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Antimicrobial Properties of Native American Herbal Tea: *Hypericum hypericoides*
Senior Project

In partial fulfillment of the requirements for
The Esther G. Maynor Honors College
University of North Carolina at Pembroke

By

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Biology
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## Abstract

Patients with obesity and type II diabetes are characterized by an altered gut microbiome. Antimicrobial agents may be a possible avenue to restore normal gut microbiota. These experiments were designed to test *Hypericum hypericoides* or St. John the Worker, a traditional medicine of the Lumbee Native American tribe of North Carolina, for antimicrobial properties on thirteen different bodily bacteria. Experiments from Spring 2018 suggested that plant-paste made from *Hypericum hypericoides* had antibiotic properties. For further experiments, the thirteen bacteria were streaked over three Mueller-Hinton agar plates per bacteria, and each plate was divided into four sections with three different antibiotics and one disc of water per plate as a negative control. The third plate per bacteria had two different antibiotics, one water disc, and 75% plant-paste to test *Hypericum hypericoides* antibiotic properties against seven standard antibiotics. The plant-pastes for all experiments were made with approximate concentrations of 100% and 75% with 0.5g of plant to 0.5mL of water and 0.375g of plant to 0.5mL of water respectively. Experiments on Mueller-Hinton agar plates instead of bacteria specific growth media illustrated inhibition on three of five bacteria. Since both experiments yielded antibiotic inhibition to some degree, we also performed the same tests done on the Mueller-Hinton agar plates on Tryptic Soy Agar and Luria Broth Agar plates to compare the antibiotic standard inhibition zones to the inhibition results seen from the plant-pastes in Spring 2018. The results suggest growth inhibition of five of the thirteen studied species: *Corynebacterium xerosis, Micrococcus luteus, Bacillus subtilis, Staphylococcus aureus,* and *Neisseria sicca.* Our experimental data suggest a possible avenue of therapy by using traditional medicines to target altered microbiomes in obesity and type II diabetes.

# Antimicrobial Properties of Native American Herbal Tea: *Hypericum hypericoides*

## Introduction

Traditional plant-based medicines are a primary source for new treatments within pharmacology. However, some potential treatments remain understudied, particularly those from Native American tribes. The Lumbee American Indians from Pembroke, North Carolina have many traditional remedies that potentially house new treatments for various illnesses. Particularly, a plant in the same family as St. John's Wort (*Hypericum perforatum*) named St. Andrew's Cross (*Hypericum hypericoides*), is traditionally used in Lumbee medicine as a tea to treat various infections and metabolic disorders. St. Andrew's Cross is a native perennial plant found in the southeast United States, and is more specifically referred to as St. John the Worker by the Lumbee people.

Since St. Andrew's Cross is used to treat metabolic disorders, our lab was curious in its potential usage as a treatment for Type II Diabetes, a metabolic disorder that disproportionately affects Native American communities. One key characterisitic in individuals with Type II Diabetes is a markedly decreased diversity of bacteria within their gut microbiome (Sankaranarayanan et al., 2015). Our lab was interested in the antibiotic properties of the St. Andrew's Cross plant as a way to restore a normal more diverse gut microbiome to individuals with Type II Diabetes. Since this plant is used to treat metabolic disorders, perhaps it holds antibiotic properties to help normalize the microbiome of the Type II Diabetic gut.

To test this, we made a hot tea from the roots, stems, and leaves of St. Andrew's Cross and tested the aqueous tea extract and the leftover moist plant-paste against different bacteria that live commonly on the skin of the human body. From these experiments, we can determine if the plant has antibiotic properties and will be able to move forward into anaerobic gut bacteria inhibition tests. We will also be able to perform different extraction methods in the future to further isolate the antibiotic compound within the plant. Our results show that traditional medicines could be a possible avenue to help treat the altered gut microbiomes in Type II Diabetes.

## Methods and Procedures

*Plant Collection and Processing*

To collect the *Hypericum hypericoides* plant, we travelled to Sampson's Landing in Robeson County, North Carolina with Dr. Lisa Kelly who helped us identify the plant. We found the plant near a lake and dug up the entire plant to place in a Zip-Loc bag to bring back to our lab. We dated and placed the bags in a freezer at - 80° Celsius until needed for further use.

Prior to performing the experiments with aseptic technique, we would take a bag of plant from the freezer and pull out one whole plant of *Hypericum hypericoides.* The rest was placed back into the freezer. Using scissors sterilized by flame, we chopped up the root, stem, leaves, flowers, and seed-pods of the entire plant into a clean mortar and pestle. We ground the plant as fine as possible until we could weigh 0.5g and 0.375g of the ground plant matter. Any excess plant left unground was placed back into the bag and placed into the freezer again. Excess plant that was ground was placed into a 50mL tube labelled "Pre-Ground *Hypericum hypericoides*" and placed in the freezer to be used for later spring 2018 experiments. Excess ground plant in fall 2018 and spring 2019 were thrown away so that freshly ground plants were used each time.

