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Supercritical Fluid Extraction of Native Appalachian Plants for Antibacterial Compounds

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Abstract

Novel antibacterial compounds are in high demand due to antibacterial resistance creating a gap in viable treatment for various bacterial infections. These compounds can be developed synthetically, requiring several steps to design a lead compound, or they can be discovered through screening of compounds from natural sources. Synthetic development can be costly and time-consuming, thus the screening of natural compounds is favored. A wide diversity of unexplored natural sources exist, suggesting a method is needed to narrow the screening selection to plants most likely to contain antibacterial compounds. Plants used in traditional medicine, for conditions we know now as bacterial infections, can be explored for novel compounds. This research applies the screening of various traditional Appalachian medicinal plants using supercritical fluid extraction (SFE) to optimize the extraction of candidate compounds. Supercritical fluid extraction is highly efficient at extracting compounds from a matrix with exceptional precision. Extractions are varied by temperature, pressure, and polarity to extract a range of compounds from plant matter. The crude extractions are tested for inhibition of gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli*. For extractions showing antimicrobial activity, SFE parameters were further refined and re-evaluated to identify the exact parameters that extract the bioactive compound(s) to then be purified and characterized. Refined extractions with bioactive compounds were analyzed using LC-MS/MS to determine the specific compounds showing activity. Black Walnut (*Juglans nigra*), Sycamore Maple (*Acer pseudoplatanus*), White Oak (*Quercus alba*), and Joe-pye Weed (*Eutrochium purpureum*) were screened using these methods. Joe-pye Weed extracts showed significant inhibition of both *Escherichia coli* and *Staphylococcus aureus,* and was characterized by LC-MS/MS to identify the concentration of the known antibacterial compound, salicylic acid, in the inhibitory fractions.

Introduction

The world population is increasingly in need of novel antibacterial compounds to combat the spread of antibacterial resistance. Since 1987, no new classes of antibiotics have been successfully released to the market.[1] Of the antibiotics developed since 2017, 9 of 11 are derivatives of antibiotics that bacteria have developed drug resistance to, and as such are expected to be overcome by bacterial resistance soon after being released to market.[2] *Staphylococcus aureus* (S. aureus) and *Escherichia coli* (E. coli) are two common pathogens in humans that have developed resistance to a wide range of commonly used antibiotics. Infections of these pathogens, along with many others, have been encountered and treated throughout history long before they were known to be caused by bacteria. Traditional medicinal remedies used plants as a method of treating ailments without quantifiable evidence to prove their effectiveness and people relied on generations of personal accounts for the reliability of natural medicine. In

some cases, these medicinal remedies may have been effective due to antimicrobial compounds in the plant matter.[3] Plants have almost unlimited potential for undiscovered natural compounds, and phytochemicals, the active compounds within plants, are naturally complex in design and often capable of a spectrum of antimicrobial activity. Many native plants are reputed in Appalachian medicine as having antibacterial activity, helping guide the exploration of uncharacterized compounds toward plants with known potential. Even though some traditional medicine may prove to be simply a placebo effect, the many plants historically used have enough potential to raise interest in the pharmaceutical industry. One of the most widely used anti-inflammatory agents originated from the willow tree (*Salix alba L*); the compound salicin made the bark extract act as a natural pain reliever for Native Americans and is a prodrug of aspirin, Salicylic acid. Antibiotics often originate from microorganisms and their derivatives, but as the isolation of natural products continues, more plant compounds are being discovered with antibacterial activity.[4]

The Appalachian region is home to many native plants that indigenous people would use for traditional medicine. Our goal is to determine if the selected plants for this research from Native healing remedies show any significant antibacterial properties. Plants used for conditions implied as bacterial in nature are prioritized to increase the likelihood of discovering active antibacterial compounds. Plants such as Black Walnut (*Juglans nigra*), White Oak (*Quercus Alba*), and Joe-Pye Weed (*Eutrochium purpureum*) are native to Western North Carolina and show promise in medicinal value as potential sources of antibiotics. Black Walnut was traditionally used as a miscellaneous disease remedy, for colic, toothache, sores, and skin conditions. The application of black walnut for sores implies a possible connection to antibacterial properties.[5] White Oak was used as an astringent and antiseptic, to clean and treat burns and sore mouth. Joe-Pye Weed was used for urinary infections.

