis, method development and bioactivity testing of the plant. For this research, the leaves were used instead of the rhizomes because of the following reasons: (1) since one of the goals of this study was to look for constituents other than the alkaloids or any of the already identified constituents from goldenseal, using the leaves would probably yield new compounds, which have not been reported yet in the literature since this source is least investigated; and (2) harvesting mature rhizomes would mean killing the entire plant; since goldenseal is considered as a threatened species due to overharvesting, it would be best to use the leaves. The leaf extracts, fractions and sub-fractions were tested for their potential synergistic activity using a combination assay. The enhancement in the antibacterial activity of the test samples was evaluated by the change in the minimum inhibitory concentration (MIC) of berberine. This was done through co-administration of known concentrations of berberine with a single concentration of each of the test samples against wild type Staphylococcus aureus. The combination, which resulted in the reduction of the MIC of berberine, was designated to
have either synergistic or additive effect. Preliminary experiments on the leaf extracts, its fractions and sub-fractions yielded results indicating additive or synergistic effect. In a separate experiment, leaf extract was also found to be more active than the rhizome extract in combination with berberine against wild type *S. aureus* using the checkerboard assay. As we have shown in an earlier published work, the FIC index calculated for the combination of crude goldenseal leaf extract with berberine was 0.19, which indicated that the observed activity was due to synergy.35

The extraction scheme that was employed starting with dried *H. canadensis* leaves is shown in Figure 8 (Materials and Methods, Section 2). The leaves were air-dried before processing to remove moisture. The presence of moisture in the extracts will encourage mold growth, which would compromise the samples. The air-dried leaves were processed for extraction by homogenizing with a coffee grinder to physically break down the cell wall of the leaves and then soaked in methanol, which is capable of penetrating the cell walls to extract both polar and non-polar constituents from the organelles. The methanol extract was dried in vacuo and subjected to partitioning with solvents of different polarities as a broad-first pass method of separation. Fats, oils and other non-polar compounds were extracted with the hexane layer. Polar compounds including some proteins and pigments and hydrosoluble tannins were extracted with water. A chloroform-water combination was used to extract all other constituents, which would include pigments, saponins and polyphenols. Solvent partitioning is a broad way of separating compounds in the complex plant leaf extract. This is largely based on polarity. In this sense, the distribution of polar and non-polar compounds into each
The tannin-free organic extract had a dark, tar-like appearance due to the presence of the leaf pigments with the other components. To reduce the complexity of this extract, a separation process was undertaken in which the first stage was normal phase flash chromatography (Materials and Methods, Section 3). There are 11 fractions collected for the first stage chromatographic separation after eluates were pooled based on the UV profile for the entire run. Supporting information from thin-layer chromatography profile was also used as basis for pooling. The pooled fractions were tested using the combination assay with varying concentrations of berberine against wild type *S. aureus*. Since the interest of this study was not in the activity of berberine by itself but rather the combined activity of other *H. canadensis* constituents with berberine, a microdilution experiment (Materials and Methods, Section 7.2) was devised where known concentrations of berberine were combined with a single concentration of each of the fractions. The minimum inhibitory concentration (MIC) of berberine was established to be 75 to 150 µg/mL against the wild type *S. aureus* in this microdilution assay. The
result of the combination assay is as shown in Figure 12. Based on Figure 12, all of the fractions displayed better antibacterial activity (lower MIC) in combination with berberine than was observed for berberine alone. The combination of fraction 4 and 4.7 µg/mL of berberine showed the greatest inhibition of bacterial growth as displayed by the lowest optical density at 600 nm (OD600). The MIC of berberine with fraction 4 is 15 times less than the MIC of berberine alone at 75 µg/mL. Berberine combinations with fraction 5 and 6 also showed reduced MIC of berberine at 9.4 µg/ml. Since fraction 4 showed the best inhibitory activity against S. aureus in combination with berberine, it was prioritized and forwarded to the next stage of separation. The second stage of separation (Materials and Methods, Section 3) of F4 resulted in eight (8) sub-fractions. As with the previous stage of the separation, the sub-fractions were tested for antibacterial activity in combination with berberine against the wild type S. aureus. The result of the combination assay is as shown in Figure 13. Based on Figure 13, all of the sub-fractions displayed better antibacterial activity (lower MIC) in combination with berberine than was observed for berberine alone. The combination of sub-fraction 2 and sub-fraction 8 with 9.4 µg/mL berberine showed greater inhibition of bacterial growth as again displayed by the decrease OD600.
Figure 12. Minimum inhibitory concentration of berberine in combination with each of the fractions.

Each fraction was added at a constant concentration (75 µg/mL) to a series of assay wells containing a range of berberine concentrations (4.7 to 300 µg/mL). The MIC of berberine was measured alone (berb) and in combination with each of the fractions (F1-F11) and the starting material (SM), the tannin-free organic extract from which the fractions were derived. Berberine combined with fraction 4 (F4) demonstrated the lowest MIC (4.7 µg/mL), which is 15 times lower than the MIC of berberine by itself (75 µg/mL). Fraction 5, 6, and the starting material (F5, F6, and SM) also decreased the MIC of berberine (MIC at 9.4 µg/mL). Fraction 10 had the highest berberine concentration but did not demonstrate the lowest MIC value, indicating that the activity of the crude extract is a result of more than just berberine. Each treatment was done in triplicate wells.
Figure 13. Minimum inhibitory concentration of berberine in combination with each of the sub-fractions.

Each sub-fraction was added at a constant concentration (75 µg/mL) to a series of assay wells containing a range of berberine concentrations (4.7 to 300 µg/mL). The MIC of berberine (berb) was measured alone and in combination with each of the sub-fractions (sF1-F8), fraction 4 from which the sub-fractions were derived, and the starting material (SM), the tannin-free organic extract from which fraction 4 was derived. Berberine combined with sub-fractions 2 (sF2) and 8 (sF8) demonstrated the lowest MIC (9.4 µg/mL), which is 16 times lower than the MIC of berberine by itself (150 µg/mL).
In order to explain the results of the microdilution assay, it is important to identify the compounds present in the *H. canadensis* fractions and sub-fractions, which are responsible for the observed activity. Furthermore, it is also important to correlate the concentration of these constituents in the fractions and the sub-fractions with the MIC values obtained from microdilution assay. A powerful technique to look at compounds present in complex mixture such as leaf fractions is liquid chromatography with mass spectrometry (LC-MS). In LC-MS, the complex mixture is de-convoluted through solvent gradient separation via LC, and each simplified component is scanned to identify the mass (via the mass-to-charge ratio) of the compounds present through MS. In order to construct a constituent profile, each of the fractions and sub-fractions was analyzed with LC-MS with ion trap mass analyzer (Materials and Methods, Section 4) using electrospray ionization both in the positive and negative ionization mode. The positive ionization mode is ideal for alkaloids, which contain the nitrogen in the isoquinoline ring that can be easily protonated to produce the positively charged species. In the case of alkaloid berberine, it is a naturally occurring quaternary ammonium compound in solution, which makes the positive mode ideal for its detection. The mass to charge ratio (m/z) of the berberine cation (M⁺) is 336.1, while both canadine and β-hydrastine undergo protonation resulting in MH⁺ ions with m/z of 340.1 and 384.0, respectively. The base peak chromatogram of 3.92 \times 10^{-6} \ M standard alkaloid mixture and the mass spectrum of each alkaloid are shown in Figure 14. Unlike the positive ionization mode, which was ideal for basic compounds that are easily protonated or for compounds that have inherent positive charge in solution, the negative ionization mode would be suitable.
for compounds, which naturally exist with negative charge or, which have acidic hydrogen that can be easily deprotonated. This mode of ionization allowed for the identification of other compounds in the fractions and sub-fractions, which most likely does not include alkaloids. By using the negative ionization mode, the search for compounds in H. canadensis is expanded to acidic compounds as well. It is also likely that the acidic compounds detected in this mode will be new in goldenseal since previous studies focused on alkaloids, which are detected in the positive mode.