*Hypericum hypericoides* that was used in spring 2018 was harvested in the winter of 2017-2018, while plant used in fall 2018 and spring 2019 were harvested in the summer of 2018.

*Preparation of Aqueous Extracts*

Using the rule of 1mL of water is equivalent to 1g of mass, we knew that a 100% tea concentration would be 0.5g of plant matter to 0.5mL of distilled deionized water. Accordingly, a 75% tea concentration would have 0.375g of plant to 0.5mL of distilled deionized water. We weighed and allotted the plant samples into separate 1.5mL Eppendorf microfuge tubes labelled with the desired tea concentration and pipetted the appropriate volume of water into the tubes afterwards. In some instances, the water would not sink through the entire compacted plant mass. When this happened, we only placed half of the plant matter into the tube and added 0.250mL of water so that the first half would be fully moistened. Then, we added the rest of the plant and another 0.250mL of water to finish the setup. After the plant and water mixture was created, each tube was placed in a hot box at 62° Celsius for twenty minutes to brew. This procedure was used in all experiments.

For the spring 2018 experiments, after brewing, plant matter was pressed deeper into the tube with a sterile plunger and the tubes were centrifuged to pull plant particulate from the hot water extract. We labelled two clean separate Eppendorf 1.5 mL microfuge tubes with 100% $H_2O$ Extract and 75% $H_2O$ Extract to place the separate tea extracts in after centrifugation. Once the liquid was separated from the plant matter, we pipetted the aqeous extract off the top and placed it in the proper 100% or 75% Extract tube. Then, these tubes were all centrifuged again to pull any more plant particulate found in the liquid extracts to the bottom of the tube. Finally, we had four separate Eppendorf 1.5 mL microfuge tubes, one 100% Plant Matter tube with moist plant matter that was used to make the tea, a 100% Extract tube with the liquid from the 100% Plant Matter tube, a 75% Plant Matter tube, and a 75% Extract tube. Teas were made in the same day experiments were performed to maintain the freshness of the tea.

For the fall of 2018 and spring of 2019, the same procedure was performed, although since we were no longer testing aqueous extracts it was unnecessary to centrifuge the plant particulate to the bottom of the tube. We only used the 75% plant-paste in the fall 2018 and spring 2019 experiments as there was no significant difference between the inhibition seen with the 100% or 75% plant paste in the spring 2018 experiments. The distilled deionoized water was left on the plant to maintain moisture so that potential aqueous extract could diffuse in the agar, unlike the plant-pastes from spring 2018 that would have dried out faster due to centrifugation.

*Bacterial Strain Culturing*

Bacterial cultures were taken from pre-made glycerol stocks from the same freezer the frozen plant was kept in. We took thirteen bacteria from the cultures: *Corynebacterium xerosis, Micrococcus luteus, Bacillus subtilis, Staphylococcus aureus, Neisseria sicca, Enteroccoccus faecalis, Kelbisella pneumoniae, Escherichia coli, Proteus vulgaris, Proteus mirabilis, Enterobacter aerogenes, Pseudomonas aeruginosa,* and *Staphylococcus epidermidis.* Originally, we plated *C. xerosis, M. luteus, B. subtilis, N. sicca, E. faecalis,* and *K. pneumoniae* on Tryptic Soy Agar (TSA). *S. aureus, E. coli, P. vulgaris,* and *P.* mirabilis were all placed on Luria Agar (LA). *E. aerogenes, P. aeruginosa*, and *S. epidermidis* were placed on Nutrient Agar (NA). We kept these plates in a cold room at 4°Celsius after one overnight incubation for the entire lab to use for their own experiments.

One day prior to the actual experiment, we took bacteria from these starter plates and, with aseptic technique, transferred the bacteria from the plates into 3 mL of appropriate liquid culture that was the broth form of the media from the starter plates. Preliminary experiments ruled out eight bacteria, therefore for these experiments we only grew these five bacteria: *Corynebacterium xerosis, Micrococcus luteus, Bacillus subtilis, Staphylococcus aureus,* and *Neisseria sicca.* Four grew in Tryptic Soy Broth, and *S. aureus* grew in Luria Broth (LB) for all experiments. The cultures were left overnight to grow in a shaking incubator set at 37° Celsius.

