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Separation and Identification of Antibacterial Compounds Found in Botanicals Used in Traditional Medicine By Supercritical Fluid Extraction and High-Performance Liquid Chromatography-Mass Spectrometry

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Abstract

The increasing prevalence of antibiotic resistant bacteria poses a global health crisis and is worsened by the over prescription of antibiotics as well as the lack of funding for novel antibiotic research. New antibiotic compounds can be found by researching botanicals, specifically those used in traditional medicine which contain a variety of phytoactive compounds, some of which possess antibacterial, antiviral, and or antifungal properties. In the present study three botanicals used in traditional Cherokee medicine were screened for antibacterial activity and the presence of known antibacterial compounds: *Hydrastis Canadensis* (Goldenseal), *Achillea millefolium* (Yarrow), and *Geranium maculatum* (Geranium). These botanicals were selected due to their traditional uses for treating ailments likely caused by bacterial infections. Essential oils were extracted and collected in fractions using supercritical fluid extraction with carbon dioxide as solvent and a 95% ethanol cosolvent modifier. A broth microdilution assay was used to test if the extract fractions inhibited the growth of gram-positive bacteria, *Staphylococcus aureus* (SA), or gram-negative bacteria, *Escherichia coli* (EC). After identifying a fraction that inhibits growth; the pressure, temperature, and solvent flow rates for a second extraction are modeled around the active fraction. This process can be repeated to find the parameters yielding the highest concentration of antibacterial activity. These fractions will be tested using the same microbroth dilution assay, and if antibacterial activity is demonstrated again, further research will be conducted to identify the active compound using liquid chromatography-mass spectrometry (LC-MS). Standards of seven known active compounds in goldenseal were purchased and characterized using LC-MS to compare with the mass spectra of the active extracts. These comparisons will help determine if the antibacterial activity is due to a known molecule or a novel one, and if it is a novel compound LC-MS will be used in attempt to characterize it.

1. Introduction

In 2019, more than 35,000 people in the US died due to antibiotic resistant bacteria.[1] Additionally, the World Health Organization stated that resistant bacteria lead to over 700,000 deaths occur each year. Further, it is predicted that by 2050 it could be responsible for 10 million deaths.[2] Although this prediction is disputed due to lack of information from middle to low income countries.[3] Antibiotics are classified as organic molecules that kill bacteria; these can be natural or synthetic molecules. Antibiotic resistance occurs naturally, although this problem is much-worsened by over prescription in healthcare and overuse in livestock.[4,5] Seeking out novel antibacterial compounds is an effective approach for combating bacteria that are resistant to modern antibiotics. In addition to antibiotics becoming less effective due to overuse, the production of them has often been delayed due to economic and regulatory obstacles. A severe drop in profit led to 15 of the largest producers of antibiotics to abandon the field; production of antibiotics was not as profitable as other pharmacueticals.[6]

Phytoactive compounds used alone, or combined with existing antibiotics, may be an effective approach to combat antibiotic resistance.[7] Botanicals used in traditional medicines have shown a variety of biological activities such as, but not limited to, antibacterial, antiviral, antioxidant, anti-inflammatory, cardioprotective, and neuroprotective activites.[8] Medicinal plants are readily available, cheap, and are a great source of novel antibacterial molecules.[9] Phytochemical compounds such as alkaloids, phenolics and polyphenols have all been found to contribute to antibacterial activity in medicinal plants.[10] Alkaloids are heterocyclic nitrogen containing molecules found in plants, and some have the ability to impair cell division and kill cells. Berberine, a phytoactive compound found in goldenseal, has been used in tandem with other drugs to treat *H. Pylori* bacteria, which can cause stomach ulcers.[11] It has shown antimicrobial, hepatoprotective, anti-hyperlipidemic, anticancer, anti-diabetic, anti-inflammatory, and anti-arrhythmic properties.[12-8] Phenolics and polyphenols are a large group of molecules containing flavonoids, quinones, tannins, and coumarins, which have all shown biological activites.[7-13] Phenolics have shown antiulcer, anti-inflammatory, antidiabetic, antioxidant, cytotoxic, and antitumor properties.[14] Since the research field of phytoactive compounds is growing, many medicinal plants have not been studied.[13]