Sample analysis in the positive mode was utilized to identify and quantify the presence of berberine, canadine and β-hydrastine in the fractions and the sub-fractions. The presence of the alkaloids can be easily correlated with the MIC values since berberine is reported to have an MIC range of 120 to 240 µg/mL\textsuperscript{29,62} against wild type S. aureus; canadine is only mildly active compared with berberine, and β-hydrastine\textsuperscript{32} has no antibacterial activity at all. However, our earlier work showed that both canadine and β-hydrastine had no antibacterial activity (MIC > 300 µg/mL)\textsuperscript{35} in the microdilution assay. Each of the alkaloid was quantified in order to determine if each was present in inhibitory concentration. The results for the constituent profiling in the positive ionization mode for the fractions and sub-fractions are shown in Figure 15 and 16 respectively. Based on the quantitative results in Figure 15, the alkaloid β-hydrastine was found to be more abundant than berberine in the leaf fractions. Berberine was found in the later fractions; with fraction 10 (F10) having the most abundant berberine content while β-hydrastine and canadine were highest in F3. It would make sense since berberine is naturally occurring as a quaternary nitrogen compound, which would be eluted with a
more polar solvent since it has the highest affinity with the polar silica stationary phase used for the first stage separation. Fraction 3 (F3) had the highest abundance of both canadine and β-hydrastine relative to other fractions as shown in Figure 15. For the sub-fractions, only β-hydrastine was detected at a significant level (Figure 16). Berberine was not detected in the sub-fractions and canadine was only detected in sub-fraction 2 (sF2) albeit at low level (< 0.1 %).
Figure 14. Base peak chromatogram of $3.25 \times 10^{-5}$ M standard alkaloid mixture and the mass spectrum of each alkaloid in the positive ionization mode.
Figure 15. Quantitative analysis of alkaloids in the fractions.

Each fraction was analyzed for berberine, canadine and β-hydrastine content using calibration curve from a standard alkaloid mixture. Fraction 3 (F3) contained the most abundant canadine and β-hydrastine and fraction 10 (F10) contained the highest berberine concentration. Each fraction was analyzed at 1.0 mg/mL and at 10, 100, 1,000 and 10,000-fold dilution of this initial concentration. Reported concentrations are based on the dilutions that fell within the linear range of the calibration curve. Fraction 4 (F4) showed the best MIC value in combination with berberine.
Of the three alkaloids, only hydrastine was detected at significant levels in the sub-fractions. It was most abundant in the latter sub-fractions, sub-fraction 5 (sF5), sub-fraction 6 (sF6) and sub-fraction 7 (sF7). Although these sub-fractions had the highest β-hydrastine content, sub-fraction 2 (sF2) and sub-fraction 8 (sF8) showed the lowest MIC in combination with berberine (Figure 18), suggesting that β-hydrastine was not responsible for the activity of the fractions.
If the antibacterial activity of each of the fractions and sub-fractions is solely based on the presence of alkaloids, then we can make predictions on the outcome of the microdilution assay based on the quantitative information provided by Figures 13 and 14. We can expect that only fraction 10 (F10) would show antibacterial activity alone against wild type S. aureus since it contained inhibitory concentration of berberine. Fraction 3 (F3) would be ineffective against the bacteria since its most abundant alkaloid is β-hydrastine. Based on Figure 17, F3 displayed no inhibitory activity by itself. The same is true for fraction 4 and the starting material (F4 and SM), both of which contained significant levels of β-hydrastine. These results clearly showed that β-hydrastine does not possess antibacterial properties. Fractions 10 and 11 (F10 and F11) both displayed slight inhibitory activity relative to the control, which can be attributed to the presence of berberine. By identifying and quantifying the alkaloids in each fractions, the following salient points were established: (1) the alkaloids berberine, canadine and β-hydrastine were indeed present in the leaves of goldenseal at detectable concentration, with β-hydrastine as the most abundant alkaloid; (2) based on the concentration of the alkaloids, the fractions most likely to have direct antimicrobial activity against S. aureus (Fractions 10 and 11), which contain berberine could be identified.
Figure 17. Direct antibacterial activity of the fractions against wild type *S. aureus*.

Each of the fractions was incubated with *S. aureus* at 75 µg/mL final well concentration. The optical density of the bacteria at 2% DMSO in Müeller-Hinton broth was used as the solvent control (CTRL). Fraction 10 and 11 (F10 and F11) showed significant change in the OD600, albeit borderline compared with the solvent control. As expected, fraction 3 (F3) did not show inhibitory activity. The rest of the fractions, including the starting material (SM) showed no inhibitory activity relative to the control. Error bars represent standard deviation of the optical density of triplicate wells.
Based on the premise that the alkaloids are solely responsible for the antibacterial activity of goldenseal, the result of the combination assay can be preempted using the quantitative information shown in Figures 15 and 16. Since known concentrations of berberine are added to a single concentration of the fraction, then the activity can be extrapolated based on the final berberine concentration in the combined solution. Again, based on Figure 15, fractions 10 and 11 (F10 and F11) would likely be the most effective in arresting the growth of the bacteria in combination with berberine. Due to the inherent inhibitory concentration of berberine in the fraction (F10 and F11), less concentration of the berberine added is needed to inhibit the growth of the bacteria in the wells. But these assumptions are not supported by the result of the microdilution assay as shown in Figure 12. All of the fractions displayed better inhibitory activity relative to pure berberine. Except for fractions 10 and 11 (F10 and F11), all the fractions have sub-inhibitory concentrations of berberine. Fraction 4 (F4) in combination with 4.7 µg/mL berberine was 16 times better than berberine by itself. Moreover, β-hydrastine, its most abundant alkaloid, cannot account for this activity since it was already established that β-hydrastine has no antibacterial activity. Fractions 5, 6 and the starting material (F5, F6 and SM) also displayed significant inhibitory activity in combination with 9.4 µg/mL berberine. Like F4, the alkaloid contents of F5, F6 and SM could not account for the observed MIC values. Furthermore, the MIC values do not reflect a combined effect of the internal and the external berberine added but rather showed an enhancement effect.

The second stage chromatography of fraction 4 (Materials and Methods, Section 3) enabled the separation of the alkaloids berberine and canadine. Only β-hydrastine was
detected in the sub-fractions at significant levels as shown in Figure 16. The direct antibacterial activity of the sub-fractions is shown in Figure 18. Based on Figure 18, sub-fraction 2 (sF2) displayed antibacterial activity by itself as shown by the reduction in the OD600 by 50% compared with the solvent control (CTRL). The other fractions, including fraction 4 and the starting material (F4 and SM) showed no intrinsic antibacterial activity. Again, β-hydrastine cannot account for the observed activity of sF2 because if β-hydrastine was responsible, then sF5 should be the most active since it contains the highest level of β-hydrastine based on Figure 16. The result of the microdilution assay for the combination of the sub-fractions and the berberine in Figure 13 cannot be correlated to the presence of the alkaloids as well. Since the active compounds berberine and canadine are not found in the sub-fractions (except for the sub-inhibitory concentration of canadine in sF2), then the MIC values should be dependent only on the added external berberine, i.e., the MIC values of the combination should be the same MIC as that of berberine alone. However, as shown in Figure 13, this supposition does not match with the experimental result. Sub-fractions 2, 8 and fraction 4 (sF2, sF8 and F4) showed the best activity in combination with only 9.4 µg/mL berberine. Although this is a two-fold increase in the MIC of F4 in Figure 12 (where sF2 and sF8 were derived, and the same F4 tested), it is still 16 times better compared with the MIC of berberine by itself as shown in Figure 13. The two-fold variation in the MIC values is common since the MICs are derived from 2-fold dilutions. Again, the MIC values of sF2 and sF8 do not correlate with the levels of alkaloids. These results
underscore the enhancement effects of sF2 and sF8 on the activity of berberine as well as F4 from which the sub-fractions were derived.
Figure 18. Direct antibacterial activity of the sub-fractions against wild type *S. aureus*.