*Agar Diffusion Assay*

For spring 2018 experiments, we gathered five pre-made plates; four Tryptic Soy Agar (TSA) plates and one Luria Agar (LA) plate. Each plate was divided into six defined testing sections and labelled with the date, initials, bacteria to be tested, and the lab name. Zone 1 was 100% Plant Matter, Zone 2 was 100% Hot Aqueous Extract (Tea), Zone 3 was 75% Plant Matter, Zone 4 was 75% Hot Aqueous Extract (Tea), Zone 5 was 70% Isopropanol Rubbing Alcohol as a positive control (90% Isopropanol Rubbing Alcohol for *C. xerosis* tests), and Zone 6 was a distilled deionized water sample as a negative control. We also hole-punched Whatman filter paper to make the 6mm disks used to place samples on the bacteria-coated agar for

our experiments and kept them in another sterile 50mL Falcon Tube labelled "Sterile Filter Disks."

Using cotton swabs and aseptic technique, bacteria were spread in a lawn across the appropriate agar plate. Then, filter disks were placed first into the 100% Plant Matter Eppendorf 1.5 mL microfuge tube with flame sterilized tweezers and a sample taken out of the tube on the sterile 6mm disk. The disk was then placed in Zone 1 labelled for 100% Plant Matter. The process was repeated for Zones 2, 3, 4, 5, and 6 with each Zone having the set sample mentioned above whether it was plant-paste or the tea . The positive controls 70% isopropanol and 90% isopropanol, and the negative control distilled deionized water were stored in and taken from sterile 50 mL Falcon Tubes.

After all the disk samples were placed in the appropriate section, we placed the assays for overnight incubation at 37°Celsius. Plate results were recorded the following day. Zone of inhibition diameters were measured with a ruler in millimeters, and assays were photographed before and after diameter measurements. Measurements were placed in an Excel spreadsheet, and the 6mm size of the disk was subtracted from the diameter to leave the true diameter of inhibition for each value in the results section.

For fall 2018 and spring 2019 experiments, the above procedures were followed, only all bacteria after the overnight broth incubation were plated on Mueller-Hinton (MH) agar plates and spread with an L-spreader instead of cotton swabs. The Mueller-Hinton plates are standard plates employed in the Kirby-Bauer disk diffusion assay (Bauer, Kirby, Sherris & Turck 1966). There were nine plates for each bacteria and the experiments were performed in sets of three. These experiments prioritized testing the average inhibition of seven different antibiotics against each bacteria and how it compares to the average 75% *Hypericum hypericoides* plant-paste inhibition on the same plates. Each plate was split into four quadrants, and there were a total of twelve quadrants for each individual experiment for inhibition against a bacteria. The first plate for each experiment had Amoxicillin (20μg/10μg Clavulanic acid) in zone 1, Chloramphenicol (30μg) in zone 2, Erythromycin (15μg) in zone 3, and distilled deionized water as a negative control in zone 4. The second plate had Streptomycin (10μg) in zone 5, Ciprofloxacin (5μg) in zone 6, Sulfamethoxazole (23.75μg/1.25μg Trimethoprim) in zone 7, and distilled deionized water as a negative control in zone 8. The last plate for each experiment had Neomycin (30μg) in zone 9, a 75% *Hypericum hypericoides* plant-paste in zone 10, a randomly selected antibiotic in zone 11, and distilled deionized water as a negative control in zone 12. These three plates were considered one experiment for each tested antibiotic and paste against a specific bacteria. There were at least three duplicate trials for each of the five bacteria for each antibiotic and plant-paste on the Mueller-Hinton plates.

This same setup was also used for the testing of each antibiotic against the bacteria on their respective agar plate such as those seen in spring 2018 for comparison. All

five bacteria except for *S. aureus* were placed on TSA plates, and *S. aureus* was tested on LA plates. The only difference in the setup was that the last plate in zone 10 where 75% plant-paste was there was now another random antibiotic for extra testing.

Each antibiotic disk size was 6mm in diameter. To control for this, a paper wrapped sterile plastic restaurant straw with a diameter of 6mm was snipped short to use as a "cookie-cutter" to control the amount of 75% *Hypericum hypericoides* plant paste that was placed on each Mueller-Hinton agar plate. The 75% paste was spread onto flame sterilized aluminum foil and cut to the 6mm diameter with the straw and placed onto the MH plate with flame sterilized tweezers.

## Results

The goal of our experiments was to illustrate the antimicrobial properties of our plant in comparison to various antibiotics. The antibiotics were amoxicillin, chloramphenicol, erythromycin, streptomycin, ciprofloxacin, sulfamethadoxizole, and neomycin. Values illustrate the diameter of the zone inhibition minus 6mm to account for the size of the antibiotic discs.