Effective extraction and purification systems are still progressing in the isolation of phytochemical compounds which limits research in this field.[13] In past research, a variety of methods have been used to extract phytoactive compounds from plants, such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), accelerated solvent extraction (ASE), and supercritical fluid extraction (SFE).[15] SFE is a relatively new extraction method that is effective at extracting phytochemicals from plant matter; it uses supercritical carbon dioxide (SC-CO_2) as a solvent, which is nontoxic and inexpensive.[16] When CO_2 is held at or above its supercritical point, 31.0ºC and 73.8 bar, it can take on properties of both liquids and gasses. SC-CO_2 has the solvating capability of a liquid but has a diffusivity and viscosity similar to a gas. [17] This allows for the solvent to pass through the plant material more easily while still holding solvating capabilities. SFE can be performed with a co-solvent, or modifier, to improve the extraction of polar compounds. Adding a modifier, such as ethanol or methanol, increases the solubility of the analyte in CO_2, which improves the yield of polar compounds.[16] Some target compounds, such as alkaloids and phenolics, have poor solubility in CO_2 alone, and using a polar co-solvent such as ethanol can increase solubility and yield.[18] In a study done by X. Ruan el al., SFE was proven to be a successful extraction method for alkaloids. In their research, the SFE parameters such as extraction time, temperature, pressure, and ethanol modifier flow were all studied to find the optimal conditions to yield a higher percentage of alkaloids. The optimal parameters for alkaloids were an extraction time of 3 hours, temperature of 60.4º, pressure of 26.5MPa, and an ethanol concentration of 89.3%.[19] These extractions can be done in fractions, where each fraction collected is done at a different set of parameters.

A suitable way to test these fractions for antibacterial activity is a bacterial cell death assay using the broth microdilution method. This method uses a plate with columns and rows of wells filled with broth medium and bacteria cultures, and then antibacterial agents are added. The plate is inoculated with the test organism, after incubation, absorbance of the wells is read at 590nm, which can be used to determine percent inhibition as well as the minimal inhibitory concentration (MIC) is measured.[20] The MIC is the lowest concentration of sample that prevents growth of bacteria. Another common method similar to this is agar microdilution which also is used to measure MIC.[13] Samples demonstrating antibacterial activity can be researched further using LC-MS to characterize the antibacterial phytochemicals.

Active compounds in botanicals have been characterized in multiple ways in previous research. LC-MS^n was determined to be an appropriate method for characterizing compounds in botanical extracts.[21] Alsheikh et al. refers to gas chromatography-mass spectrometry as the gold standard of identifying phytochemicals.[13] The characterization of goldenseal alkaloids was performed by Avula and Wang using ultra-performance liquid chromatography (UPLC) with UV detection. In their study, seven alkaloids were identified using UPLC-UV-MS, with a single quadrupole mass filter and electrospray ionization. This method proved to be effective for the identification of berberine and ß-hydrastine.[22] In another study by Le et al. the alkaloids of goldenseal were identified using ultra-performance liquid chromatography coupled with an electrospray ionization quadrupole time of flight mass spectrometer (UPLC-QTOF-MS) in MS^E mode.[21] Using this method they identified 1.5 times more alkaloids using UPLC-QTOF- MS^E than using Orbitrap LC-MS^n. UPLC-QTOF- MS^E is less expensive and much more available than Orbitrap MS^n and it was found to be a suitable replacement.[21] After collecting mass spectra data, databases with known mass spectra can be used as help for identification.[23]

In summary, this research started by the selection of plants that were: used in traditional Cherokee medicine, had traditional uses that suggested presence antibacterial properties, and non-endangered. Next supercritical fluid extraction was performed to extract phytochemicals from plant matter in fractions, where each fraction is done at a different set of parameters. LC-MS was performed to characterize eight standard compounds, all found in goldenseal and known to possess antibacterial activity, to build a database to compare plant extracts to in the future.