Each of the sub-fractions was incubated with *S. aureus* at 75 µg/mL final well concentration. The optical density of the bacteria at 2% DMSO in Müller-Hinton broth was used as the solvent control (CTRL). Sub-fraction 2 (sF2) showed significant change in the OD600 compared with the solvent control. The alkaloid levels in sF2 do not correlate with observed activity. The rest of the sub-fractions, including the starting material and fraction 4 (SM and F4) showed no inhibitory activity relative to the control. Error bars represent standard deviation of the optical density of triplicate wells.
The result of the microdilution assay for the fraction (Figure 12) and sub-fraction (Figure 13) showed that there is no direct correlation with the presence of the alkaloids and the MIC values as shown in Figures 13 and 14, respectively. In fact, the MIC values of fractions 4, 5, and 6 (F4, F5 and F6; Figure 12) and sub-fractions 2 and 8 (sF2 and sF8; Figure 13) showed enhancement in the activity of berberine as displayed by lower MIC values compared with the MIC of berberine by itself. It is considered as enhancement effect since berberine is present at sub-inhibitory concentrations in F4, F5 and F6, and was not detected in sF2 and sF8, and yet the samples showed significant inhibitory activity at concentrations that are 16 times (F4, sF2 and sF8) and 8 times (F5 and F6) less than the known inhibitory concentration of berberine alone. These results suggest the presence of other constituents, likely present at highest concentration in F4, F5, F6, sF2 and sF8, which enhance the antibacterial activity of berberine in an additive or synergistic fashion.

Figure 19 shows the base peak chromatogram of the starting material (SM), fractions 4, 5 and 6 (F4, F5 and F6) in the positive ionization mode. It is shown in this figure that the alkaloids are the most abundant compounds, which can be detected in this mode. Meanwhile, Figure 20 shows the base peak chromatogram for the same samples in the negative ionization mode. The compounds detected in this mode seem to be different from those detected in the positive mode. Comparison of the two chromatograms shows that there are additional compounds, which can be detected in the negative mode. These additional compounds in Figure 20 are likely to be acidic compounds, which could have
not been reported as a constituent of goldenseal since previous studies focused on the presence of alkaloids.
Figure 19. Base peak chromatogram for the starting material (SM), fractions 4, 5 and 6 (F4, F5 and F6) using positive ionization mode.

SM is the tannin-free crude organic extract from which F4, F5 and F6 were derived. F4 was 15 times better while F5 and F6 were 8 times better in combination berberine compared with berberine alone against the bacteria. The most abundant compounds detected in these samples in the positive ionization mode are the alkaloids with β-hydrastine (m/z 384.0), berberine (m/z 336.1) and canadine (unlabeled; m/z 340.1).
Figure 20. Base peak chromatogram for the starting material (SM), fractions 4, 5 and 6 (F4, F5 and F6) using negative ionization mode.

The compounds detected in the negative ionization mode were different from those detected in the positive mode (Figure 19) for the SM and the fractions as shown by the different peaks observed in the negative ionization mode relative to the positive ionization mode.
Figure 21. Base peak chromatogram and selected ion chromatogram (m/z 297.1 and 311. 1) for fraction 4 (F4, top panel) and sub-fraction 2 (sF2, bottom panel) in the negative ionization mode.

Difference in the retention time of the peaks was due to pressure variation from one analysis to the other. The identities of the peaks were confirmed by the fragmentation pattern of the ions. Orbitrap mass analyses of F4 and sF2 verified that the peaks in F4 and sF2 are the same. The compound with m/z 297.1 is a mixture of two flavonoids, 6-desmethyl-sideroxylin (23) and 8-desmethyl-sideroxylin (24) that coeluted with the C-18 column. The other flavonoid, sideroxylin, has m/z 311.1 (25).
Two compounds with mass to charge ratio (m/z) of 297.1 and 311.1 were detected to be present in fraction 4 (F4) and sub-fraction 2 (sF2) (Figure 21). The compounds with m/z 297.1 and 311.1 were isolated and purified through a series of steps that will be discussed later (Results and Discussion, Section 2). The peak corresponding to the ion with m/z 297.1 was determined to be a mixture of two (2) structural isomers, 8-desmethyl-sideroxylin and 6-desmethyl-sideroxylin, while the ion with m/z 311.1 was sideroxylin (Figure 22). All of these compounds are flavonoids.

The concentration of flavonoids in all the fractions and sub-fractions was quantified by constructing calibration curves from standards obtained in the isolation process. The chromatogram for the pure flavonoids are shown in Figure 23. Since the structural isomers with m/z 297.1 co-eluted during the LC-MS analysis (Figure 21), the isomers were quantified as a mixture and not as individual constituents in the fractions (Figure 24). Figure 24 showed that both the desmethyl-sideroxylin mixture (23 and 24) and sideroxylin (25) were most abundant in fraction 4 (F4), which also exhibited the lowest MIC value in combination with berberine as indicated in Figure 12. The flavonoids identified here have not been published as constituents of goldenseal, but were previously identified from two different plant species, 24 and 25 from *Eucalyptus* spp. 66, 67 and 23 (which was referred to as 4’, 5-dihydroxy-7-methoxy-8-methylflavone) from *Dracaena cochinchinensis*. 68
Figure 22. New flavonoids isolated from goldenseal.

The base peak with m/z 297.1 corresponds to two isomeric flavonoids, 6-desmethyl-sideroxylin (23) and 8-desmethyldesideroxylin (24), which co-eluted with the Prevail C-18 column used in the LC-MS. The isomers were quantified as a mixture. A Luna PFP column was able to resolve the isomers (Results and Discussion, Part III). Compound 23 was the minor constituent while 24 was the major constituent of the mixture. The flavonoids sideroxylin with m/z 311.1 was resolved from the isomers with the Prevail C-18 column.
Interestingly, as shown in Figure 24, fraction 5 contained less than 0.010% of compound 23, but none of the flavonoids were detected in fractions 6. However, both fractions 5 and 6 displayed significant activity in combination with berberine (Figure 12). As shown in Figure 25, there are other compounds, which were observed in the chromatograms for fractions 5 and 6 that were not present in the other fractions, including fraction 4. It is, therefore, likely that the activity observed for fraction 4 is due to compounds different from those present in fractions 5 and 6. These compounds have yet to be identified, and are considered as future work for this project. Fraction 4 was prioritized because of the correlation between activity and concentration of the flavonoids. It was moved to the next stage of separation because it showed the best antibacterial activity in combination with berberine as evident from lowest MIC relative to the other fractions from the first stage of separation (Figure 12).
Figure 23. Base peak chromatogram of the purified flavonoids.

After a series of chromatography, the flavonoids were isolated and analyzed with LC-MS. The retention time of the isomeric 23 and 24 were almost identical with the Prevail C-18 column. The flavonoid 25 was easily resolved from the isomers.
Figure 24. Quantitative analysis of flavonoids in the fractions.

All three flavonoids (23, 24 and 25) were present at the highest abundance in fraction 4, which also exhibited the lowest berberine MIC against the wild type *S. aureus*. Isomers 23 and 24 were quantified as a mixture because they co-eluted with the Prevail C-18 column used for LC-MS analysis. Based on the quantification result, the flavonoids in goldenseal are present at low concentration compared with the alkaloids.
Figure 25. Unknown constituents in the fractions 5 and 6 (F5 and F6) detected in the positive and negative mode.

There were other peaks detected in the fractions but only the compounds with m/z 397.89 (positive ion mode) and 313.0 (negative ion mode) showed correlation with the antibacterial activity of F5 and F6 (Figure 12). No compounds have previously been reported in goldenseal with masses that correlate with the detected m/z values.
Based on the result of the quantification of the flavonoids in the sub-fractions (Figure 26), sub-fraction 2 (sF2) had the highest flavonoids concentration while none were detected in sub-fraction 8 (sF8). Both sub-fractions had the best MIC value as seen in Figure 13 and both contained none of the known alkaloids as well. Thus, it appeared that the activities of these sub-fractions are due to different compounds. It seems likely that the activity of sF2 was due to the flavonoids. Meanwhile, the presence of other ions in sF8 was investigated to rationalize the activity of this sub-fraction. Only one compound with m/z 578 detected in the positive mode was observed to correlate with the activity of sub-fraction 8 (data not shown). However, it is not possible to conclude with certainty that this compound is responsible for the activity of the fractions 4, 5 and 6. It is also possible that sub-fraction 8 contains active constituents that are not detectable by the LC-MS analysis employed. Further purification and quantification are needed in order to investigate the potential role of the ions with m/z 578 in the activity of fractions 4, 5, and 6. Investigation of the constituents responsible for the activity of sub-fraction 8 is considered as future work. Subfraction 2 was advanced to the next stage of separation because it was one of the two most active fractions.

