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Isabella Cavagna April 10, 2023

Extraction and Bioactivity Testing of Alkaloids from Ethanolic Leaf Extract of *Amsonia tabernaemontana* Walter (Apocynaceae)

by

Isabella Cavagna

Dr. Cassandra Quave

Adviser

Biology

Dr. Cassandra Quave

Adviser

Dr. Kathleen Campbell

Committee Member

Dr. William Wuest

Committee Member

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Isabella Cavagna

Dr. Cassandra Quave

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An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Sciences

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Abstract

Extraction and Bioactivity Testing of Alkaloids from Ethanolic Leaf Extract of *Amsonia tabernaemontana* Walter (Apocynaceae)

By Isabella Cavagna

Amsonia tabernaemontana Walter, also known as the eastern bluestar, belongs to the Apocynaceae family and is native to North America. It is frequently found in yards and gardens as a low-maintenance and disease-resistant flowering plant. Several other species within the *Amsonia* genus have uses in traditional medicine and have been followed up with an analysis of the chemical profiles of the plants and their respective bioactivity. Few studies of the alkaloid content have been conducted on this species. The plant was collected, ground, and macerated before it underwent acid-base extraction. The resulting partitions were screened for alkaloid content using normal-phase TLC and Dragendorff's spraying reagent as a color indicator for heterocyclic nitrogen, revealing an alkaloid-rich partition. An initial screen of the partitions for antimicrobial activity against *Staphylococcus aureus* revealed inconclusive results. Further evaluation of the bioactivity of this fraction and purification of the partitions are needed to determine any potential bioactivity.

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Chapter 1:

Introduction

1.1 Antibiotic Resistance

Bacteria have evolved to become resistant to almost every category of antibiotic that has been developed. While the discovery and use of antibiotics have changed the medical industry and saved millions of lives, the misuse of these drugs has led to unnecessary exposure and thus increased resistance. Antibiotics have had life-altering effects on the global medical community, treating and curing life-threatening infections. They also assist in treating infection in patients recovering from surgery or chemotherapy as well as increasing life expectancy from 56 years old to almost 80 years old (Ventola, 2015). However, the positive effects of these drugs may become obsolete as bacterial resistance becomes more prevalent. Easily transmissible diseases such as tuberculosis, gonorrhea, typhoid fever, and Type B *Streptococcus* have become more difficult to treat as community-acquired antimicrobial resistance is increasingly common across the world (Llor and Bjerrum, 2014). Microbes can resist antibiotics by four different mechanisms: decreasing drug penetration, pumping drugs out of the cell, modifying the target component of the membrane, or rendering the drug inactive. The genes that enable these bacteria to resist antibiotics are selected by exposure to even the smallest amount of a drug. It kills the non-resistant strains leaving behind only the hypermutable, resistant bacteria that can exchange genetic information through lateral gene transfer (Reygaert, 2018). This process leads to even more concerning multi-drug resistant bacteria, which lack susceptibility to several antibiotics and prove even more dangerous for those who are infected by these strains.

Table 1.1: Bacterial Mechanisms of Antibiotic Resistance (Reygaert, 2018)

Mechanism	***Method of Reaction***	***Common Species***	***Drug Resistance***
Limit Drug Uptake	1. Changes to outer layer (cell wall thickness, LPS[1] layer) 2. Reduce quantity or increase specificity of membrane porins 3. Biofilm formation	- *Staphylococcus aureus* - *Enterobacteriaceae* - *Pseudomonas aeruginosa* - *Neisseria gonorrhoeae*	- Vancomycin - Carbapenems - Beta-lactams - Tetracycline - Imipenem
Drug Efflux	1.Efflux pumps upregulated - ABC[2] - MATE[3] - SMR[4] - MFS[5] - RND[6]	- *Vibrio cholerae* - *Vibrio parahaemolyticus* - *Staphylococcus epidermidis* - *Acinetobacter baumannii*	- Fluoroquinolones - Tetracycline - Aminoglycosides - Ampicillin - Erythromycin - Vancomycin
Modify Drug Target	1. Altering membrane receptors 2. Genes coding for a structural change to peptidoglycan	- *Staphylococcus aureus* - *Enterococci*	- Vancomycin - Daptomycin - Oxazolidinones - Streptogramins
Inactivate Drug	1. Degradation of drug 2. Group transfer to drug	- *Enterobacteriaceae* - *Staphylococcus aureus* - *Enterococcus faecalis* - *Enterococcus faecium*	- B-lactamases - Tetracycline

[1]Lipopolysaccharide layer; [2]ATP Binding Cassette; [3]Multidrug and Toxin Exclusion; [4]Small Multidrug Resistant; [5]Major Facilitator Superfamily; [6]Resistant Nodulation cell Division

Initially, one of the most forceful drivers of resistance comes from the overuse and misuse of antibiotics. Inappropriate use of antibiotics comes from misdiagnosis of viral infections which are unable to be treated by antibiotics, as well as the increasing prevalence of antibiotic use in agriculture and livestock. Antibiotics are often added to livestock food to prevent infections and stress-related diseases and also help promote growth (Isaacson and Torrence, 2002). This use has contributed to the pool of resistant pathogens which can increase the emergence of resistant pathogens in humans. A variety of factors contribute to this issue including demographic characteristics, such as socioeconomic statistics of different countries, as well as attitudes and expectations from doctors and patients (Housby and Mann, 2009).

More recent studies estimate the global disease burden of antimicrobial resistance as 1.27 million deaths directly attributable to antimicrobial resistance and 4.95 million deaths associated with antimicrobial resistance (Murray *et al.,* 2019). Increased use of antibiotics is associated with increased resistance, and countries with higher rates of income inequality have high higher rates of resistance (Kirby and Herbert, 2013). The income inequality used in the study is defined by the Standardized World Income Inequality Database based on a comparative analysis of disposable income, market income, absolute redistribution, and relative redistribution. Furthermore, even though the antimicrobial resistance crisis disproportionately affects populations in under-developed countries, it is growing more prevalent in more developed countries. Primary care physicians in certain countries in Europe prescribe 80-90% of all antibiotics, and 80% of antibiotic use in the United States comes from agricultural use (Llor and Bjerrum, 2014). Antibiotics are often incorrectly prescribed to treat respiratory infections caused by viruses, such as common colds, bronchitis, and ear and throat infection. In light of this crisis,

there is a dire need for alternative and complementary drugs and therapies to replace the common use of antibiotics.

1.2 Ethnobotany and Phytochemistry

One area of promising research initiatives for alternative and complementary treatments to antibiotics includes investigating the chemical components of plants or natural products. This research path has already become a leading source of innovation in drug development. Between the years 1981 and 2019, there have been 1881 approved natural product agents across therapeutic areas of study (Newman and Cragg, 2020). Many of the drugs used today such as anticancer treatments, immunosuppressants, painkillers, and more are derived from plants. Within plants, chemicals used for defense make up almost 10% of plant mass, signifying that there is unlimited potential to study the interactions of these chemicals (Abelson, 1990). The study of plant-derived compounds and their mechanisms of action within their host organism can be used as a guide for natural protective mechanisms from bacteria. To find a more targeted method to search for novel drugs within the natural world, many scientists have turned to an ethnobotanical approach. By studying traditional uses of plants as medicine, researchers can reduce the time it would take to screen thousands of plants for bioactivity. Instead, the historical use of plants can guide researchers toward species that already have evidence of medical use. Modern analytical techniques can identify novel compounds that account for the plant's bioactivity.