Previous research from Panda et al. performed a large-scale screening encompassing 222 plant species of both random selection and known medicinal use from India. Antibacterial activity was discovered across all parts of plants, though most noticeably in leaf extracts. This study explored the idea of aqueous extracts versus methanol extracts, as traditional healers typically used aqueous extraction methods but methanol extractions are more likely to permeate the cell membrane. The results from their study determined that methanol extraction was more effective and capable of extracting more active compounds than traditional water-based extraction would obtain.[6]

Supercritical fluid extraction is the separation of a component from its matrix using a supercritical fluid, a substance above its critical point of temperature and pressure. The substance, supercritical CO_2 in this extraction, behaves with properties between the gas and liquid phases. The gas-like viscosity and liquid-like density provide an enhanced solubility for organic compounds.[7] Supercritical fluid CO_2 is considered a clean method of extraction, as it does not require chemical solvents. When using a co-solvent, ethanol is relatively safe and easily evaporated out of the extraction. CO_2 is a nonpolar solvent, thus extraction of polar compounds requires the addition of ethanol as a polar co-solvent.[8]

Various studies have explored supercritical fluid extraction of individual plant species. Supercritical fluid extraction has been used to assess plants of the Artemisia and Pulicaria genera with varying but promising activity.[9] Celery and parsley fruit showed moderate to strong antibacterial activity.[10] Green cardamom and common guava were extracted and evaluated against oral pathogens, showing significant activity.[11] Supercritical fluid extraction of plant compounds is not as widely used as other extraction methods, so large-scale screenings like the 222 plant species in India are not available. Collections of small-scale studies have developed a superficial understanding of the general antimicrobial activity of supercritical fluid extractions.

Table 1. MIC Results of Plant Extracts in Selected Literature

Plant [# of species]	Extraction method	S. Aureus MIC	E. Coli MIC	P. aeruginosa MIC	B. subtilis MIC	Reference
Various genera [65]	Methanol	62-5000	--	--	--	Panda, S.K. et al.[6]
Artemisia abyssinica	SFE	6250	12500	NE	6250	Al-Maqtari, et al.[9]
Large Wormwood (Artemisia arborescens)	SFE	3100	6250	NE	3100	
Pulicaria jaubertii	SFE	1550	1550	50000	390	
Pulicaria petiolaris	SFE	6250	3130	NE	390	
Celery (*Apium graveolens* L.)	SFE	320-640	--	--	--	Misic, D., et al.[10]
Parsley (*Petroselinum crispum* (Mill.) Fuss)	SFE	>2560	--	--	--	
Green Cardamom (Elettaria cardamomum)	SFE	390-780	--	--	--	Kumari, R., et al.[11]
Common Guava (Psidium guajava)	SFE	670-1250	--	--	--	

MIC (Minimum Inhibitory Concentration) Values: μg/ml; NE: Not effective.

The parameters of extraction are important for optimizing the extraction of particular compounds from the plant matrix. Santoyo et al. performed an extraction of oregano oil (Origanum vulgare L.) varying pressure, temperature, and ethanol concentration. The antibacterial properties of oregano oil were already known by the time of this research, but antibacterial activity of oregano oil obtained by SFE was not yet determined and provided the goal of this research, optimizing the extraction and antimicrobial activity of the plant matter. The results of this research showed the variation of concentration of compounds by different extraction parameters and subsequent MBC values (Table 2). Camphene concentration variations show most clearly the importance of optimizing parameters for extracting trace concentrations of a compound, as introducing low pressure and a polar co-solvent decreases the total yield of camphene to undetectable amounts. The MBC values show that Experiment 5 produced the most antibacterial extraction across all tested pathogens, with slight improvement over the other experiments. The composition of the extracts and the parameters can provide a glimpse into what types of compounds may be responsible for bioactivity. Such as in this case, the lower temperature, lower pressure, and polar co-solvent appear to have a higher affinity for trans-sabinene hydrate, terpinen-4-ol, p-cimen-9-ol, carvacrol, spathulenol, abietatriene, and 2,3-dihydro-1,1,3-trimethyl-3-phenyl-1H-indene. Results of testing isolated standards for antimicrobial activity confirmed carvacrol as the most effective antibacterial compound, matching the highest concentration of carvacrol to highest antibacterial activity in experiment 5.[12]

Table 2. Comparison of parameters, sample of identified compounds, and MBC values (Santoyo et al.)