Subfraction F2 (sF2) was subjected to third stage separation (Materials and Methods, Section 3), which resulted into six (6) semi-pure isolates. These included the mixture of 23 and 24, and compound 25. At this stage, no alkaloids were detected in the semi-pure isolates. A fourth stage separation using Luna-PFP® (pentafluorophenylpropyl) column was done to separate the mixture into 23 as the
Flavonoids 24 and 25 were assayed against the wild type S. aureus both alone and in combination with berberine as was done for the fractions and sub-fractions. Flavonoid 23 was not tested because there was not enough sample available. The results showed that the growth of the wild type strain was not inhibited by the presence of either 24 (Figure 27) or 25 (Figure 28) alone. This was shown by the absence of a notable decrease in OD600 up to 300 µg/mL of flavonoid concentration. This showed that each of the flavonoids tested alone does not have any direct antibacterial activity. Comparing Figures 24 and 25, flavonoid 24 exhibited improved antibacterial activity in combination with berberine (decrease in MIC from 150 µg/mL to 75 µg/mL), while sideroxylin did not elicit any improvement in the MIC relative to the activity of berberine by itself. Berberine alone has an MIC of 150 µg/mL, and in combination with reserpine, a known efflux pump inhibitor, berberine MIC is at 37.5 µg/mL as shown in Figure 27. These results lend merit to the effectiveness of the new approach to bioassay-guided isolation based on the following points: First, constituent profiling using HPLC-ESI mass spectrometry allowed for the detection of an array of ionizable compounds in the complex fractions. These could then be easily quantitatively analyzed, also using HPLC-ESI mass spectrometry to correlate their presence with observed bioactivity. Second, setting-up the microdilution assay as a combination experiment for the antibacterial activity allowed for the discovery of 8-desmethyl-sideroxylin, which do not have bioactivity by itself but works to enhance the
activity of berberine. A conventional antibacterial assay designed to directly associate bacterial growth inhibition with an active compound would have missed this compound. The modified approach to bioactivity-guided fractionation enabled the identification of two compounds that work synergistically even when one compound is inactive by itself.
Figure 26. Quantitative analysis of flavonoids of sub-fractions derived from fraction 4.

All three flavonoids were most abundantly found in sub-fraction 2 (sF2). The presence of the flavonoids in sub-fraction 8 (sF8) did not show correlation with the activity of the sub-fraction in combination with berberine. There could be other compounds present in sF8 other than the flavonoids responsible for the observed reduction in the berberine MIC (Figure 13).
Figure 27. Broth microdilution assay with 8-desmethyl-sideroxylin (24).

The flavonoid showed no activity against wild type *S. aureus* even at the highest concentration tested (300 µg/mL). The combination of 60 µg/mL 24 with berberine showed complete inhibition at 75 µg/mL of berberine, a 2-fold decrease in MIC compared to berberine alone. Reserpine, the positive control at 50 µg/mL final well concentration, showed complete inhibition in combination with 37.5 µg/mL berberine.
Figure 28. Broth microdilution assay with sideroxylin (25).

The flavonoid showed no activity against wild type *S. aureus* even at the highest concentration tested at 300 µg/mL. The combination of 60 µg/mL 25 with berberine showed inhibition at 150 µg/mL, which was identical to the MIC of berberine by itself. Reserpine showed complete inhibition at 37.5 µg/mL, which is 3 times less than berberine MIC.
1.2. Improved solubility of reserpine and 8-desmethyl-sideroxlin in DMSO-broth solution resulted in improved activity against the NorA overexpressed *S. aureus* strain (K2378)

It is to be pointed out that although there is an observed MIC for berberine in combination with 24, this MIC is greater than the MIC for the combination with fraction 4 and sub-fraction 2 from which the flavonoids were derived. However, the attenuation and, sometimes at worst, the total lack of activity of a purified compound is common in natural products.\textsuperscript{37, 54, 69} Figure 13 showed that the activity of F4 is not solely due to the presence of the flavonoids since all the sub-fractions showed activity in combination with berberine. The presence of yet-to-be identified compounds with direct antibacterial activity in sub-fraction 1 (Figure 25) also contributed to the overall activity of fraction 4. Also, the actual process of simplifying the complexity of the fractions down to the sub-fraction up to a single compound breaks interaction among the constituents, which could be vital in the observed bioactivity. Such is the case for the reduced solubility of purified compounds including flavonoids 24 and 25. During the bioassay of the fractions and sub-fractions, samples were dissolved in 100% DMSO then diluted with Müeller-Hinton broth to achieve the desired stock solution with 2% DMSO at the final well volumes. The isolated flavonoids were easily solubilized by 100% DMSO but immediate formation of precipitate (“crashing out” of solution) was observed upon dilution with Müeller-Hinton broth. The flavonoids tested in Figure 28 and 29 were reported to be at a 60 µg/mL final assay concentration but the actual concentration of the flavonoids was definitely lower than 60 µg/mL. The
presence of flavonoid particulates clearly indicated the limitation in the bioavailability of the purified compounds.

Reserpine, a reported efflux pump inhibitor isolated from the plant *Rauwolfia serpentina*\(^70\), was used as the positive control because it is commercially available and relatively inexpensive. As with the flavonoids, precipitation upon mixing of the stock solution with broth was observed. Although this observation has never been actually reported in the literature, it has been mentioned through personal communications with a collaborator\(^71\) and was brought-up at discussions during presentations at conferences. Reserpine is reported as an effective efflux pump inhibitor against NorA at a concentration range of 20 to 25 µg/mL\(^72, 73, 74\.) Through the course of this work, reserpine was ineffective in combination with berberine against the bacteria, which could probably be due to the same solubility issues observed as mentioned above. Solubility issues resulted in varying MIC values of berberine in combination with reserpine with the microdilution assay. At best, 50 µg/mL of reserpine showed efflux pump inhibition, albeit inconsistently, to give a berberine MIC range of 37.5 to 75.0 µg/mL. This is just a two-fold decrease from the MIC range of berberine of 75 µg/mL to 150 µg/mL by itself against wild type *S. aureus* (Figures 27 and 28). Other solvents were tested in an attempt to improve the solubility of reserpine. Glycerol and broth were initially tested but barely dissolved reserpine. Other organic solvents such as acetonitrile, ethyl acetate and chloroform showed little to no effect on the solubility as well. The surfactant Tween 20 (Sigma) at 1, 2 and 5% in broth solubilized reserpine, but
greatly affected the growth of the bacteria in the solvent control wells (likely due to disruption of the bacterial cell wall of the surfactant). Reducing the percentage to 0.1 % in broth, Tween 20 was not able to solubilize reserpine as effectively thereby no lowering of MIC of berberine was observed. Based on these results, DMSO was still the best solvent of those tested even with the undesirable precipitation of reserpine when mixed with broth.