Many ethnobotanists ideally want to bring the traditional medicines they study from their native regions toward becoming FDA-approved botanical drugs. As of 2023, there are only two approved botanical drugs, sinecatechins (Veregen®) and Crofelemer (Mytesi™) (FDA, 2022). Sinecatechin is an extract made from the green tea leaves of the *Camellia sinensis* (L.) Kuntze

(Theaceae) plant. It is used as a topical treatment for genital and perianal warts (Tyring, 2012). Crofelemer is a formulation made from the red latex of the *Croton lechleri* Müll.Arg. (Euphorbiaceae) tree from South America. Crofelemer is used to treat diarrhea in people who have HIV by blocking chloride-ion channels in the intestines (Chordia and MacArthur, 2013). *C. lechleri* has an interesting history of ethnobotanical use in the Western Amazon Basin, where several indigenous groups used the red latex from the tree to treat many different ailments ranging from preventing bleeding from cuts, wound healing, skin infections, and gingivitis (Carlson, 2004). Ethnobotanists realized the medicinal potential of the plant and began the necessary steps required to gain the approval of the compound as a Botanical Drug from the Food and Drug Administration.

Using this ethnobotanical approach to drug development has led to a vast collection of plant compounds to investigate. Phytochemicals are natural chemicals that aggregate in specific parts of plants and can have specific bioactivities. There are over 4,000 types of phytochemicals with a range of bioactivity that enables the plants to defend themselves from environmental dangers (Saxena *et al.,* 2013). They are also commonly referred to as plant secondary metabolites; the molecules are nonessential for growth, development, and reproduction in plants, but aid in protection from the environment, communication with other plants and non-harmful microorganisms, and attract pollinators (Yang *et al.,* 2018). Some important phytochemicals include phenolics, terpenoids, and alkaloids. Phenolics are among the most numerous secondary metabolites, mainly functioning as a defense compound in plants. Their role in humans is slightly different as they have been found to possess antioxidant properties.

Another phytochemical class of interest is alkaloids. They are the largest group of plant natural products and have a wide range of pharmacologic properties (Porras *et al.,* 2021). They

are heterocyclic compounds that are typically alkaline in nature due to their secondary or tertiary amine groups. Alkaloids can also have quaternary amines that exist as salts or amide groups, both of which render the molecule neutral (Heinrich *et al.,* 2018). These chemical properties enable alkaloid compounds to have an impact on various human systems. When alkaloids are protonated in acidic conditions, they can be transported throughout the body, and in basic and neutral conditions, they become neutral, lipid-soluble compounds that can pass through biological membranes. Alkaloids with tertiary nitrogen can interact with neurotransmitters and affect the central nervous system (Verpoorte, 2005). They can act as stimulants or anesthetics such as caffeine, morphine, and nicotine. Plants with these compounds have been used in traditional Chinese, Indian, and Islamic medicines such as antiseptics, analgesics, and for many other purposes (Bribi, 2018).

1.3 Alkaloids

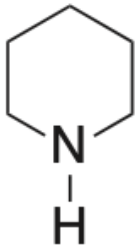


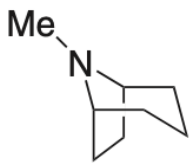
Figure 1.2: Basic Piperidine Alkaloid Structure

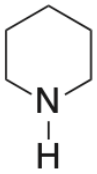
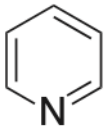
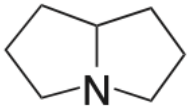
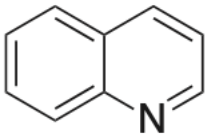
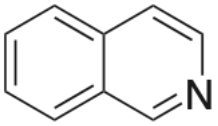
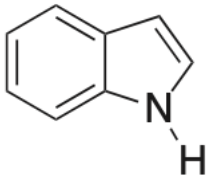
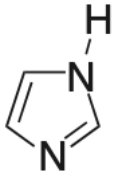
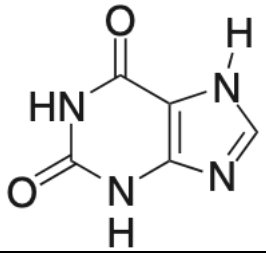
Alkaloids are primarily found within plants but have been discovered in fungi, bacteria, and even some animals. Their role is still being studied, but they are used for self-defense, communication, and as a deterrent for species that would eat the plant. Alkaloids can act as both proton donors and acceptors, thus giving them the ability to hydrogen bond with many biomolecules such as enzymes and other proteins. There are three main general classes of

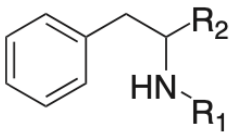
alkaloids based on how they are derived. There are amino-acid-derived classes true alkaloids and protoalkaloids, and then also psuedoalkaloids which are synthesized from a side product in the amino acid synthesis pathway (Dey *et al.,* 2020). Within these classes, there are various subclasses relating to their chemical structures and properties.

Alkaloids can function as feeding deterrents against herbivores due to their inhibition of certain metabolic pathways. They also act as phytoanticipins and phytoalexins in plants as a form of an antimicrobial innate plant immune system. The difference between phytoanticipins and phytoalexins is that the former are compounds present in plants before the presence of a pathogen is detected and provide chemical barriers, and the latter are compounds that begin to be expressed after the pathogen is detected to induce the secretion of antimicrobial compounds at the infection site (González-Lamothe *et al.,* 2009). While there are many ways to classify alkaloids, the main technique of classification is based on their structure, mainly focusing on the placement of the nitrogen within the heterocycle.

Table 1.3: Alkaloids Classes and Bioactivity (Heinrich *et al.,* 2018)

Alkaloid Class	**Molecule Structure**	**Bioactivity**	**Alkaloid Example**
Tropane		Stimulant, anticholinergic, hallucinogenic, mydriatic	Atropine, Cocaine, Scopolamine, Hyoscyamine

Piperidine		Poison, stimulant, addictive	Coniine, Arecoline
Pyridine		Stimulant	Nicotine
Pyrrolizidine		Hepatotoxic	Senecoinine
Quinoline		Anti-malarial	Quinine, Chinchonine
Isoquinoline		Analgesic, opiates, anesthetic	Morphine, Berberine,
Indole		Antihypertensive, hallucinogenic, anticonvulsant	Reserpine, Inogaine, Strychnine
Imidazole		Cholinergic, pupillary constrictor	Pilocarpine
Purine (xanthine)		Stimulant, vasodilatory	Caffeine, Theobromine

Phenykalkylamine		Stimulant, hallucinogenic, vasoconstrictor, bronchodilator	Ephedrine, Mescaline

Alkaloids have been the structural inspiration for many modern medicines. Much of current drug development has focused on synthesizing products using natural products as the structural scaffold (Cushnie *et al.,* 2014). One example of this is the drug quinine, a derivative of the quinolone alkaloid. Quinine has been used as an anti-malarial for many years, first isolates from the bark of the *Cinchona calisaya* Wedd. (Rubiaceae) tree in 1820 by Pierre Joseph Pelletier and Joseph Bienaime Caventou. There was a folk tale of the bark being used by native groups in the Andean jungle by creating a decoction of the bark in water. They took extracts of the bark to cure fevers and other ailments (Achan *et al.,* 2011). Discovering new alkaloids can aid in the process of drug discovery not only in that the compounds themselves can have direct biological activity, but also because they can contribute to the scientific understanding of how molecules interact with the body and provide starting blocks for more complex compounds for novel drugs.