Exp	Parameters	α-Pinene	Carvacrol	Camphene	Linalool	δ-3-Carene	E. coli MBC	B. subtilis MBC	P. aeruginosa MBC
1	40°C 350 barr	3.05%	39.89%	0.58%	3.25%	1.40%	2050 ± 50	2250 ± 400	2100 ± 50
2	60°C 350 barr	3.40%	39.08%	0.34%	2.54%	0.90%	2250 ± 90	2240 ± 100	2120 ± 100
3	40°C 250 barr 4% EtOH	0.72%	44.62%	0.02%	2.62%	0.28%	2050 ± 100	2250 ± 50	2080 ± 110
4	60°C 250 barr 4% EtOH	0.85%	43.67%	0.07%	2.72%	0.12%	2090 ± 80	2240 ± 800	1750 ± 100
5	40°C 150 barr 7% EtOH	0.64%	49.43%	--	2.50%	0.24%	1950 ± 50	1950 ± 50	1750 ± 50

MBC (Minimum Bactericidal Concentration) Values: µg/ml

The focus of this research is on supercritical fluid extraction and analysis of the antibacterial compounds in plants in Western North Carolina. Depending on the known properties of the plant matter, as well as results from exploratory trials, the parameters for the supercritical fluid extraction will be varied for optimization of compound extraction. Temperature, pressure, extraction time, flow rate, sample size, co-solvent, and particle size of the sample are all parameters that can influence the ability of specific compounds to be extracted from the matrix.[8] Parameters that show antibacterial activity on the antibacterial assay will be refined to smaller increments of variation around the specific parameters that extracted the most active compounds and re-evaluated for isolating the source of activity.

Methodology

Apparatus

All SFE experiments were performed on a Waters MV-10 ASFE system using 5 mL to 25 mL extraction vessels. The components of this system include a fluid delivery module, ThermoCube chiller unit, extraction oven module, automatic back pressure-regulator module with heat exchanger module, fraction collection module, and ChromScope software. Antibacterial assays were analyzed using a microplate absorbance reader. Compounds were identified using Shimadzu liquid chromatography-mass spectrometry (LC-MS/MS).

Chemicals

SFC-grade carbon dioxide was used as an extraction solvent. Nitrogen gas was used for turbo-vapping the samples. 95% ethanol was prepared for co-solvent use from EMPLURA® ethanol absolute. Black walnut and Sycamore maple were purchased from the NY spice shop ground and dried. Sycamore maple was accidentally received instead of the intended plant, American Sycamore (*Platanus occidentalis)*. White Oak and Joe-Pye Weed were purchased from Starwest Botanicals. All plant material was further ground using a coffee grinder to achieve a finer consistency as required. DMSO and chloramphenicol were used for the antibacterial assay. Methanol and water with 0.1% formic acid were used as mobile phases in LC-MS/MS.

Supercritical fluid extraction

Table 3. Extraction Parameters

Plant	Temperature	Pressure	Vessel
Black Walnut	50-70°C	100-250 barr	5 ml
White Oak	40°C	100-300 barr	5 ml
Sycamore Maple	40°C	100-300 barr	5 ml
Joe-Pye Weed	60°C	125-185 barr	5 ml 15 ml

Carbon dioxide (CO_2) was pressurized to a range of 100-300 barr and temperatures varied from 40-70°C. 95% ethanol was used as a co-solvent. The sample weight was scaled to approximately half the selected vessel's volume capacity. Samples were subjected to 20-minute dynamic extractions per each unique set of sample parameters. Pressure was increased at 15-barr intervals. Extractions were collected in 100-ml fraction collection flasks. All fractions collected were immediately turbo-vapped at 55°C until all ethanol was evaporated.

Antibacterial assay of SFE fractions

The Fractions of plant extracts were tested in a 96-well microplate antibacterial assay against *Staphylococcus aureus* and *Escherichia coli* using chloramphenicol as the positive control. Before being added to the assay plate, 15 µL of DMSO was added to each sample. Tryptic soy broth (TSB) was used as the medium for the bacteria, with 89 µL added in each well. A total of 10 µL of each bacteria and 1 µL of fraction samples were then placed into appropriate wells. Chloramphenicol was used in place of fraction samples in the positive control. The negative controls were wells containing tryptic soy broth and bacteria, and wells containing only tryptic soy broth. Wells were performed in quadruplicate for Black Walnut and Joe-Pye Weed. White Oak and Sycamore Maple were performed in duplicate and discontinued before replicating results. The assay was incubated for approximately 16 hours and analyzed using a microplate absorbance reader. Absorbance values were quantified by the absorbance reader and analyzed for percent inhibition.