To minimize the precipitation of reserpine in DMSO-broth, and at the same time improve the MIC of reserpine-berberine combination, a two-prong approach was taken. The **first** is by spiking the reserpine into the wells at a higher concentration while delivering final well concentration of 20 µg/mL in 2% DMSO rather than diluting the stock solution prior to adding into the wells; and **second** is by using an *S. aureus* strain that expresses the NorA efflux pump to a greater extent than the wild type strain to improve the sensitivity of the microorganism to reserpine. Reserpine was tested using the new preparation against the NorA over-expressed strain (K2378). It was tested at 50 µg/mL final concentration to compare the effect of the new preparation (spiking reserpine at high concentration in 100% DMSO) versus the old preparation (diluting reserpine stock solution) against NorA overexpressed *S. aureus*. By visual inspection, the new preparation showed minimal precipitation in the wells prior to incubation. The result shown on Figure 29 showed that the new preparation of reserpine at 50 µg/mL was also better in combination with berberine with an MIC value of 75 µg/mL. For the over-expressed strain, berberine showed no MIC value even at the highest
concentration tested (300 µg/mL), which indicated that berberine was effectively effluxed out of the cell and did not accumulate at a toxic level. The result in Figure 29 clearly indicated that reserpine was able to potentiate the activity of berberine against the NorA over-expressed S. aureus strain by inhibiting the efflux pump. However, the precipitation was still observed using the new solution preparation method (spiking with high concentration reserpine), albeit to a lesser extent. The solubility experiment with spiking reserpine into the wells at 50 µg/mL (2% DMSO) final well concentration against the NorA overexpressed S. aureus as shown in Figure 29 was reproduced and provided the basis for preparing the flavonoids, which exhibited precipitation as well.
The effectiveness of reserpine as an efflux pump was tested using new preparation by spiking high concentration of reserpine dissolved at 100 % DMSO into the wells. The percent DMSO is maintained at 2% at the final well volume. The effect of spiking (new) versus diluting (old) reserpine (50 µg/mL final concentration) in combination with berberine was compared. The new preparation showed better inhibition than the old preparation with an MIC value of 75 µg/mL berberine.
In the succeeding microdilution assay for testing the flavonoids against K2378 using the new dilution method, reserpine was tested at 20 µg/mL instead of 50 µg/mL in accordance with its reported range of active concentration. This is also in the hope that the reduced concentration would result in reduced precipitation. A 5 µL minimum volume was set for spiking the concentrated reserpine because this is the smallest volume, which can be dispensed without compromising accuracy even if the repeater pipette was set to dispense as small as 1 µL solution. There were no immediate precipitation observed in the wells treated with reserpine prior to incubation and there was little to no precipitate in the wells after 18-hours incubation with the bacteria. Based on the MIC curves below in Figure 30 or 31, it takes 75 µg/mL berberine combined with 20 µg/mL reserpine to inhibit the growth of S. aureus with over-expressed NorA efflux pump. The result seemed counterintuitive because of the enhanced berberine activity at a reduced reserpine concentration. It is usually expected that increasing the concentration would result in increased activity. But lowering the reserpine concentration probably allowed for the 2% DMSO to keep the reserpine in solution even after dilution with the broth in the well. More of the reserpine interacted with the efflux pumps rather than crashing out of the solution to form the precipitate, which contributed to the optical density of the solution. Optimal solubility in the 2% DMSO in broth solution decreased precipitation and increased bioavailability of reserpine resulting in the overall increase activity, and decreased in the optical density in combination with berberine.
Using the conditions that were most effective for solubilizing reserpine, the flavonoids were then tested at three (3) concentrations of 5, 10 and 20 µg/mL. The lower concentrations were also tested in the event that the highest concentration would prove to be problematic in terms of solubility. And the results on Figures 26 and 27 showed that this was indeed the case. For both flavonoids, precipitation was observed in the wells combined with 20 µg/mL of each, albeit minimal. After 18-hr incubation with the bacteria, most of the wells, except solutions with 5 µg/mL of the flavonoids without the bacteria, showed precipitation. Wells containing 20 µg/mL flavonoids had the highest amount of precipitate. This was more evident for 25 compared to 24 as exhibited by the relatively higher optical density at zero berberine concentration. The change in the optical density of the wells untreated with bacteria compared with the wells treated with bacteria in the presence of the 25 was not just due to the growth of the bacteria but to the precipitate that increased the optical density significantly. Furthermore, the precipitation of the flavonoids in the wells also supported the precipitation of some of the berberine, which also exhibited low solubility in broth. This resulted in pronounce light scattering effect in the wells giving marked variability in the optical density of the wells, thus the huge error bars for the combined berberine and flavonoid 25 at 20 µg/mL curve. Background subtraction minimizes this effect but significant variation in the OD600 of the wells with the same flavonoid treatment were still observed. However, 25 still showed no activity against the NorA overexpressed strain as shown in Figure 30 at 10 and 5 µg/mL.
concentrations, which had lesser precipitation compared with the 20 µg/mL. The result for 24 was more promising in spite of the problem with its solubility as shown in Figure 31. Comparing the curves for the 20 and 10 µg/mL, it is apparent that 24 enhanced the activity of berberine. Although there is no MIC value displayed for berberine at 10 µg/mL of 24, the observed decreased in the optical density is a good indication of activity given the inherent limitation in solubility at this concentration. It is interesting to note that optimizing the flavonoid solubility could potentially make it at par or even better than reserpine as a synergist of berberine.
Figure 30. Broth microdilution assay with sideroxylin (25) against NorA over-expressed S. aureus.

The top panel is the result for 5 and 20 µg/mL of 25 with berberine. Bottom panel is the result for 10 µg/mL of 25. All concentrations did not show inhibitory activity. Low solubility of 25 resulted in the formation of precipitate in the wells. The precipitate caused well-pronounced light scattering effect as indicated by the large error bars shown in the first panel for the 20 µg/mL of 25. The combination of sideroxylin with increasing berberine concentration exhibited no synergistic action relative to berberine by itself.
Figure 31. Broth microdilution assay with 8-desmethyl-sideroxylin (24) against the NorA over-expressed S. aureus (K2378).

The top panel is the result for 5 and 20 µg/mL of 24 with berberine. Both concentrations did not show inhibitory activity. The lower panel is at 10 µg/mL of 24 with berberine, which displayed improvement of the berberine activity. Flavonoid 24 exhibited low solubility in DMSO-broth solution.
A new method, synergy-directed fractionation, is proposed as an alternative to bioactivity-directed fractionation

Testing for the bioactivity of berberine and flavonoid as separate constituent as well as testing the combination effect of the berberine-flavonoid mixture revealed the presence of compounds that potentiated the activity of berberine while not having activity themselves. This was displayed by the lowered MIC of the berberine–flavonoid mixture against wild type *S. aureus* relative to the MIC of berberine alone. The work accomplished above in purifying the most active fraction and sub-fraction down to three individual flavonoids guided by quantitative constituent profiling allowed for the identification of these minor constituents. However, there are still other constituents that have not been identified and quantified in the active fractions and sub-fractions that could contribute to the overall activity of the leaf extract either in synergistic or additive manner. It is not possible when testing at just a single concentration of each fraction or sub-fraction to distinguish synergistic from additive effects. For this reason, a synergy assay was conducted (the checkerboard assay, Review of Related Literature, Section 2.5), which involved varying both the concentration of berberine and the concentration of the crude leaf extract, fraction 4 and sub-fraction 2. The results of these experiments showed that the crude extract, fraction 4 and sub-fraction 2, indeed, have synergistic antimicrobial activity with berberine as shown by their FIC indexes of 0.19, 0.13, and 0.03, respectively.35, 75
The results above validates the need to take a multi-component approach as an option to the single-compound paradigm in studying as complex a material as plant extract in terms of its phytochemistry. Only by considering that there might be other constituents in *H. canadensis* responsible for its reported activity that a suitable experimental strategy was devised in order to investigate this assumption. This work has shown that quantitative constituent profiling should be an integral part of this strategy in order to correlate activity not only with the presence or absence of particular compounds in the complex plant mixture, but most importantly the concentration of the said compounds. It is hereby proposed that synergy-directed fractionation method as an alternative technique to study herbal plants. The work flow developed as a result of this research is as shown in Figure 32. In this fractionation scheme, the extracts collected from solvent-partitioning (Materials and Methods, Section 2) were analyzed using liquid chromatography-mass spectrometry (LC-MS) (Materials and Methods, Section 4). The collected extracts were then tested for their potential synergistic activity using a combination assay (Materials and Methods, Section 7.2). The extract that reduced the MIC of berberine in the combination assay was assumed to have either synergistic or additive activity, and moved forward to the first stage of separation. The resulting fractions were subjected to the same LC-MS analysis and synergy testing and the fraction with the most pronounced effect in combination with berberine was moved to the next stage of separation. This iterative process was performed until the suspected synergistic constituents were isolated and purified.
Techniques such as $^1$H and $^{13}$C NMR and high resolution mass spectrometry are used to identify the structure and the accurate mass of the pure samples.