1.1 Goldenseal

The first botanical chosen for this study is goldenseal, a plant native to eastern North America.[24] Goldenseal has been used in traditional Cherokee medicine to treat a variety of ailments such as wounds, digestive disorders, ulcers, skin and eye ailments, and cancer. In a study by Milande et al., goldenseal root powder was extracted using an ultrasonic bath, and the major alkaloids were characterized by 1D and 2D-qNMR. UHPLC-UV and UHPLC-MS-MS performed on stock solutions of ß-hydrastine and canadine for comparison. Their research found that NMR was an appropriate method for characterizing compounds in medicinal plants.[25] The leaves and rhizomes of goldenseal both show antibacterial properties. Alkaloid compounds such as berberine, hydrastine, and canadine all contribute to goldenseal's antibacterial properties, shown in figure 1.[8] Berberine is an isoquinoline alkaloid whose antibacterial action mechanisms are: DNA intercalation, targeting RNA polymerase, gyrase and topoisomerase IV, and the inhibition of cell division.[9] Berberine displays antibacterial activity against *Staphylococcus aureus, Escherichia coli*, and H_1N_1 influenza A virus.[12] As well as isolated berberine, goldenseal extracts inhibit the growth of H1N1 influenza A.[26] Berberine on its own may not be a suitable replacement for antibiotics, but with modification it may lead to new effective synthetic antibiotics.[12] Yellow root shares many similarities to goldenseal and is also known to contain the alkaloid berberine.[27]

1.2 Yarrow

The second plant, Yarrow was selected due to its traditional uses in Cherokee medicine, such as treating wounds and gastrointestinal complaints. Some known phytoactive compounds in yarrow are the alkaloids adinene and pinene, as well as flavonoids luteolin and apigenin.[24] Apigenin is thought to be the compound attributing the most to yarrow's activity, and has shown *in vivo* wound healing abilities.[28] In a study by Verma et al. essential oil extracts of yarrow demonstrated antibacterial activity against *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, Klebsiella pnumoniiae, Pseudomonas aeruginosa, Escherichia coli,* and *Salmonella typhimurium.* The best activity reported was against *Staphylococcus aureus* and *Salmonella typhimurium,* both having a MIC value of 125 µg/mL.[29] SFE parameters of 100 Bar and 60°C were found to be a suitable starting point for collecting yarrow essential oils.[30]

1.3 Geranium

The third botanical studied was geranium, which was used in traditional Cherokee medicine to treat cuts and sores.[24] Traditional uses also include the treatment of hemorrhoids, inflammation, gallbladder problems, and gastric ulcers.[31] Although there is little research on *geranium maculatum,* there is some literature published on different species within the geranium genus. Bigos et al. found that geranium oil extracted from *Pelargonium graveolens* demonstrated antibacterial activity against multidrug resistant *S. aureus,* at concentrations of 0.25-2.5 µg/mL.[32] Geranium oil is commonly used in the cosmetic industry due to its rose like scent. Gomes et al. optimized the SFE parameters of *Pelagonium sp.* for the highest yield of geranium oil for the purpose of creating perfume. The optimal SFE parameters found were pressure of 90-100 bar, temperature of 40 °C, and an extraction duration of 15-30 min.[33] These parameters served as a starting point for the extractions conducted in this study.

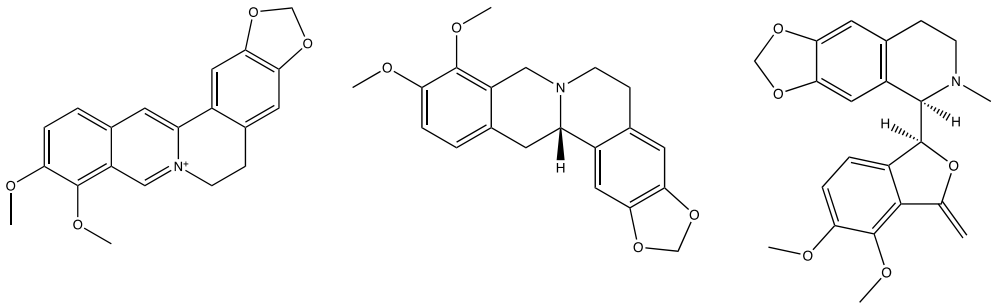

Figure 1: most prominent phytoactive compounds in goldenseal: berberine, canadine, and hydrastine from left to right.