LC-MS/MS screening of extract fractions

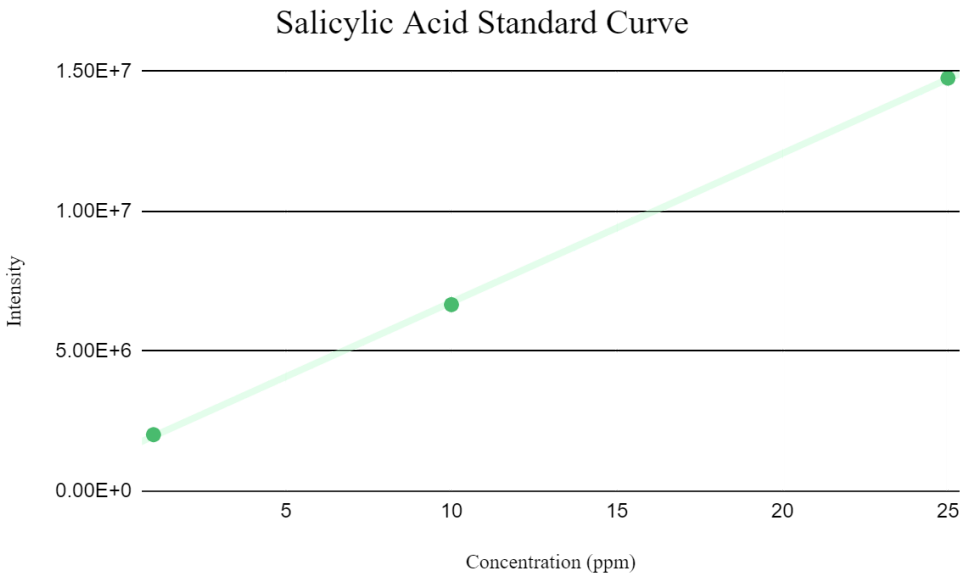

Figure 2. Calibration curve of Salicylic acid.

LC-MS/MS was used for analysis without a column for direct insertion. Methanol and water with 0.1% formic acid were used as solvents. Salicylic acid, a metabolite of the known component of Joe-Pye Weed, methyl salicylate, was prepared as a standard to identify a component of the composition of the inhibitory fractions. The extraction parameters showing antibacterial activity were put in solution using 100 uL of methanol and 1.4 mL of water and placed in vials for LC-MS/MS analysis. Standards of salicylic acid were developed at 1, 10, and 25 ppm to produce a standard curve (Figure 2). The standard curve was produced using a mobile phase gradient of 100% water to 100% methanol over 20 minutes. Selected ion monitoring (SIM) for the negative ion of salicylic acid (137 m/z) was used to identify the intensity of salicylic acid in each of the standards. The retention time of salicylic acid was approximately 14 minutes.

Results

The results of the bacterial inhibition assays showed varying amounts of antibacterial activity in each of the selected plants. Table 4 shows the greatest inhibitory activity of each plant and the associated parameters of extraction. Of the initial exploratory extractions of Joe-Pye Weed (Table 5), the highest inhibition rates of 54.46% of S. aureus growth and 21.70% of E. coli growth were discovered from fraction J7.

Table 4. Bacterial Inhibition Assay Results

Plant	Fraction Parameters	S. aureus	Fraction Parameters	E. coli
Black Walnut	--	0%	50°C 100 barr	18.51%
White Oak	40°C 200 barr	2.63%	40°C 300 barr 95% EtOH	5.98%
Sycamore Maple	--	0%	60°C 100 barr 95% EtOH	2.92%
Joe-Pye Weed	60°C 170 barr	54.46%	60°C 170 barr	21.70%

Table 5. Joe-Pye Weed Fractions and Parameters

Fraction	Parameters	Fraction	Parameters
J1	125 Barr	J7	170 Barr
J2	125 Barr 95% Ethanol	J8	170 Barr 95% Ethanol
J3	140 Barr	J9	185 Barr
J4	140 Barr 95% Ethanol	J10	185 Barr 95% Ethanol
J5	155 Barr	CL	Chloramphenicol
J6	155 Barr 95% Ethanol		

Fractions J6 and J9 were the next most inhibitory extractions at 46.50% inhibition of S. aureus and 15.44% inhibition of E. coli respectively (Figure 1). Chloramphenicol, used as a control, showed 71.90% inhibition of S. aureus and 85.64% inhibition of E. coli. The other selected plants showed only mild inhibition in comparison, with Black Walnut being the second most inhibitory extract identified at 18.51% inhibition of E. coli (Table 4).