The enhancement in the antibacterial activity of the samples was evaluated by the change in the minimum inhibitory concentration (MIC) of berberine. This was done through the co-administration of known concentrations of berberine with a single concentration of each of the test samples against the wild type Staphylococcus aureus. The combination, which resulted in the reduction of the MIC of berberine, was designated to have either synergistic or additive activity. To verify whether the observed effect was indeed synergistic and not additive, a synergy test was conducted using the checkerboard assay and an isobole was constructed to classify whether the effects are synergistic, additive or antagonistic (Review of Related Literature, Section 2.5). All synergy test results were correlated to the LC-MS analysis, which was used to identify and quantify the constituents present in the extracts, fractions and sub-fractions. The observed potentiation effect on the berberine antibacterial activity by the sideroxylin and its analogue flavonoids (23, 24 and 25) against S. aureus clearly showed that this method was effective in identifying synergists. This synergy effect was shown to be through the inhibition of the NorA efflux pumps of S. aureus as displayed by increased percent fluorescence of ethidium bromide inside the cells. Synergistic effect can also be attributed to better solubility of the more complex mixture versus the isolated compounds. The reduction in the solubility was concomitant with the fractionation process indicating that other compounds deemed as impurities
possibly helped with the solubility of the flavonoids in aqueous media. By being present in the mixture, compounds such as saponins or polyphenols acted synergistically by increasing the solubility of the flavonoids, thus resulting in increased bioavailability, and leading to greater interactions with the NorA efflux pumps.
Figure 32. Schematic diagram for synergy-directed fractionation.

This method is an iterative process of LC-MS analysis and synergy tests at each stage starting from the crude extract until the compounds with synergistic activities were identified, isolated and purified. A compound with no direct antibacterial activity present in goldenseal that is capable of enhancing the activity of berberine can be identified using this method by virtue of the combination assay. Compounds with direct antibacterial activity can also be identified as with this technique.
2. **Separation of 6-desmethyl-sideroxylin and 8-desmethyl sideroxylin using fluorinated solid stationary phase**

As a result of the third stage of separation, the flavonoids 8-desmethyl-sideroxylin (m/z 297.1) and sideroxylin (m/z 311.1) were obtained at 0.00064% and 0.00096% yield, respectively. Initially, both HPLC-PDA chromatograms and LC-MS spectra indicated that the desmethyl-sideroxylin isolate was a single compound with M-H⁻ m/z 297. However **Figure 33A** showed the presence of minor signals, which although small (<10%), were considered significant. Initially, the extra signals were thought to be due to other impurities. However, after re-isolation of more crystals, the extra signals were strong enough to be analyzed as the minor form of the compound with m/z 297 as seen by the pairing of major and minor peaks in the \( ^1H \) NMR spectrum (**Figure 33B**). The small differences in the chemical shifts between the paired peaks indicated a slight difference in the structure of the compounds. Analysis of major signals in the \( ^1H \) NMR showed the presence of aromatic rings, one of which is fused to a benzopyranone, typical for flavonoid-type structures. The positions of the substituents such as a methoxy, methyl and two hydroxyl groups were all determined by analysis of 2D NMR data and by comparison with published literature. Together with the mass spectroscopic data, the major compound was then identified as 8-desmethyl-sideroxylin. A plausible reason for the chromatogram peak as opposed to two peaks (one each for major and minor component), consistent with the NMR data would be that the two compounds co-eluted. It was hypothesized that isomers with
possibly one or two substituents attached at different positions to the fused rings would be likely to coelute and would have similar $^1$H NMR spectra. We proposed to separate the possible isomers using a Luna-PFP® column
Crystals collected from the third stage separation that showed a single peak in the HPLC-PDA trace and LC-MS trace (m/z 297) were analyzed using JEOL ECA-500 spectrometer (500 MHz) using DMSO-d$_6$. The spectra of the crystals showed the presence of peaks marked above (shown expanded in B) indicating the presence of another compound. The “impurity” was eventually identified as the minor flavonoid, 6-desmethyl-sideroxylin.
Luna-PFP® is an example of stationary phase with pentafluorophenylpropyl ligand attached to the silica base to facilitate selective interactions with the solute through hydrogen bonding, dipole-dipole, aromatic and π-π interactions, and hydrophobic interactions. This stationary phase can separate compounds that are not separated with the more common C-18 stationary-phase, which relies on hydrophobic interactions for separation. Although the precise structure of the Luna-PFP stationary phase is proprietary, the structure is based on the typical phenyl phase shown in Figure 34.

![Figure 34. PFP stationary phase diagram.](image)

The Luna-PFP® has an ultra-pure metal-free silica base, which is prepared with proprietary silica treatment and bonding to reduce silanol interactions. Typical phenyl phase vary the acidity of the silica depending on the metal-content, acid-washed or pure new generation silica. The bonding technology could be either monomeric or polymeric. The R-groups could be OH, Me or i-Pr. Spacer is typically C₀ to C₆, which depending on the number of carbons, imparts varying degrees of hydrophobic and π-π interaction with the solute. There could be a group X between the spacer and the phenyl ligand, which could either be a heteroatom or CH₂. The presence of the phenyl ligand imparts aromatic and hydrophobic characteristic to the phase. Some columns have two phenyl moieties attached.
The chromatogram shown in Figure 35, resulted from separation of the isolate with m/z 297 from the third stage of separation (corresponding to the NMR spectrum in Figure 33A) using the Luna-PFP® column. It is clear from this chromatogram (Figure 35) that the isolate was, indeed, a mixture of two compounds with the minor constituent (t_R = 17.92 min.) eluted earlier than the major constituent (t_R = 20.16 min.). The minor and the major compounds were later identified to be 6-desmethyl-sideroxylin (23) and 8-desmethyl-sideroxylin (24). The isomeric compounds differ by the absence of a methyl group at either position C6 (6-desmethyl) or C8 (8-desmethyl) on the A ring. Sideroxylin (25) has methyl groups at positions C6 and C8 (Figure 37). The Luna-PFP® at the same optimized gradient condition was also effective for separating a mixture of all of the three H. canadensis flavonoids (Figure 37) as shown in Figure 36.
Using Luna-PFP®, the mixture was resolved into two compounds, with 6-desmethyl-sideroxylin ($t_R = 17.92$ min.) being the minor constituent and 8-desmethyl-sideroxylin ($t_R = 20.16$ min.) the major constituent. The sample was analyzed at 1.0 mg/mL concentration.
Figure 36. Chromatogram for a semi-pure isolate containing the three flavonoids (23, 24 and 25) at 330 nm.

A semi-pure isolate analyzed containing the three flavonoids (23, 24 and 25) showed good baseline separation using the same gradient and column employed to obtain the separation shown in Figure 35. These results demonstrate the advantage of the pentafluorophenylpropyl phase over the conventional reversed-phase C-18 column for separating isomeric flavonoids.
Sideroxylin (25) was easily separated from the isomers at the third stage of separation using the YMC C-18 column. The extra methyl group at the C8 position made the molecule more hydrophobic relative to the other isomers, thereby increasing its interaction with the C-18 phase. Consequently, it was retained in the column longer than the other two isomers. However, the hydrophobicity of the regioisomers, 6-desmethyl-sideroxylin (23) and 8-desmethyl-sideroxylin (24) was so similar, that they co-eluted in the separation with C-18 stationary phase. With the PFP column, the highly electronegative fluorine enables hydrogen bonding, dipole-dipole and induced-dipole interactions with the -OH, -CH$_3$ and -OCH$_3$, respectively. In the case of desmethyl-sideroxylin isomers, the most significant interaction is the aromatic/π-π interaction, which is afforded by the presence of the phenyl moieties in the stationary phase and the solutes. The difference in the position of the methyl group on the A-ring among the various isomers is likely to cause them to have varying degrees of interaction with the delocalized electrons in the p-orbitals of the phenyl ring. The flavonoids 24 and 25 with their substituents close to one another as shown in Figure 37, were retained longer in the column. This would indicate a stronger interaction with the stationary phase. Flavonoid 23 was eluted first because it has weaker interaction with the stationary phase because its substituents were localized in two areas. Varying degrees of stacking interactions with the stationary phase could also result from differences in substituent positions. However, these assumptions are only based on the molecular structures and the retention times of the flavonoids and further studies to determine
the distribution coefficient and capacity factor of each of the flavonoids in order to explain their order of elution.