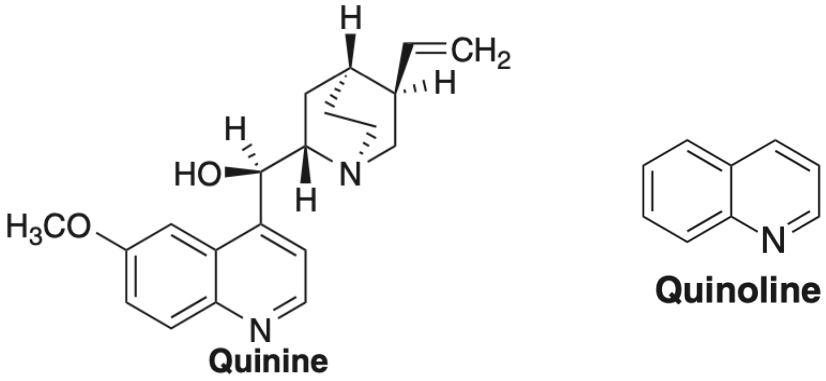


Figure 1.4: Example of the structure of quinoline (right) as a structural base for the more complex compound quinine (left) that has antimalarial activity (Heinrich *et al.,* 2018).

1.4 *Amsonia* Genus

Figure 1.5: Examples from the *Amsonia* genus; Top Left: *Amsonia ciliata* Walter or Fringed Bluestar (Garland, 2023); Top Right: *Amsonia ciliata* Walter var. *texana* (A. Gray) J.M. Coult. Or Texas Bluestar (Garland, 2023) (Rechenthin, 2023); Bottom: *Amsonia tabernaemontana* Walter or Eastern Bluestar (Mohlenbrock, 1989)

The *Amsonia* genus belongs to the Apocynaceae family and species in this genus are referred to as Bluestars. These taxa are characterized by blue and white flowers that cluster together and bloom for several weeks during the spring and summer. Twenty-two species within this family are native to a variety of regions within North America, as well as Southern Europe, Turkey, Japan, and China. There are only a few documented uses of this genus in traditional

medicine. One species native to China, *Amsonia elliptica* (Thunb.) Roem. & Schult, has been used in a decoction to cure chills and induce sweat as a treatment for illnesses (Murray, 1819). Another species, *Amsonia tomentosa* Torr. & Frém., has been utilized by the Native American Zuni tribe, where it was ground into a poultice and used to treat rattlesnake bites in conjunction with several other plants (Stevenson, 1915).

While *Amsonia* plants have not become the most highly sought after source of novel compounds within the world of drug discovery, there have been many discoveries of bioactive compounds, especially alkaloids in addition to other phytochemicals within the plant. There are several studies on the alkaloids of the two aforementioned *Amsonia* plants in addition to *Amsonia brevifolia* A. Gray. Studies have shown that indole alkaloids have been isolated and undergone pharmacological testing (Sharma *et al.,* 1988) (Ozaki, 1989).

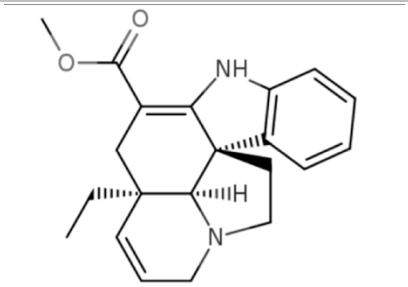
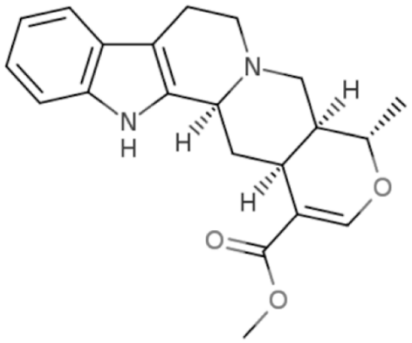
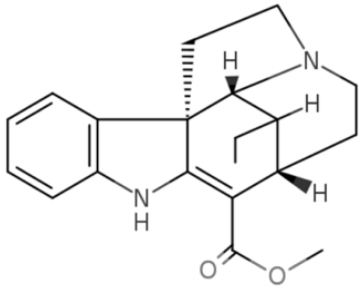
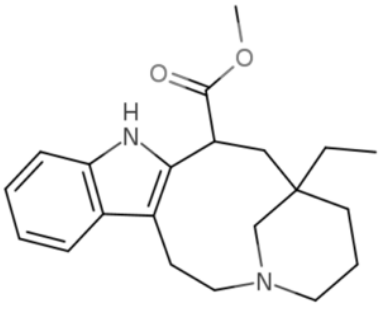
1.5 *Amsonia tabernaemontana* Walter (Apocynaceae)

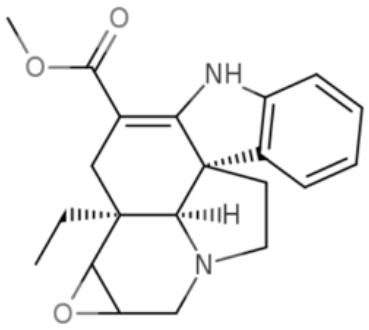
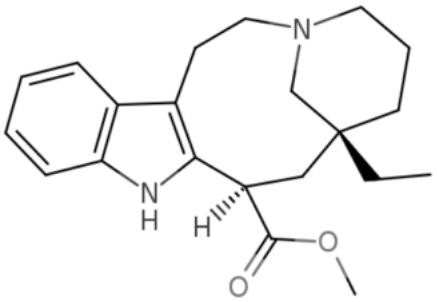
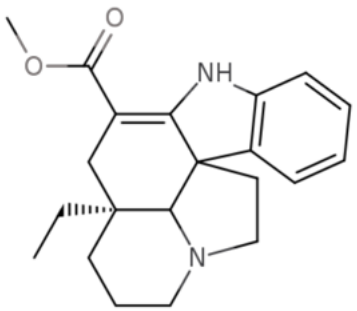
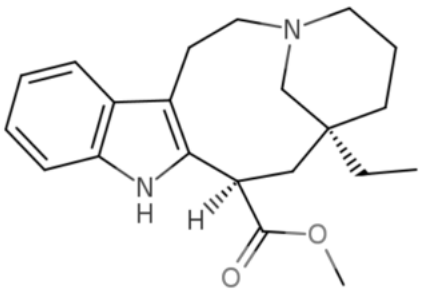
Figure 1.6: Photo of *Amsonia tabernaemontana* Walter (Apocynaceae) plant specimen before collection for the Emory Herbarium and Quave Lab in Baker County, Georgia, 2019 (Emory Herbarium, 2023).

Amsonia tabernaemontana Walter (Apocynaceae) is more commonly referred to as the Eastern Bluestar. It is native to North America and can be found in several eastern regions in the United States. Of the 22 *Amsonia* species, *A. tabernaemontana* is the most common. It has bright blue flowers that grow in small clusters and bloom in the springtime. This is a perennial plant that is commonly found in North American regions where there are wet or moist woods. More specifically, this includes areas of coastal plains from the United States in New Jersey toward more southern interior regions in states of central Illinois, southern Indiana, and Kansas (World Flora Online, 2023).