**Table 1** illustrates the antibiotic standards obtained by placing each antibiotic disk on the MH plates against all five of the chosen bacteria. Overall, 16 of 35 antibitoics tested on the MH plates were significantly different from the same antibiotic experiments done on TSA or LA plates seen in **Table 2** based on a two-tailed t-test. This shows that 19 of the 35 antibiotic values cannot be compared as easily to the standardized antibiotics on the MH plates, and overall have a different range of inhibition on the TSA and LA plated bacteria. Notably, all antibiotics that were tested against *C. xerosis* on both TSA and MH agar plates were not statistically different. *B. subtilis* only had statistically different inhibition ranges between the erythromycin and streptomycin antibiotics. *S. aureus* only had statistically different inhibition ranges with streptomycin and neomycin between LA and MH plates.

For the bacteria that antibiotics did not show a significant difference in inhibition, **Table 3** shows the average zones of inhibition seen when the 75% plant-paste and the 100% plant-paste were placed on the MH, TSA, or LA plates. The spring 2018 plates were all TSA except for *S. aureus* which was plated on LA. The fall 2018 and spring 2019 experiments show decreased inhibition in comparison to the spring 2018 experiments, and the 75% plant-paste on the MH plates only inhibited *M. luteus, B. subtilis,* and *S. aureus* bacteria.

**Table 1:** Table showing the average zone of inhibition of each antibiotic against five bacteria on Mueller-Hinton Agar plates with standard deviation. Antibiotics are Amoxicillin (Amo), Chloramphenicol (Chl), Erythromycin (Ery), Streptomycin (Str), Ciprofloxacin (Cip), Sulfamethadoxizole (Sul), and Neomycin (Neo). Numbers with a * represent a ($p<0.05$) between antibiotics on MH plates and TSA or LA plates.

| | **Average Zone of Inhibition (mm) (Mueller-Hinton Agar Standardization)** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Bacteria** | **Amo** | **Chl** | **Ery** | **Str** | **Cip** | **Sul** | **Neo** |
| ***C. xerosis*** | 19.8 ± 1.7 | 18.8 ± 1.7 | 17.0 ± 0.8 | 12.3 ± 1.2 | 20.3 ± 0.6 | 22.0 ± 0.0 | 10.3 ± 2.5 |
| ***M. luteus*** | 0.0 ± 0.0* | 16.0 ± 2.0* | 14.3 ± 0.6* | 9.0 ± 1.8* | 13.3 ± 1.0* | 10.3 ± 2.2* | 11.3 ± 3.2 |
| ***B. subtilis*** | 16.8 ± 1.5 | 18.5 ± 1.3 | 19.0 ± 1.7* | 13.3 ± 0.6* | 22.7 ± 1.2 | 21.0 ± 1.7 | 16.0 ± 2.2 |
| ***S. aureus*** | 24.0 ± 0.0 | 13.3 ± 0.6 | 13.5 ± 0.6 | 8.0 ± 0.8* | 18.3 ± 1.0 | 17.0 ± 2.7 | 11.7 ± 1.2* |
| ***N. sicca*** | 34.8 ± 3.1* | 21.7 ± 4.9* | 16.3 ± 2.5* | 3.0 ± 1.0* | 17.0 ± 1.0* | 10.8 ± 4.6 | 6.5 ± 1.3* |

**Table 2:** Table showing the average zone of inhibition of each antibiotic against five bacteria on Tryptic Soy Agar (TSA) and Luria Agar (LA) plates with standard deviation. *S. aureus* was the only bacteria grown on LA media. Antibiotics are Amoxicillin (Amo), Chloramphenicol (Chl), Erythromycin (Ery), Streptomycin (Str), Ciprofloxacin (Cip), Sulfamethadoxizole (Sul), and Neomycin (Neo). Numbers with a * represent a ($p<0.05$) between antibiotics on MH plates and TSA or LA plates.