2. Experimental Methods

2.1 Materials

Goldenseal root powder 5:1 extract was purchased from Bodi 4 Life Inc. TSB media, staphylococcus, and E. coli were provided by Dr. Wolfe. 200 proof ethanol, EMPLURA, was purchased from VWR International. The ethanol was diluted with DI H_2O to make 95% ethanol. LC-MS grade water was purchased from J.T.Baker. LC-MS grade acetonitrile was purchased from OmniSolv. LC-MS grade methanol was purchased from Honeywell. A BETASil Phenyl column, 150mm x 2.1 mm, was purchased from Thermo Scientific. Standard compounds purchased: Canadaline (TRC Canada), Palmatine (LKT Laboratories, Inc), Hydrastine (The Nature Network), Berberine chloride (Alfa Aesar), Jatrorrhizine (AmBeed). Acetic Acid was purchased from Macron Fine Chemicals. Ammonium Acetate was purchased from Fisher BioReagents.

2.2 Supercritical Fluid Extraction (SFE)

SFE was performed using a MV-10 ASFE System (Waters, Milford, MA, USA), a system composed of a Thermo Cube, fluid delivery module, column oven, back pressure regulator, heat exchanger, and a fraction collection module. In preparation for SFE, a cleaning procedure was conducted prior to all extractions. (The cleaning method had the following parameters: CO_2 flow:9mL/min, duration: 1.5min, extraction temperature: 40ºC, extraction pressure: 150 Bar, makeup flow: 0.2mL/min, ethanol 100% flow: 1mL/min. A three-step cleaning method was conducted to purge the lines from vessel 3. All steps of the method have a CO_2 flow of 4mL/min, ethanol 100% flow of 1mL/min. Each step was done at 35ºC and lasted 1 minute. The first step was done at 100barr, the second to 150barr, and the third to 200 Bar.) During extraction the oils that are carried out are collected in fractions and these fractions are all extracted at different temperatures, pressures, and co-solvent flow rate. This allows determination of what SFE parameters are responsible for carrying out the antibacterial phytochemicals.

2.3 Antibacterial Assays

General Sterilization Methods: All bacterial work was performed under a flame using sterile conditions. All media were either autoclaved (121 °C) or filtered through a 0.2 μM polyethersulfone (PES) filter. Overnight liquid cultures were made by inoculation 8 mL of sterile Tryptic Soy Broth (TSB, 30g TSB powder in 1L of DI water) with one colony of bacteria and incubating at 37 °C for 20 hours. Pathogenic bacteria used included *Escherichia coli* (EC, ATCC 25922) and *Staphylococcus aureus* (SA, ATCC 29213).

Before preparing samples for the antibacterial assay, ethanol in the products was evaporated using a TurboVap. To prepare the samples 10 μL of DMSO was added to each vial, vials contained approximately 10 mg of essential oil. A sterile 96-well plate antibacterial assay was set up by adding 89 μL of TSB media, 10 μL of overnight bacteria culture (rows A and B used SA, rows C and D used EC) to all wells in columns 2-11. Column 1 only had chloramphenicol (1 mg/mL DMSO) and was the positive control. Column 12 of the microwell plate was only bacteria which was used as the positive control, row A and B had SA bacteria and row C and D had EC bacteria. The plate was incubated at 37 °C for 24 hours. Using a Synergy HTX multimode plate reader (BioTek), the assay plate was read for absorbance at 590 nm to determine antibacterial activity. This method was followed for all bacteria inhibition assays.

2.4 Liquid Chromatography-Mass Spectrometry

Liquid chromatography and mass spectrometry were performed on LCMS-2020 (Shimadzu, Kyoto, Japan). A C18 column was used for liquid chromatography (Thermo Fisher). Eight standards of alkaloids found in the studied plants, and known to have antibacterial activity, were purchased. Initially, stock standards were made at 1000 ppm, but these were too concentrated to provide accurate absorbance data on the mass spectrometer. After several dilutions, it was found that the maximum concentration we could accurately measure was 1 ppm. The mass to charge ratio was found for all standards and can be seen in table 4. After identifying the m/z for each standard, a mixture of four standards was made to analyze through a LC column and find a proper solvent gradient to make them elute at different times.

The solvents used were A: water with 10 mM ammonium acetate adjusted to 4.8 pH with acetic acid and B: Acetonitrile. The gradient used that provided separation of analytes can be seen in table 5.