There have been several studies conducted on this species, however, none of them focused on the alkaloid content of the plant as a potential antimicrobial. There was one study that suspended root cell cultures of *A. tabernaemontana* in different conditions and measured organogenesis and alkaloid production. The study references that tabersonine was previously isolated from the seeds of the plant. Using thin-layer chromatography and Dragendorff reagent, alkaloids were identified in the root cell cultures. The study confirmed the presence of alkaloids tabersonine from seeds and dehydroaspidospermidine from roots (Furmanowa and Rapczewska, 1981). Several other studies have focused on this extraction of tabersonine from the seeds, however very little has been done in studying other parts of the plant concerning alkaloid content. There have been other studies of *A. tabernaemontana* that focus on the bioactivity of cell cultures of the plant. Two studies specifically used suspended cell cultures of the seedlings and found that the cultures had aminopeptidase activity as well as α-galactosidase activity (Poór, 1997) (Stano *et al.,* 2005). The known compounds in *Amsonia tabernaemontana* Walter are in the **Table 1.7**.

Table 1.7: Known compounds in *Amsonia tabernaemontana* Walter

Chemical	Structure	Molecular Weight (g/mol)
Tabersonine/Tabersonin		336.4
Tetrahydroalstonine		352.4
Tubotaiwin		324.4
Vincadin		340.4

Lochnericine		352.4
(+/-)-epivincadine		340.5
Ervamin		338.4
(+/-)-vicadine		340.4

1.6 Research Aims and Hypothesis

Considering the growing issues around antibiotic resistance, it is important to focus on alternative therapies and innovative pathways for drug discovery. This pressing need to find new antimicrobials can be relieved by using natural products for new drug synthesis. The main goal of this project is to isolate alkaloids from the Quave Natural Products Library (QNPL) to determine the presence of antimicrobial properties. The QNPL is a vast collection of 2,500 traditional medicinal plant extracts collected from plants growing in the Mediterranean, North Africa, the Caribbean, the USA, the Balkans, and Asia. A literature review on the species in the collection was performed by postdoctoral fellow, Dr. Gina Porras, and plants known to be rich in alkaloids were identified. From this point, samples with larger material mass were chosen to begin a more detailed screening process. The focus will be on optimizing the alkaloid extraction methods made by Dr. Porras and isolating these secondary metabolites from *Amsonia tabernaemontana* Walter (Apocynaceae). I hypothesize that alkaloids from *A. tabernaemontana* will have antimicrobial properties because many other species in the *Amsonia* genus show evidence of containing alkaloids.

Chapter 2:

Methods

The methods for the extraction and bioactivity testing of the alkaloids from leaves of *Amsonia tabernaemontana* Walter (Apocynaceae) are as follows: Plant collection and processing; Ethanolic maceration; Acid-Base partitioning; Chemical analysis; Bioassays. The final goal is to repeat the chemical analysis and bioassays until pure alkaloid isolation.

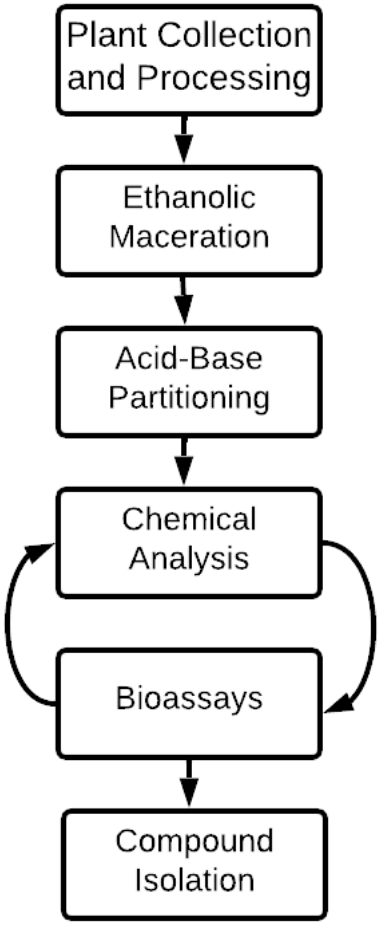


Figure 2.1: Schema of the methods for the extraction and bioactivity testing of alkaloids in *Amsonia tabernaemontana* L. Walter

2.1 Plant Collection

Amsonia tabernaemontana was collected on June 26, 2019, in Baker County, Georgia at the Ichauway Plantation. The roots and leaves were separated and brought back to the Quave Laboratory for processing.

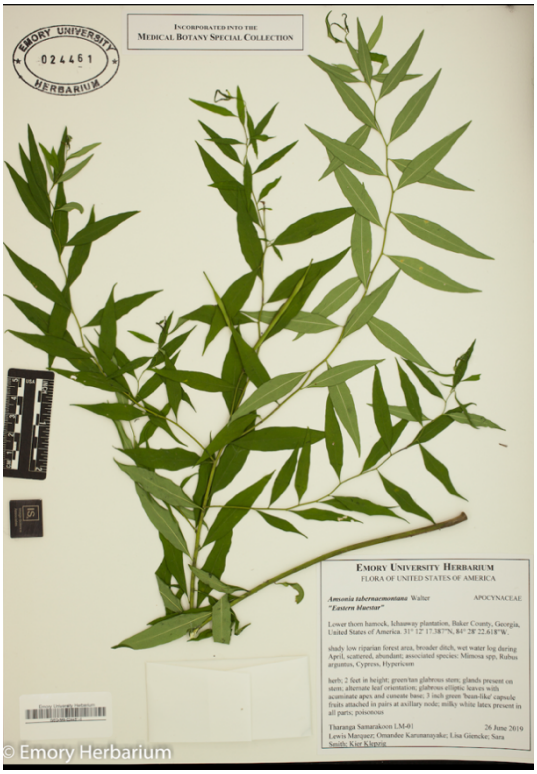


Figure 2.2: Digitized Emory Herbarium *Amsonia tabernaemontana* L. Specimen (Emory Herbarium, 2023)

The materials were dried and then ground in a Thomas-Wiley Mill at a 2 mm mesh size to create a fine powder. The plant voucher was made and used for identification.

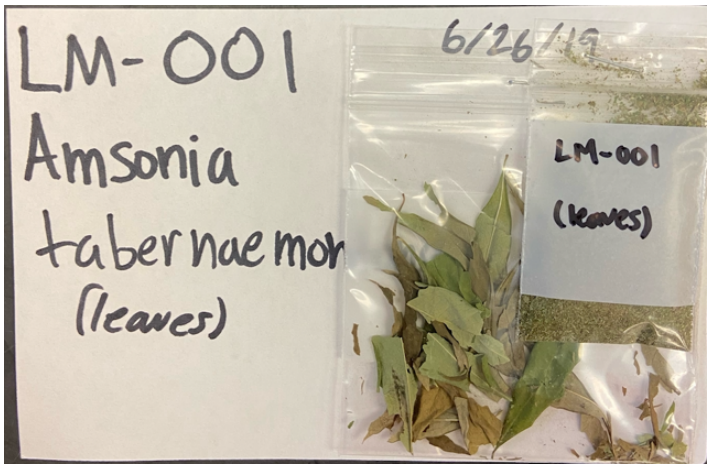


Figure 2.3: *Amsonia tabernaemontana* Plant Voucher

2.2 Plant Maceration

To prepare the crude plant extract, the ground leaves underwent maceration in 80% ethanol. Using a 1:10 ratio of grams to milliliters, 40 g of plant parts were placed in 400 mL of 80% aqueous ethanol, stirred, and allowed to soak in the solvent for 72 hours. The filtrate was separated from the plant parts using coarse and fine vacuum filtration with Fisherbrand P8 and P2 filter paper respectively. The filtrate was set aside, and another 400 mL of 80% methanol was combined with the plant parts for a second maceration. The same steps were followed again before the combined filtrates were condensed and dried using a rotary evaporator at 38°C. The remaining extract was dissolved in 200 mL of deionized water for resuspension and freezing in a round-bottom flask. The water content was removed using a lyophilizer before the leftover plant extract was removed and stored in a -80°C freezer for storage.