Figure 37. The flavonoids with corresponding substituent distribution.

Variation in the number of substituent and the arrangement in the A-ring of the flavonoid influenced retention times because of electrostatic interactions with the phenyl ligand of the column. The flavonoids are ordered according to their retention time, with the more substituted sideroxylin being retained longer.
3. HPLC-ESI MS & UPLC-ESI HRMS profiles and the fragmentation pattern of the pure flavonoids

The base peak chromatogram of fraction 4 obtained from low resolution LC-MS in the negative ionization mode using Prevail® C-18 column is shown in Figure 38. Molecular ions 297.1 and 311 are clearly the most abundant ions that can be detected in the negative mode. However, as mentioned earlier, the chromatogram only displayed a single peak for m/z 297, indicating that 23 and 24 co-eluted and were unresolved with the solvent gradient used in the LC separation (Figure 21 and 33 A). Attempts to optimize the LC-MS separation to separate the minor and the major desmethyl-sideroxylin isomers were unsuccessful. The flavonoids were thus quantified as a mixture using LC-MS.

The application of high-resolution mass spectrometer (HRMS) with orbitrap mass analyzer provided the opportunity to further profile the constituents present in the fractions and sub-fractions. HRMS gives the accurate mass (to ~ ± 0.0001) of each ion detected as it elutes from the LC. Corresponding molecular formulae can be obtained from these accurate mass measurements, which in turn can be used to search for the possible identity of the constituent in online databases. This information can be used to ascertain whether the compounds being analyzed are known compounds from goldenseal or from other plants or organisms. The accurate mass of each of the pure flavonoids was also obtained at 2.2, 2.5 and 2.6 parts per million (ppm) mass accuracy for flavonoid 23, 24 and 25, respectively. The mass accuracy calculations are based on the difference between
the theoretical mass and the measured mass of each ions, as shown in Figure 39. Additionally, our HRMS is coupled with ultra performance liquid chromatography (UPLC), which affords enhanced resolution as compared to conventional HPLC. The chromatogram of fraction 4 obtained with UPLC and HRMS is as shown in Figure 40. Compared with the analysis of the same fraction using HPLC coupled to low resolution MS (Figure 38), the UPLC-HRMS (Figure 40) was able to resolve the peaks corresponding to 23 (t_R=23.88 mins.) and 24 (t_R= 24.18 mins.) in the same mixture. The reason for this improvement in separation is the decrease in the particle size of the stationary phase of the column used from 3 µm (HPLC) to 1.7 µm (UPLC). Smaller particle size increases the number of theoretical plates (N), thereby improving resolution by the following equation (Equation 3):

\[
R_s = \left(\frac{N^{1/2}}{4}\right) \left(\frac{\alpha-1}{\alpha}\right) \left(\frac{k_2}{1+k_2}\right) \quad (\text{Equation 3}),
\]

where N is the theoretical number of plates, \(\alpha\) is the selectivity factor, and \(k\) is the capacity factor. An increased in the number of theoretical plates means increased interaction of the analyte with the stationary phase, as a consequence of greater stationary phase surface area. This increased interaction improves the efficiency of separation of the two compounds, resulting in better resolution between the two peaks as shown in the chromatogram in Figures 36.
Figure 38. Base peak chromatogram of fraction 4 in the negative ionization mode with low resolution LC-MS.

The column used was Prevail C18® with 3 µm particle size and 2.1 × 50 mm column dimension. The peak with m/z 297.1 is a mixture of flavonoids 23 and 24.
Figure 39. Mass spectra of the flavonoids with the orbital mass analyzer.

The accurate masses of the flavonoids were calculated by adding the accurate mass of hydrogen (1.007825032 Da) with the m/z parent ions obtained from the mass spectra of the pure flavonoids analyzed with the orbitrap mass analyzer.
Figure 40. Base peak chromatogram of F4 analyzed with Acquity UPLC®ESI-HRMS with orbitrap mass analyzer in the negative ionization mode.

The column used was BEH C18 with 1.7 µm particle size and 2.1 × 50 mm column dimension. The small particle size of the column allowed for separation of the isomeric flavonoids, 6-desmethyl-sideroxylin (23) and 8-desmethyl-sideroxylin (24).
**Figure 41.** Base peak chromatogram of a mixture flavonoids 23 and 24 analyzed with Acquity UPLC® ESI-HRMS with orbitrap mass analyzer in the negative ionization mode.

The peaks corresponding to compound **23** (t<sub>R</sub>=5.54 mins.) and **24** (t<sub>R</sub>=5.83 mins.) were resolved to display the presence of two compounds.
Another valuable piece of information derived from LC-MS is the fragmentation pattern of the parent ion of the compound of interest. This is useful in differentiating compounds having the same molecular mass but with different chemical structure, such as isomers. If unique fragments are generated at sufficient abundance, the fragments can be used to distinguish isobaric compounds and aid in structure elucidation. The fragmentation pattern of the parent ion of 23 was expected to be different from that of 24 because of positional variation in substituent attached to the A ring of the flavonoids as shown in Figure 37. The mass spectra of the fragments of the parent ions (MS/MS or MS²) generated through collision-induce dissociation (CID) at 35% normalized collision energy shown in Figure 42. A 35% normalized collision energy meant using 35% of the energy from the 5V supply normalized to the mass of the ion undergoing collision. The 35% collision energy is the same for 23 and 24 because they have the same mass whereas 35% collision energy for 25 requires more energy than for 23 or 24 because 25 has higher mass. Tentative structure assignment of fragments can be accomplished with computer software such as ACD/MS Fragmenter® (ACD Labs, Version 12.01). This software package predicts the possible fragments of a molecule ionized in the positive or negative mode. Fragment prediction for negative ionization uses deprotonation [M-H⁺], electron capture [M⁺], and hydride attachment [M+H⁻] to generate the negatively-charged species. Other parameters for predicting the fragments are the number of fragmentation steps involved and the maximum number of fragments generated at each fragmentation
steps. The prediction, however, does not consider the amount of energy needed to generate the fragments. Comparing the mass spectra of the isomers (Figure 42 B), the two most abundant fragments for both desmethyl-sideroxylin isomers are m/z 297.0 and 282.0. However, a fragment with m/z 178.8 is unique to 23 and a fragment with m/z 182.9 is unique to 24, albeit both at low abundance relative to 297.0 and 282.0. The parent ion with m/z 297.0 corresponds to a loss of hydrogen, which indicated a possible deprotonation of acidic hydrogen. The m/z 282.0 corresponds to a loss of a methyl group at position C8 for 23 or C6 for 24 or from the methoxy group attached at position C7 for both flavonoids. With the ionization mode set at deprotonation, and by limiting both the maximum fragments generated each step and the fragmentation step to one, ACD/MS Fragmenter® predicted only 3 possible fragments (m/z 297.1, 205, & 92) from the neutral molecules of flavonoids 23 and 24. Only the ion with m/z 297.0 was observed in the actual MS-MS spectra of both flavonoids as shown in Figure 42 B. By increasing the maximum fragments generated per step to twelve, the Fragmenter® was able to predict the structure of the fragment at m/z 282. The fragmentation pathway for the two most abundant fragments for 23 and 24 as predicted by the Fragmenter® in the deprotonation ionization mode is as shown in Figure 43 and 44, respectively. The minor fragments with m/z 253.1 and 112.8, meanwhile, were generated by the Fragmenter® using the hydride transfer ionization mode and set with at least 4 fragmentation steps with at most 380 fragments generated per step. The remaining
fragments m/z 178.8 unique to 23 and m/z 182.9 unique to 24 were not predicted by the software with the three modes of ionization.
Figure 42. (A) Base peak chromatogram of the pure flavonoids with their corresponding (B) fragmentation pattern using ion trap mass analyzer with full scan orbitrap mass analysis.

The fragmentation patterns were obtained using 35% normalized collision energy. The flavonoids were dissolved in 2% DMSO in methanol to improve solubility.
Figure 43. Fragmentation pathway for 6-desmethyl-sideroxylin (23).