3. Results and Discussion

3.1 Cell Growth Inhibition Assays

The results from the cell growth inhibition assays reports the light absorbance from the plate reader. Higher absorbance values indicate a larger amount of bacteria in the well. A negative control of bacteria only shows what the absorbance is without the presence of any compounds. The positive control, chloramphenicol, is known to inhibit the growth of E. coli and Staphylococcus. The absorbance values of chloramphenicol are used to see what absorbance is detected from near total inhibition of the bacteria. All extracts and controls were done in triplicate, with the absorbance values being averaged. Absorbance values below that of the positive control indicates that the extract did inhibit the growth of bacteria.

3.2 Goldenseal

Absorbance values showed that a goldenseal extraction (001) yielded one fraction (extract 001.6) that inhibited the growth of E. Coli. This is shown by the absorbance values of extract 001.6 compared to those of the negative bacterial control. Seeing that the absorbance values of extract 001.6 were lower than that of bacteria, it was determined that extract 001.6 inhibited the growth of E.coli.

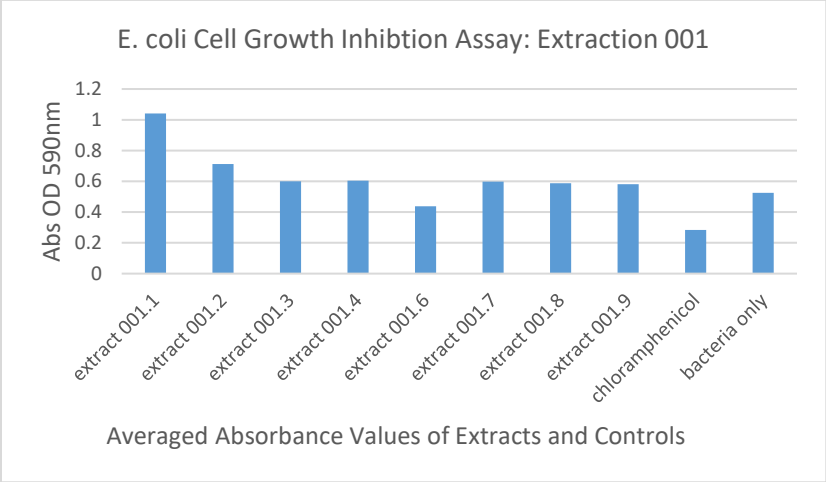

Figure 2. Results from the cell growth inhibition assay testing goldenseal extracts from extraction 001 against E. coli.

With these results in mind, an extraction (002) was performed to narrow in around the parameters of the previous fraction demonstrating antibacterial activity. Two fractions from extraction 002 demonstrated bacterial growth inhibition, (002.1 and 002.7). Fraction 002.1 demonstrated inhibition of both EC and SA while 002.7 demonstrated inhibition of SA. These results can be seen in figure 3.

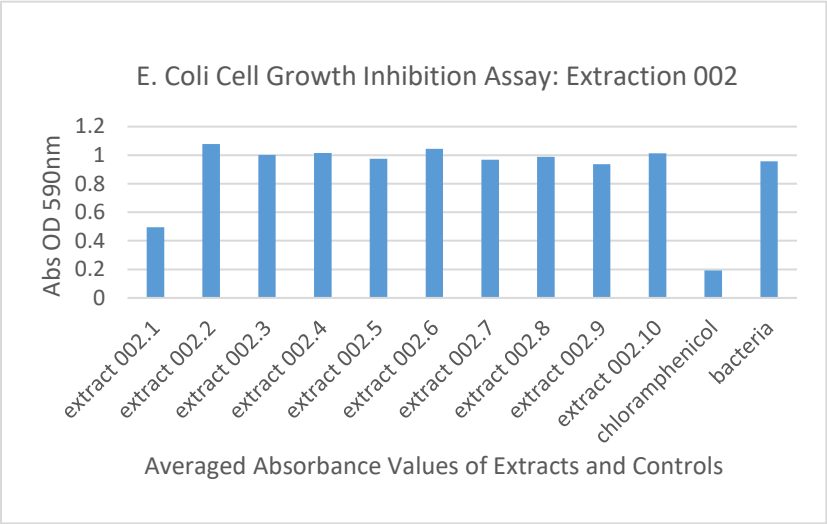

Figure 3. Absorbance values from extract 002.1 are lower compared to that of the negative bacteria control, which indicates that it did inhibit the growth of E coli.