2.3 Acid-Base Partition

To further separate the alkaloids from the other compounds in the plant extract, acid-base liquid separation was used to separate the alkaloids based on their relative acidities. Two grams

of extract from dried leaves of *A. tabernaemontana* were dissolved in 100 mL of 1 M hydrochloric acid (HCl) by stirring it with a stir bar for one hour. The solution should have a pH of 0. The HCl solution was then added to a 500 mL separatory funnel to be partitioned with ethyl acetate (EA) before changing the pH to 9 with ammonium solution and then partitioned against dichloromethane (DCM) and water-saturated *n*-butanol (BuOH). Using a 1:1 ratio of aqueous to organic solvent, 100 mL of EA was added to the funnel as the organic phase with 100 mL of HCl as the aqueous phase before the funnel was gently swirled in a figure-eight formation 10 times. The funnel was allowed to separate for 15 minutes or until two separate layers formed. The bottom layer of HCl was drained and set aside from the top layer of EA. The HCl fraction was added to the funnel with an additional 100 mL of EA and the above separatory steps were repeated until the EA layer was colorless. This took three rounds of partitioning with EA. The pH of the aqueous HCl partition was changed to 9 by slowly adding 30 mL of 25% ammonium solution in increments of 5 mL on a stir plate. The basic aqueous phase was added to the funnel with 100 mL of DCM as the organic phase and partitioned in the previously mentioned method three times. The final partition used the basic aqueous phase and water-saturated *n*-BuOH. This partition was repeated until the *n*-BuOH was colorless after separation which took four repeated partitions instead of three. The remaining 120 mL of aqueous solvent, 300 mL of EA, 300 mL of DCM, and 400 mL of *n*-BuOH were condensed and dried separately using a rotary evaporator and the final percent yields were calculated based on the initial 2 g of crude extract using the percent yield equation.

$$\%\, Yield = \frac{Mass\ (g) Extract\ from\ Partitions}{Initial\ Mass\ (g) of\ Crude\ Extract} \times 100$$

Equation 2.4: Percent Yield Equation

2.4 Solid Phase Extraction

Solid-Phase extraction was performed using a Sep-Pak C18 column. 200 mg of the *n*-BuOH dried partition was dissolved in 1mL of distilled water. The column was equilibrated by adding the following solvents 1 mL at a time: 6 mL of distilled water, 4 mL of 50% MeOH, 4 mL of 100% MeOH, and 4 mL of 100% acetone. The 1 mL dissolved *n*-BuOH extract was placed in the column and allowed to wash into the filter. The following solvents were added to the column 1 mL at a time, only adding the next milliliter when the previous one washed past the filter: 5 mL of distilled water, 3 mL of 50% MeOH, 3 mL of 100% MeOH, and 3 mL of 100% acetone. Each different solvent was eluted into separate containers. The partitions were condensed using a rotary evaporator and the percent yields were calculated using **equation 2.4**.

2.5 Thin-Layer Chromatography (TLC)

2.5.1 Alkaloid Screening TLC:

Silica thin-layer chromatography plates with foil backing were used to screen for the presence of alkaloids in the crude plant extract as well as the acid-base partitions. Samples were diluted in 100% methanol (MeOH) at a concentration of 10 mg/mL. The samples were spotted on the TLC plates using several different mobile phases to best visualize results. The different mobile phases used were either dichloromethane and methanol (DM) or chloroform, methanol, and water (CMW). The mobile phases used were as follows: 5:1 DM; 50:1 DM; 10:4:1 CMW; 25:4:1 CMW. The TLC plates were first viewed under UV light at high and low wavelengths for

compound visualization and photographed. The presence of alkaloids was identified using Dragendorff's spraying reagent, which reacts with secondary and tertiary amines and appears on the TLC plates as a dark orange color. R_f values were calculated for alkaloid bands.

$$R_f = \frac{Distance\ traveled\ by\ solute}{Distance\ traveled\ by\ solvent}$$

Equation 2.5: Calculating R_f values

2.5.2 TLC Plates Used for Bioautography

Silica thin-layer chromatography plates with glass backing were used for bioautography. The glass plates were cut to fit a standard 100 cm Petri dish. The plates were sterilized by spraying them with methanol and allowing them to dry in the sterile bacterial hood. The DCM extracts were diluted to a 10 mg/mL concentration in 100% MeOH. Three samples were spotted on each TLC plate 5 times. The mobile phase for the TLC was a ratio of 10:4:1 CMW. The plates were visualized under UV light of high and low wavelengths and certain plates were sprayed with Dragendorff spraying reagent for alkaloid visualization.

2.6 Bacteria Culturing

The bacteria were first T-streaked on Tryptic-Soy Broth Agar (TSA) plates for 18 hours at 35°C. One isolated colony was selected from the plate and was mixed into 3 mL of Tryptic-Soy Broth (TSB). The cultures were placed in an incubator overnight at 35°C with shaking to 200 rpm at a slight angle. The overnight culture was removed at 18 hours and the strain was standardized to a cell density of 0.0006 (5 × 105 CFU/mL) at 590 nm light.

Table 2.6 Bacterial Strain and Media Type Used

Strain	Species	Characteristics	Media
UAMS-1	*Staphylococcus aureus*	Methicillin sensitive strain; clinical isolate originally cultured from an osteomyelitis infection	Tryptic-Soy Broth

2.7 Bioautography

Bioautography assays were performed using the methods for the TLC Plates for Bioautography. These plates were prepared in a sterile environment after being treated with the mobile phase and sample. TSA was prepared and 10 mL was added to 100 cm Petri dishes. After cooling, the TLC plates were carefully laid on top of the agar. The UAMS-1 *S. aureus* was prepared in overnight culture in TSB before being standardized. The soft agar was prepared by adding 0.5% agar in TSB and autoclaving the mixture. Once sterilized, the soft agar was cooled until 35°C before the standardized bacteria were added. 10 mL of the inoculated soft agar was quickly poured over the TLC plates on the hard agar while being careful not to directly pour over the plate and only around it. The assay was placed in an incubator for 18 hours at 35°C.