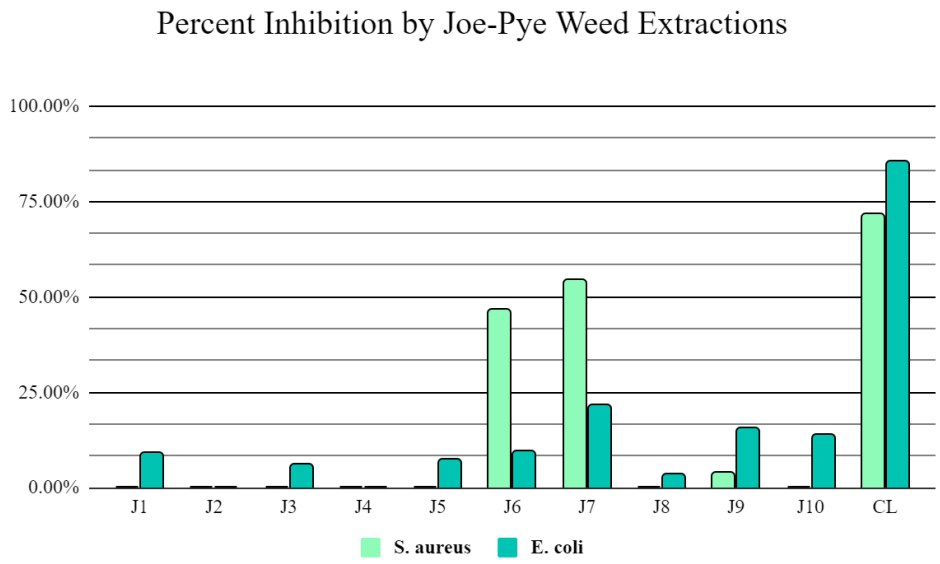

Figure 1. Joe-Pye Weed inhibition of S. aureus and E. coli across tested SFE parameters.

The 3 most inhibitory fractions, J7, J6, and J9 were then analyzed for salicylic acid concentration using the method used to obtain the salicylic acid standard curve. The presence of salicylic acid was not confirmed in these selected fractions. The chromatograms of the fractions did not show the expected peak for salicylic acid, though this may be attributed to the significant interference visible in the chromatograms.

Discussion

The bacterial inhibition assay revealed antibacterial properties from each of the selected plants for this study, though more research is required to understand the true potency of any antibacterial compounds within these fractions. Each sample likely contains multiple compounds, decreasing the overall amount of bioactive compounds in each sample well and potentially diluting its inhibition. Isolating the bioactive compounds found in these samples should increase the already significant antibacterial activity seen. Synergistic effects are also a possible factor for the significant antibacterial activity, and should be explored using an antibacterial synergy checkerboard assay in future research if isolated compounds fail to match or increase the antibacterial activity using the bacterial inhibition assay previously performed.

Due to the volatility and insolubility of much of Joe-Pye Weed's essential oil composition, GC-MS/MS and multiple reaction monitoring (MRM) may be helpful in further analysis. It is unknown how much of the volatile composition remains after extraction, though due to the vapor density of many of the compounds and the enclosed system of the MV-10 ASFE system, it is possible that volatile compounds were at least partially retained. The turbo-vap process may need to be reevaluated due to the relatively open system allowing the dispersal of evaporated compounds. The ethanol within the extractions must be fully evaporated for the antibacterial assay, as ethanol would result in a falsely increased antibacterial effect. The extractions could be left open to naturally air-dry, though this process may oxidize compounds, if they have not been oxidized during extraction, and potentially affect antibacterial activity. One of the compounds identified in Joe-Pye Weed extract, eugenol, likely confirms that oxidization already occurs from the brief exposure to air, as its degradation product appears very similar to the appearance of some of the fractions after being turbo-vapped. The degradation product of eugenol should be

considered for analysis of future Joe-Pye Weed fractions, as literature has found eugenol has antibacterial properties against S. aureus and E. coli.[13]

The analysis of each of these plants confirmed that there are antibacterial compounds within each plant, most notably in Joe-Pye Weed, though further study is required to identify the specific compounds responsible for the activity. Multiple factors, such as volatility and synergistic effects, should be explored in depth to confirm if any variations in antibacterial activity are the result of either factor. From there, extractions, preparation, and analysis can be further refined to increase the yield of these compounds, minimizing product loss and maximizing compound detection. In order to identify any novel compounds, the novel compounds should be isolated from any already characterized Joe-Pye Weed essential oil components to determine if the antibacterial activity is attributed to already known constituents or a novel compound. When isolated at a high enough yield, a serial dilution of any novel compounds can be performed to determine MIC and MBC values.

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