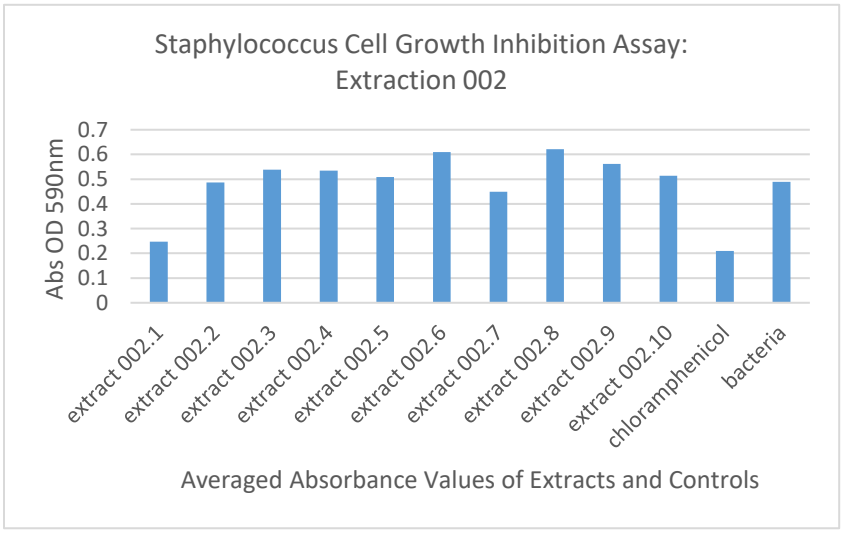

Figure 4. Absorbance values from both extract 002.1 and 002.7 are lower than that of the negative bacteria control indicating they both inhibited the growth of Staphylococcus.

Continuing the process of narrowing SFE parameters around the parameters that produced extracts with antibacterial activity, it was found that an extract (006.1) inhibited the growth of Staphylococcus with statistical significance ($p \leq 5$). It was calculated that extract 006.1 inhibited Staphylococcus by 20% compared to chloramphenicol which inhibited 73%.

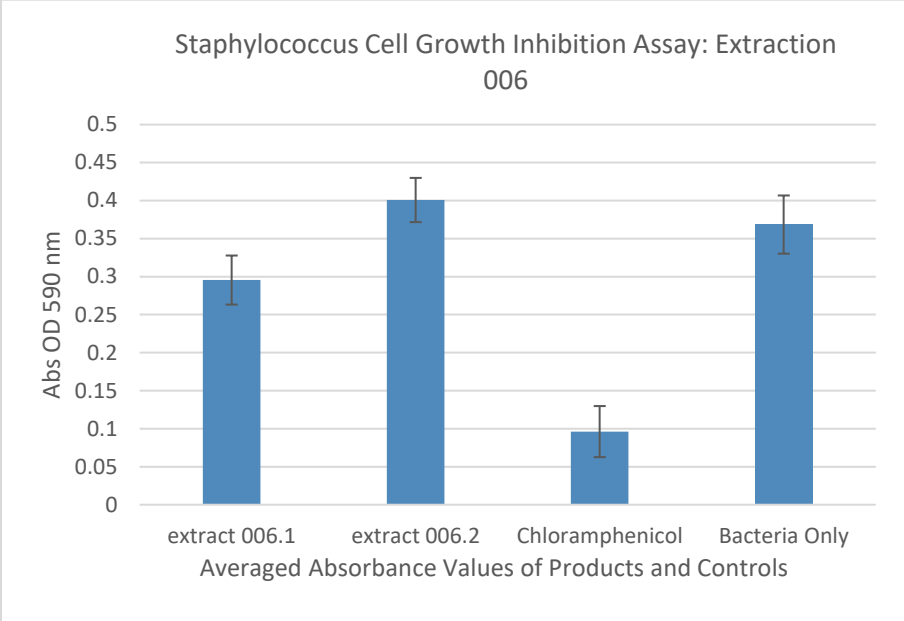

Figure 5. Absorbance values from extract 006.1 are significantly lower than that of the negative bacteria control. Whisker bars represent the standard deviation of the values which were analyzed in triplicate and averaged for depiction above.