2.8 Single Dose Response

To determine growth inhibition of bacterial strain UAMS-1, the crude DCM, EA, and BuOH extracts from the acid-base extraction experiment were diluted to 10 mg/mL in 100% DMSO as a stock solution, and then further diluted to 128 µg/mL. Media (100 µL) was added to the wells of a 96-well plate. Next, 97.4 µL of strain standardized bacteria was added before 2.6 µL of

either the crude extracts, DMSO, gentamicin, oxacillin, water, and media was added as well. The gentamicin and oxacillin functioned as positive controls, the DMSO and water as the vehicle controls, and the media blank as the negative control. The optical density of each well was documented before the plate was incubated for 18 hours at 35°C. The values read after 18 hours were used to calculate the percent inhibition of the bacteria by the extracts.

$$\% \text{ Inhibition} = 1 - \frac{OD_{t18} - OD_{t0}}{Avg(OD_{C18} - OD_{C0})} \times 100$$

OD_{t18} = Optical Density of the Extract treated wells at 590nm at 18 hours post incubation

OD_{t0} = Optical Density of the Extract treated wells at 590nm at 0 hours post incubation

OD_{C18} = Optical Density of the Control treated wells at 590nm at 18 hours post incubation

OD_{C0} = Optical Density of the Control treated wells at 590nm at 0 hours post incubation

Equation 2.7: Percent Inhibition Equation

Chapter 3:

Results

3.1 Alkaloid Screening & Extraction

Initial literature reviews and screenings of the QNPL identified the crude extract of the leaves of *Amsonia tabernaemontana* Walter as a possible source of alkaloids. To determine the presence of alkaloids, a liquid-liquid acid-base partition was performed on the *A. tabernaemontana* 80% ethanolic extract. A total of five 2 g partitions were performed. The partition tree with percent yields of each organic phase is shown in **Figure 3.1.**

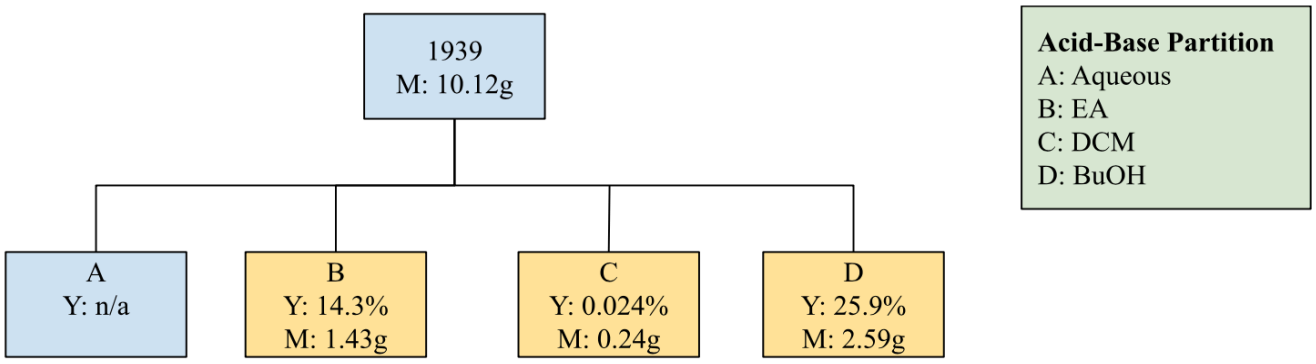


Figure 3.1: Overall partition scheme and percent yields of the total acid-base partitions.

The aqueous partition A was unable to fully evaporate due to an insoluble compound, NH_3Cl made from the basic pH change of the aqueous partition. The mass of partition A was much higher than the theoretical 59.78% yield resulting from the remaining extract. Without definitive yields, the partition was marked as n/a or not applicable. The individual percent yields for each organic partition are shown in **Table 3.2.**

Table 3.2: Percent Yields by each Acid-Base Extraction

Partition Number	Ethyl Acetate (% Yield)	Dichloromethane (% Yield)	*n*-Butanol (% Yield)
1	38.4	0.5	27.9
2	20.5	2.7	32.7
3	4.7	3.5	22.0
4	3.7	2.9	25.3
5	4.3	2.3	21.7

Variation in the percent yields of the EA fraction was most likely due to the crude extract itself. The extract had components that were wet and clung together and drier, dust-like particles. For the first few extractions, the dry, dust-like portion of the extract was used while the wet and stickier portion was used for the later extractions. The different consistency of the crude extract could have been caused by different components clinging together and thus having different percent yields in the final extraction.

To confirm the presence of alkaloids, foil-backed TLC plates were used in conjunction with Dragendorff's spraying reagent. The acid-base partition samples were diluted in 100% methanol to a concentration of 10 mg/mL. The extracts were applied to the TLC plate and different mobile conditions were tested to determine the compound composition of the plant. Various mobile conditions were used to better visualize the chemical components of the partitions. Once the TLC plate was run and dried, the spraying reagent was applied to the plate, coloring it a light orange while coloring the alkaloids a darker orange. Dragendorff's reagent reacts with heterocyclic nitrogen, precipitating a yellow/orange color. **Figure 3.3** illustrates a TLC plate under high and low wavelength UV as well as the plate with Dragendorff's reagent.

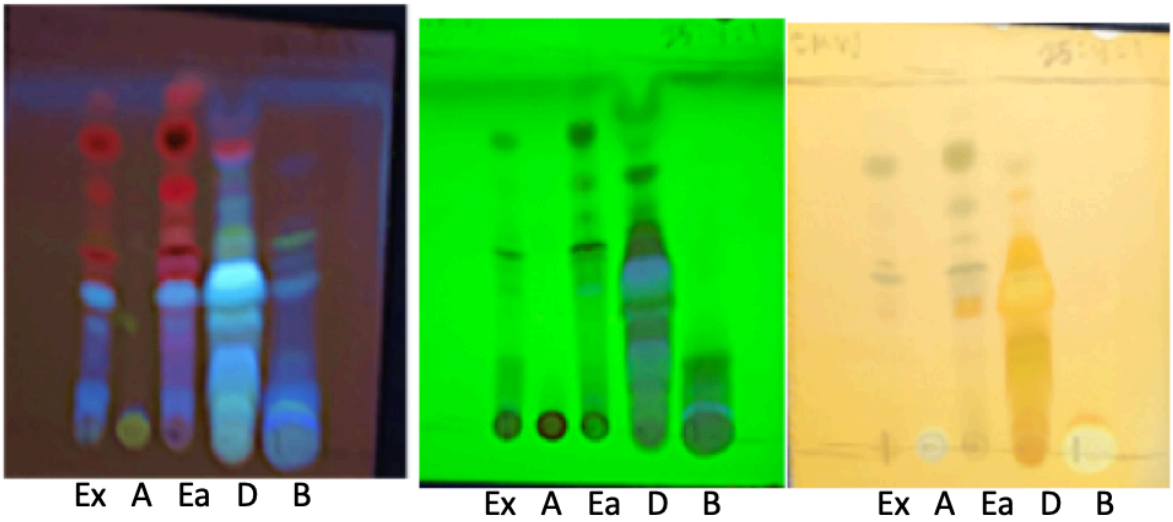


Figure 3.3: Example of Foil TLC spotting of samples with 10 mg/mL concentrations in the following order (left to right): Extract; Aqueous; Ethyl acetate, Dichloromethane, Butanol. The plates are visualized with UV rays at 254 nm (green) and 365 nm (blue), and after Dragendorff's spraying reagent was applied (orange). The mobile phase used was 25:4:1 CMW.

The DCM partition had the most intense orange color, signifying that it had the highest concentration of alkaloids within the extract. The BuOH partition had a very light-colored alkaloid spot, however it had the largest percent yield of the organic partitions. Therefore, The DCM and the BuOH partitions were further investigated. The R_f values for the alkaloids were calculated for the original extract as well as for the individual organic partitions shown in **Table 3.4** and **Table 3.5.**

Table 3.4: R_f values for alkaloids in the *A. tabernaemontana* extract on foil TLC plates. Different mobile phase conditions were tested. DM condition is the ratio of DCM to MeOH. CMW is the ratio of chloroform to methanol to water.