The fragments corresponding to m/z 282 are predicted to be formed based on this pathway where the neutral molecule undergoes deprotonation followed by the loss of the methyl group at C8 and at the methoxy connected to C7 to generate the fragments.
Figure 44. Fragmentation pathway for 8-desmethyl-sideroxylin (24).

The fragment corresponding to m/z 282 is predicted to be formed based on this pathway where the neutral molecule undergoes deprotonation followed by the loss of methyl group at the C6 position and the methoxy connected to C7 to generate the fragment.
Figure 45. Fragmentation pathway for sideroxylin (25).

The fragment corresponding to m/z 311 is predicted to be formed based on this pathway where the neutral molecule undergoes deprotonation followed by the loss of methyl group either at the C6, C8 or the methoxy connected to C7 to generate the fragment m/z 296.0.
Based on the mass spectra and the predicted structures of the fragments, it appeared that the molecules do not undergo sufficient fragmentation to generate unique fragments of high abundance at 35% relative collision energy. Higher collision energies, 50%, 70% and 100%, were used to investigate whether unique fragments with sufficient abundance can be generated from the parent ion m/z 297.0 or the fragment ion with m/z 282.0. The resulting fragments were tabulated in Figure 46. Even at high collision energies, the parent ion m/z 297.0 favored the fragmentation pathway that resulted in the fragment at m/z 282.0. Although the other fragments of the neutral molecules were very minor, the Fragmenter® was utilized to predict the fragmentation pathway resulting in these fragments. It is interesting to note that the Fragmenter® predicted that most of the minor fragments are generated from the parent ion m/z 299. This parent ion is the result of flavonoids 23 and 24 undergoing hydride transfer ionization mode (also based on the software). However, the actual fragmentation of 23 and 24 showed a parent ion of m/z 297.0, which indicated that deprotonation, is the favored ionization mode of the molecules. The Fragmenter® also predicted that deprotonation of the hydroxyl group at position C5 is the most likely form of the parent ion m/z 297.0, which undergoes fragmentation to produce the most abundant fragment at m/z 282 (Figures 47 and 48). This is also true for the flavonoid sideroxylin, which is predicted to form ions at m/z 296.0 and 311.1 (Figure 49) starting with the molecular ion deprotonated at the hydroxyl group in position C5.
Figure 46. Fragments generated from varying the normalized collision energy with an ion trap mass analyzer.

The tabulated fragments are the values labeled in the mass spectra of flavonoids 23, 24 and 25 at varying normalized collision energy. The fragments are arranged according to increasing m/z. The bold and italicized numbers are the most abundant fragments in the mass spectra.
**Figure 47.** Fragmentation pattern for 6-desmethyl-sideroxylin (m/z 297) at 50 (top panel), 70 (middle panel) and 100% (bottom panel) normalized collision energy (CE) through collision-induced dissociation (CID).

The most abundant ion is m/z 282.0 for all settings of collision energy. The neutral molecule 23 is predicted to undergo deprotonation followed by the loss of a methyl group as shown in **Figure 43.**
**Figure 48.** Fragmentation pattern for 8-desmethyl-sideroxylin at 50 (top panel), 70 (middle panel) and 100% (bottom panel) normalized collision energy (CE) through collision-induced dissociation (CID).

The most abundant fragment ion is m/z 282.0 for all settings of collision energy. The neutral molecule 24 is predicted to undergo deprotonation followed by the loss of a methyl group as shown in **Figure 44**.
Figure 49. Fragmentation pattern for sideroxylin (25) at 50 (top panel), 70 (middle panel) and 100% (bottom panel) normalized collision energy (CE) through collision-induced dissociation (CID).

The most abundant fragment ion is m/z 296.0 for all settings of collision energy. The neutral molecule 25 is predicted to undergo deprotonation followed by the loss of a methyl group as shown in Figure 45.
CHAPTER V
CONCLUSION

Synergy-directed fractionation is a technique suitable for studying the complex interactions among the different constituents present in the herbal plants such as goldenseal (Hydrastis canadensis, L.). By using this technique, three (3) new flavonoids, previously isolated from other plants, were identified in goldenseal. These closely related flavonoids, 6-desmethyl-sideroxylin, 8-desmethyl-sideroxylin and sideroxylin, were isolated from the leaves of the plant but were also detected in the rhizome. Detection, separation and identification of these structurally similar molecules proved to be one of the major challenges of this research. The isomeric flavonoids, 6-desmethyl-sideroxylin and 8-desmethyl-sideroxylin, coeluted in the conventional HPLC with a C18 column, and both exhibited the same fragmentation pattern of their mass spectra even at high collision energy. The fragmentation pathway and the structures of fragments generated at each step were predicted using ACD/MS Fragmenter®. Based on the software prediction and the actual fragmentation pattern obtained, deprotonation of the hydroxyl group attached to C5 is the preferred negative ionization of the flavonoids. This is also true for sideroxylin. A column with stationary phase derivatized with pentafluorophenylpropyl (PFP) ligand proved more effective than the commonly used C-18 because it was able to separate the isomeric flavonoids. Meanwhile, the use of Acquity© Ultra High Performance Liquid Chromatography (UPLC) was able to resolve 6-desmethyl-
sideroxylin and 8-desmethyl-sideroxylin from the flavonoids mixture even with the use of the conventional C-18 column. High resolution mass spectrometry using an orbitrap mass analyzer enabled the determination of the accurate masses of all three flavonoids (≥ 98% purity) with less than 3.0 ppm mass accuracy.

The flavonoids were identified in the fraction that exhibited the most pronounced synergistic interaction with the alkaloid berberine. The synergistic effect from dose-combinations of the extracts and berberine were investigated using broth microdilution assay. The results were validated to be real synergism based on the isobole method and the FIC index of the test samples, which were reported elsewhere. Among the pure flavonoids, only 8-desmethyl-sideroxylin showed promising synergistic activity in combination with berberine against wild type and NorA over-expressed S. aureus strains. However, the limitation imposed by low solubility of the pure flavonoids in aqueous media could be the reason for the observed decreased in activity of the flavonoids relative to the crude extract.

It was established that quantitative analysis using high performance liquid chromatography tandem with mass spectrometry (HPLC-MS) was a crucial step in the synergy-directed fractionation technique. Quantitative analyses using LC-MS allowed for the correlation of the dose-combination effect with the concentration of the constituents in fractions and sub-fractions. LC-MS allowed for the detection and identification of the alkaloids and the flavonoids present in the crude methanol extract, and in every fraction or sub-fraction collected after each level of separation. It also allowed for tracking the alkaloids and the flavonoids starting from the crude methanol extract up to the point
where pure compounds were isolated. The limitations of the mass spectrometry for identifying compounds, which are readily ionizable by electrospray, were augmented by the use of other spectroscopic techniques such as LC with a photodiode array detector and NMR. The presence of the isomeric flavonoids, 6-desmethyl-sideroxylin and 8-desmethyl-sideroxylin, were detected from H\textsuperscript{1}-NMR.

Synergy-directed fraction is an alternative way to investigate the antibacterial activity of medicinal plants as demonstrated in this study. This technique allowed the identification of flavonoids, which do not possess inherent antibacterial activity but have synergistic effect with the alkaloid berberine. The same concept can be applied to other plants to look for the presence of flavonoids that could potentially have synergistic effects with other constituents in the plants. In fact, this technique can be applied to other class of organic compounds, not just flavonoids, which have potential synergistic activity. Although the microdilution combination assay does not conclusively indicate synergistic effect, it does give a good indication of the presence of potential synergists. This is useful when searching for natural compounds to act in synergy with commercially available antibiotics to combat resistant strains of microorganisms. The use of quantitative LC-MS profiling with synergy testing makes this a robust technique to correlate constituent concentration with bioactivity in complex materials such as plant extracts. The work done here proved, through synergy-directed fractionation, that the antibacterial activity of goldenseal is due to more than one compound, which included the flavonoid 8-desmethyl-sideroxylin. The other compounds, which are yet to be identified, could also
be possible synergists of berberine. The presence of other compounds was also established through the synergy-directed fractionation.
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