3.3 Yarrow

One extraction of yarrow flower yielded a fraction that indicated antibacterial activity, (Extraction 301, extract 301.7). This extract inhibited growth of staphylococcus with statistical significance where n=4 and $p \leq 5$.

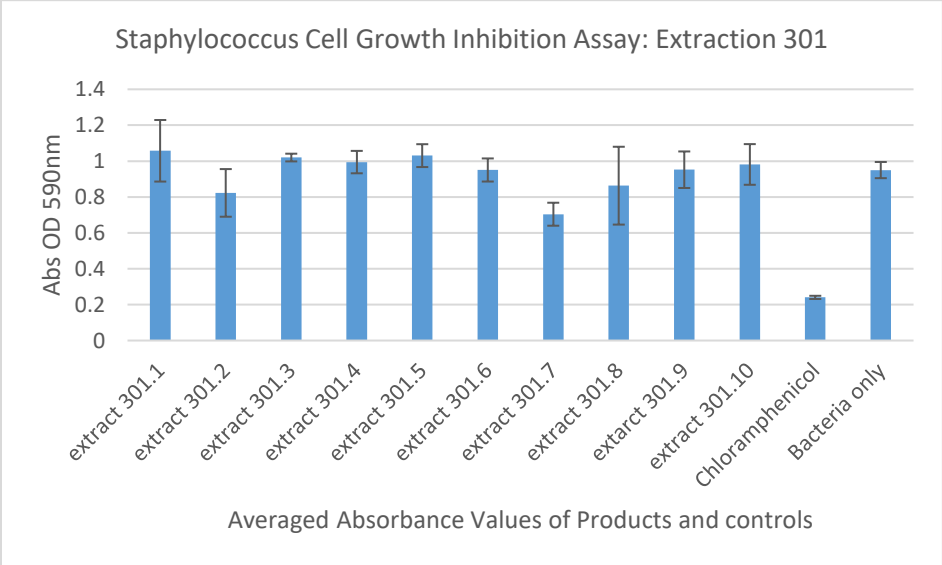

Figure 6. Absorbance values from extract 301.7 are significantly lower than that of the negative bacteria control. Whisker bars represent the standard deviation of the values.

3.3.1 geranium

Two geranium extractions were performed and all extracts were screened for antibacterial activity, which none showed.

4. Supercritical Fluid Extraction

The SFE parameters that yielded an extract demonstrating antibacterial activity can be seen in table 4.

Table 4. Parameters of plant matter extractions that yielded a extract which demonstrated antibacterial activity.

SFE Parameters of Active Extracts						
Plant	Extract	temperature (°C)	pressure (Bar)	duration (min)	CO2 flow (mL/min)	95% ethanol flow (mL/min)
Goldenseal						
	001.6	55	200	25	9	1
	002.1	35	100	15	9	1
	002.7	47	100	15	9	1
	006.1	40	100	60	6	1
Yarrow						
	301.7	50	190	20	8.5	1.5

5. Liquid Chromatography-Mass Spectrometry

The mass to charge ratio detected for the standard compounds studied can be found in table 5. The solvent gradient for liquid chromatography that provided separation between the alkaloids Berberine chloride, Jatrorrhizine, Palmatine, and Hydrastine can be found in table 6. The separation between the compounds from this column gradient can be found in figure 5. The separation from this column gradient suggests that it will provide suitable separation for the alkaloids present in the active goldenseal extractions.

Table 5. Mass to charge ratios found for standard compounds

Compound	Concentration (ppb)	m/z	Intensity
Berberine chloride	130	336.1	26,777
Jatrorrhizine	80	338.1	69,851
Coptisine	90	320.05	14,200
Palmatine	110	352.1	26,650
Canadaline	90	370.15	31,972
Hydrastine	130	384.1	30,931
Isocorypalmine	60	342.15	11,840