Mobile Phase Condition	Rf value of Alkaloids in Crude Extract
5:1 DM	0.73 0.90
50:1 DM	0.04 0.14 0.28
25:4:1 CMW	0.31 0.37
10:4:1 CMW	0.31 0.95

Table 3.5: R_f values for alkaloids in the different organic partitions of the *A. tabernaemontana* extract on foil TLC plates. Different mobile phase conditions were tested. DM condition is the ratio of DCM to MeOH. CMW is the ratio of chloroform to methanol to water.

Mobile Phase Condition	Rf value of Alkaloids in Acid-Base Partitions		
	DCM	**EA**	***n*-BuOH**
5:1 DM	0.69 0.93	0.58	0.25
50:1 DM	0.06 0.15 0.25	0.14 0.25	0.04
25:4:1 CMW	0.35 0.50 0.67 0.87	0.49	0.09
10:4:1 CMW	0.30 0.86	0.92	0.05 0.89

3.2 Solid Phase Extraction

First focusing on further purification of the BuOH organic partition, solid-phase extraction (SPE) was performed on 200mg of the BuOH extract. A Sep-Pak C18 column was used for this extraction to try to remove some accessory sugars and compounds in the partition. Distilled water, 50% MeOH, 100% MeOH, and 100% ACN were used as the mobile phase. **Figure 3.6** shows the yields from the extraction.

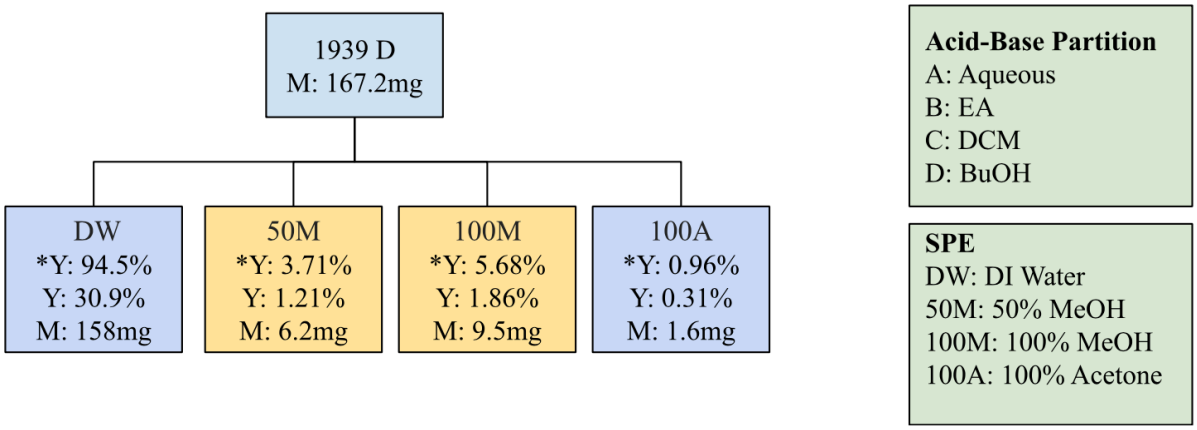


Figure 3.6: SPE of partition D (BuOH partition) and the four crude yields and percent yields in the fraction. SPDW: Sep-Pak Distilled Water, SP50M: 50% MeOH, SP100M: 100% MeOH, SP100A: 100% Acetone.

There was a yellow-colored line in the filter of the column from the initial 1 mL wash with the BuOH sample. The compounds in the filter eluted slightly during the 50% MeOH wash, but the majority came out in the 100% MeOH wash. The 100% ACN wash was to remove any remaining compounds in the filter. The different components of the SPE were analyzed using TLC and Dragendorff's spraying reagent in a mobile phase of 10:4:1 CMW. The R_f values were calculated and are presented in **Table 3.7**.

Table 3.7: R_f values for the alkaloids from the SPE of the BuOH partition

Rf value of Alkaloids from SPE			
DW	**50M**	**100M**	**100A**
n/a	0.19	0.19 0.28 0.58 0.71	n/a

Most of the alkaloids eluted in the 100% MeOH wash of the column. There were four distinct bands on the TLC plate for this extract and one band in the 50% MeOH extract. No bands appeared in the distilled water and 100% ACN extracts. The distilled water extract held most of the yield, while the MeOH extracts had very little of the final material. Due to the low yield of the SPE, the DCM partition continued to be the focus of this analysis.

3.3 Bioautography

Before proceeding with further chemical analysis, bioautography was used to determine if the alkaloids in the DCM partition had activity against bacteria. *Staphylococcus aureus,* the strain UAMS-1, was used against the extract. UAMS-1 is an oxacillin-sensitive strain of *S. aureus*, and therefore makes a good candidate for antibacterial testing. TLC plates were made for the bioautography using the consistent extract concentration of 10 mg/mL. The mobile phase used was 10:4:1 CMW because there were two distinct alkaloid bands with R_f values of 0.31 and 0.86. The distance between these bands should have allowed distinction between any possible zones of inhibition in the assay. The DCM extract was spotted three times on five glass-packed TLC plates. Two were used for the bioautography assay, two were set aside for possible scraping and chemical analysis, and one was sprayed with Dragendorff's reagent as shown in **Figure 3.8** along with the R_f values of the alkaloids in **Table 3.9**. An additional glass-TLC plate was run in the mobile phase with no sample on it to be used as a control for any effect the mobile phase may have on the UAMS growth.

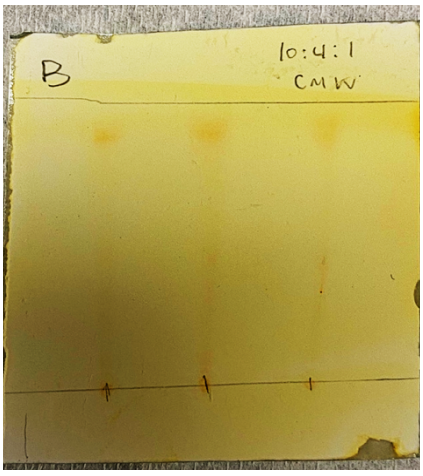


Figure 3.8: Glass-backed TLC Plate for Bioautography. The DCM partition was spotted three times at a concentration of 10 mg/mL. The mobile phase used was 10:4:1 CMW.

Table 3.9: Average R_f values for the DCM partition on the glass-TLC plates

Band	Upper Band	Lower Band
R_f Value	0.92	0.42

The R_f values on the glass-TLC plates were very similar to the ones on the foil-TLC plates. While the lower band was less distinct than the upper band, the distance between them was still far enough to be able to differentiate zones of inhibition. The TLC plates were laid in the hard agar and overlaid with the inoculated soft agar. Once cooled, the plates were set in an incubator for 18 hours at 35°C. During the second time period, they were removed from the incubator and photographed.

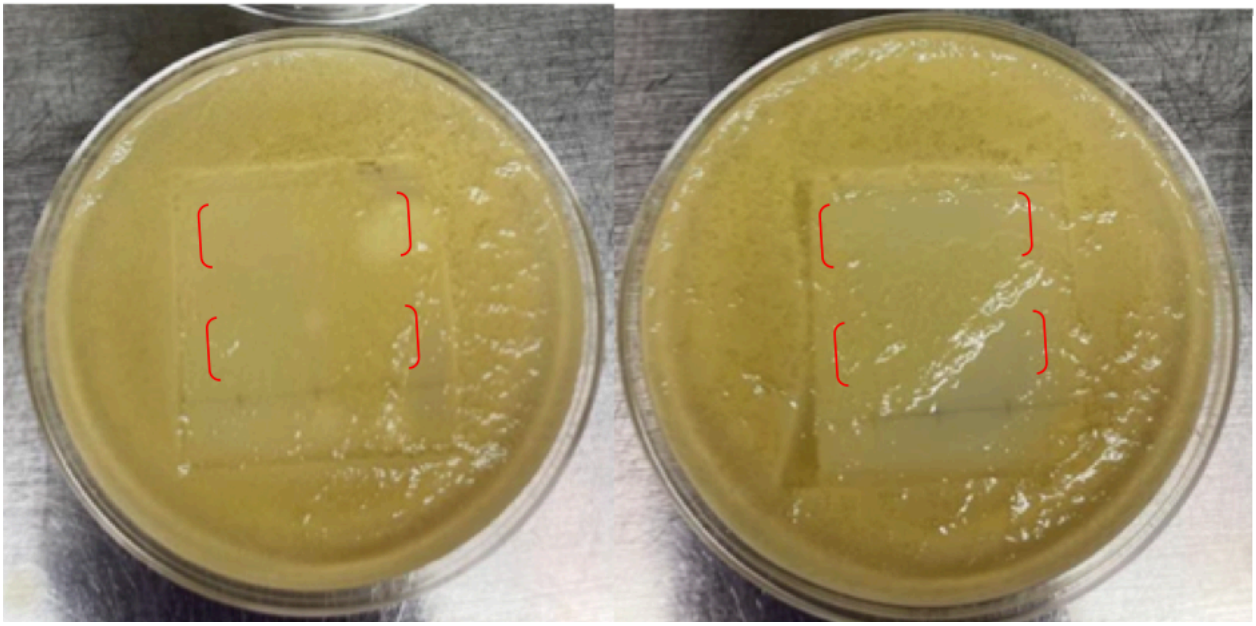


Figure 3.10: Bioautography of glass-TLC plates with DCM samples spotted three separate times. The right plate appears to have a zone of inhibition near the bottom-right and a very faint zone of inhibition across the top. The left plate has very little inhibition of growth.

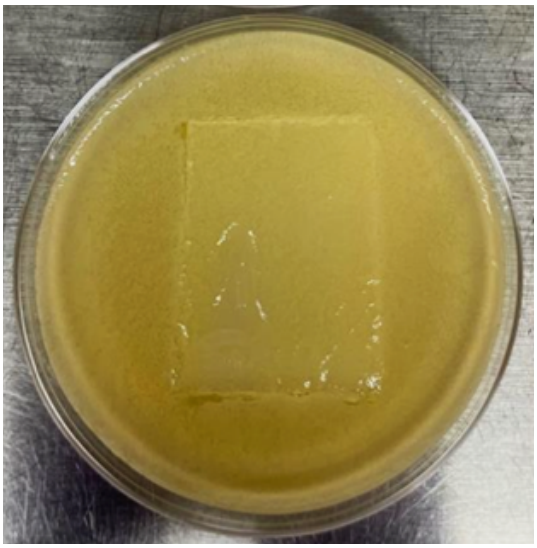


Figure 3.11: Bioautography of glass-TLC plates with only the mobile phase as the control.

The two plates with the DCM sample appeared to have some areas with less growth but it was not consistent across both assays. There seems to be some inhibition of growth by the upper band, however, more replicates of this experiment are needed to determine the antibacterial properties of the alkaloids within the DCM extract. Additionally, there is some inhibition on the control, but not everywhere. This implies that the solvent does not inhibit the growth of UAMS-1.

3.3 Single Dose Response

A single dose response assay was performed on the different acid-base extracts. The samples tested were DCM, EA, butanol, oxacillin, and gentamicin in addition to a growth control, vehicle control, and water control. The average percent inhibition of each extract against UAMS-1 is documented in **Table 3.12** in addition to their standard deviations.

Table 3.12: Average Percent Inhibition of the Acid-Base Extracts and Antibiotics

Extract	*DCM*	*EA*	*BuOH*	*OXA*[1]	*GENT* [2]
Average % Inhibition	15.80	-5.96	-12.16	100.12	99.98
Standard Deviation	6.32	5.59	5.17	0.04	0.08

[1]Oxacillin; [2]Gentamicin

The results from the single dose response illustrate that the extracts do not have activity against the *S. aureus* strain UAMS-1. The EA and BuOH have no inhibition whatsoever, however there is some inhibition of growth in the DCM extract. While this is not significant enough for further testing for antibacterial testing in *S. aureus*, further purification of the alkaloid compounds is nevertheless recommended to examine potential bioactivity in other systems or against other pathogens.

Chapter 4:

Discussion

4.1 Contributions to Science

The ethanolic extract from the leaves of *Amsonia tabernaemonata* Walter has several different alkaloids within them. The various bands on the TLC plates imply that there are several molecules to investigate and characterize regardless of their bioactivity. There is not a vast collection of literature focused on this plant regarding chemical composition as well as bioactivity. Previous experiments have focused on the roots and seeds of the plant and have not intended to extract and characterize the alkaloids. The molecules from *A. tabernaemontana* still have the potential to offer the scientific community more knowledge and understanding of the chemical makeup of plants. They can provide the structural scaffolding of other antibacterial compounds or could have inhibitory potential against other pharmacologically relevant targets. Additionally, the methods and procedures used in this project can be applied to many other species used in traditional medicine to determine new sources of bioactive natural products. With the increasing prevalence of life-threatening antimicrobial resistance, it is vital to continue efforts to find new and innovative approaches to treat bacterial infections. Using the countless resources on the use of traditional medicine will only streamline the discovery process and maintain awareness of the underused resources found in traditional medicine.

4.2 Study Limitations

This study had several limitations. The first of which was a low yield from the original liquid-liquid acid base extraction. The yields of the DCM, BuOH, and EA partitions made it

difficult to gather enough materials for chemical analysis. More plant material would have increased the yield however in 2 g increments, the extraction process would take several weeks still to gather enough of the crude extract and organic partitions. Increasing the partition amount would also be very challenging due to using HCl as the acidic aqueous phase. In larger volumes, it can be very dangerous to work with. With more time and materials, further purification steps could be taken to improve the results of the bioassay and definitively state which compounds are antibacterial, if any.

4.3 Future Directions

The next steps of this project would begin with increasing working materials and performing preparatory high-performance liquid chromatography. This analytical chemical technique would facilitate the isolation of the different compounds in the organic partitions. The EA partition could also be further tested once the alkaloids are isolated. With more refined extracts, bioautography can be repeated with a higher concentration of alkaloids. Growth inhibition assays would also contribute to ascertaining whether the alkaloids are antibacterial. Regardless of the bioactivity of the compounds, the purified alkaloids can be characterized, and known compounds identified using mass spectrometry, or unknown compounds resolved using orthogonal approaches to structural elucidation (by LC-MS/MS and NMR techniques). As the alkaloid content of this species is poorly understood, there is a potential for the discovery of novel compounds that can contribute to the scientific understanding of plant secondary metabolites and the *Amsonia* genus as a whole.

Chapter 5:
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