Table 6. Optimized solvent gradient for separation of alkaloid analytes

Time (min)	Flow % B
0-20	20%
20-21	20%
21-26	20%-70%
26-30	70%-0%

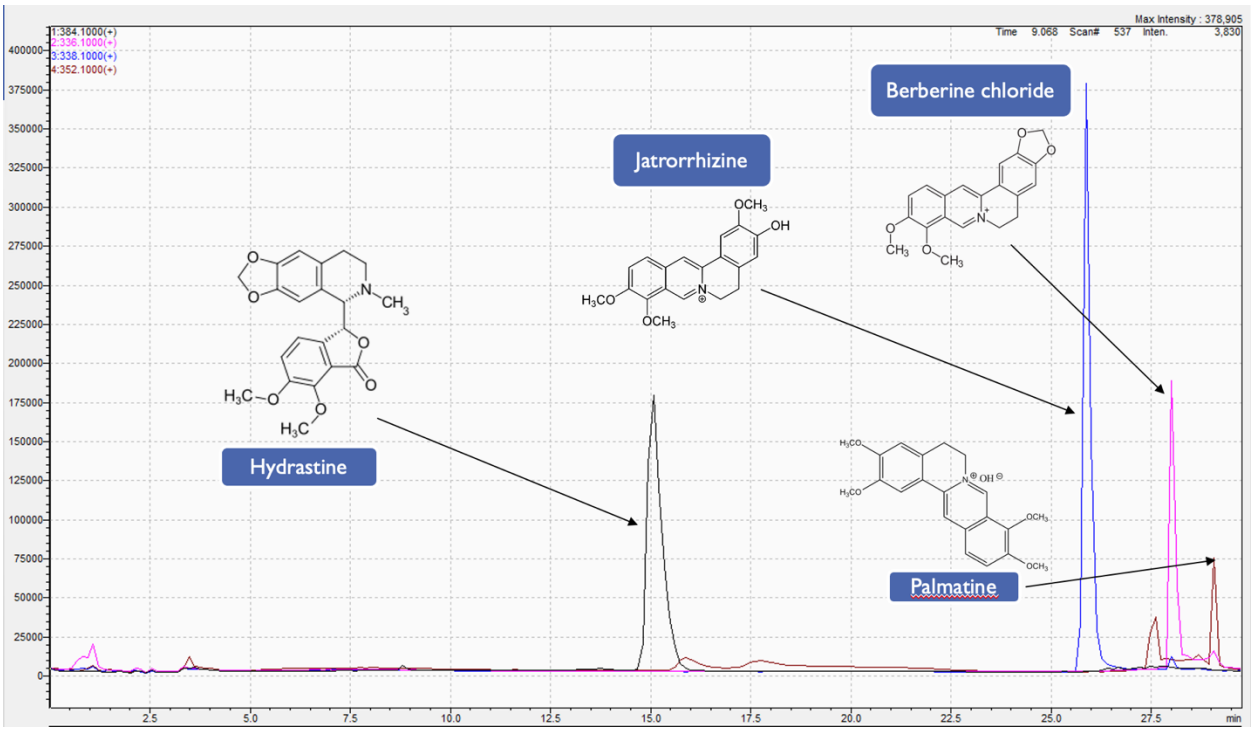

Figure 7. Separation achieved from the optimized liquid chromatography column gradient which is shown in table 6.

6. Conclusion

This suggests that goldenseal essential oils possess antibacterial compounds, due to their *in vitro* activity against *Staphylococcus aureus* and *Escherichia coli*. Three extractions of goldenseal produced fractions demonstrating antibacterial activity. One extraction of yarrow yielded a fraction that demonstrated slight growth inhibition of *Staphylococcus aureus;* However, the next yarrow extractions did not produce the same results. By characterizing four standards of known antibacterial alkaloids in goldenseal, a HPLC column gradient suitable for the separation of analytes has been determined. In the coming weeks we will use HPLC-MS to create a calibration curve to help identify the concentration of alkaloids goldenseal. After making the calibration curves for the stock standards, the fractions demonstrating antibacterial activity will be characterized and if traces of the standard compound are found in the fraction, then the calibration curve will help determine the concentration. If the standard compounds are not found in the extractions, MS will be used further to characterize the compounds in attempt to discover a novel antibacterial agent using comparisons to global databases. We will continue to research goldenseal and try to optimize the SFE parameters for yielding antibacterial compounds, as well as start to characterize the active fractions we collected. Our group will continue the rapid screening of botanicals for antibacterial properties and the plants yarrow and geranium will likely be studied further.

7. Acknowledgements

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