| | **Average Zone of Inhibition (mm) (TSA and LA Comparisons)** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Bacteria** | **Amo** | **Chl** | **Ery** | **Str** | **Cip** | **Sul** | **Neo** |
| ***C. xerosis*** | 22.3 ± 8.3 | 19.8 ± 2.2 | 19.3 ± 2.6 | 12.3 ± 1.3 | 20.5 ± 3.0 | 20.0 ± 2.5 | 13.3 ± 1.2 |
| ***M. luteus*** | 41.3 ± 2.2* | 28.0 ± 1.6* | 25.8 ± 7.3* | 18.0 ± 0.8* | 20.0 ± 0.8* | 17.5 ± 1.3* | 16.3 ± 2.5 |
| ***B. subtilis*** | 18.3 ± 1.0 | 18.5 ± 3.1 | 22.8 ± 0.5* | 17.3 ± 0.5* | 22.8 ± 0.5 | 18.8 ± 0.5 | 16.0 ± 1.0 |
| ***S. aureus*** | 23.8 ± 1.3 | 12.8 ± 1.5 | 13.5 ± 0.6 | 4.5 ± 0.6* | 16.5 ± 0.6 | 13.8 ± 0.5 | 7.7 ± 0.6* |
| ***N. sicca*** | 47.0 ± 2.6* | 32.5 ± 0.6* | 36.8 ± 1.5* | 23.3 ± 1.0* | 22.3 ± 1.5* | 13.8 ± 2.5 | 17.3 ± 1.2* |

**Table 3:** Table showing the average zone of inhibition of each antibiotic against five bacteria on Tryptic Soy Agar (TSA) and Luria Agar (LA) plates. *S. aureus* was the only bacteria grown on LA media in spring 2018. Antibiotics are Amoxicillin (Amo), Chloramphenicol (Chl), Erythromycin (Ery), Streptomycin (Str), Ciprofloxacin (Cip), Sulfamethadoxizole (Sul), and Neomycin (Neo).

| | **Average Zone of Inhibition (mm)** | | |
|---|---|---|---|
| | **75% Plant Paste** | | **100% Plant Paste** |
| **Bacteria** | **Spring 2018 (TSA Plates)** | **Fall 18/ Spring 19 (MH Plates)** | **Spring 2018 (TSA Plates)** |
| ***C. xerosis*** | 3.5 ± 1.1 | 0.0 ± 0 | 2.7 ± 1.0 |
| ***M. luteus*** | 4.7 ± 1.4 | 5.0 ± 2.7 | 5.3 ± 1.6 |
| ***B. subtilis*** | 3.3 ± 1.0 | 1.0 ± 1.7 | 3.2 ± 1.1 |
| ***S. aureus* (LB Plates)** | 4.9 ± 1.4 | 3.3 ± 0.6 | 4.4 ± 1.5 |
| ***N. sicca*** | 3.9 ± 3.3 | 0.0 ± 0.0 | 4.1 ± 3.3 |

## Discussion

Extracts of plants are a common source of new medications. For example, St. John's Wort or *Hypericum perforatum* is often employed as a medicinal treatment for infectious diseases and mental disorders. Earlier studies have shown that *H. perforatum* shows antimicrobial properties towards oral bacteria (Süntar, Oyardı, Akkol & Ozçelik 2016). For *H. perforatum,* there are multiple compounds that could be helping to inhibit the growth of bacteria. Süntar et al. also cite Brondz et al., noting that hyperforin was isolated as a main antibiotic compound in *H. perforatum* from nonpolar extracts. However, Brondz et al. also found that hyperforin must be in large concentrations to inhibit bacterial growth. Since *H. perforatum* and *H. hypericoides* are within the same family of plants, similar antibiotic compounds may be found. Further experiments could focus on extracting flavonoids, hypericins, and hyperforins from *H. hypericoides* to test against these bacteria.

Compared to the antibiotics, the *Hypericum hypericoides* 75% plant-matter aqueous extracts had a lower level of inhibition. The antibiotic inhibiting these bacteria may not be extracted well with distilled deionized water, resulting in the lower antibiotic inhibition. In the future, pure extracts from the *Hypericum hypericoides* plant utilizing different extraction methods should be tested to determine the source of antibiotic inhibition. Perhaps ethanol extractions would yield more positive and

antibiotic inhibition. Perhaps ethanol extractions would yield more positive and consistant inhibition against these bacteria. Additionally, the tests performed in spring 2018 utilized whole plants harvested in the winter of 2017 – 2018 while the experiments performed in fall 2018 and spring 2019 utilized whole plant harvested in the summer of 2018. This may have resulted in a different concentration of the antibiotic compound within the plant at the different times of year due to the types of stress the plant was under. Although many of the antibiotics showed the same type of inhibition on both MH and original growth media plates, it is difficult to compare the spring 2018 original growth media plate inhibitions to the fall 2018 antibiotic standards. These variations may be because of the different plate types utilized between the experiments, or the difference between the time of year the plants were harvested. Future experiments would consist of having the plant harvested at the same time of year and tested on the MH plates only. Experiments with *Hypericum hypericoides* harvested during different times of year may yield interesting results, as would testing specific portions of the plant such as the leaves, stem, and roots separately to isolate where and when most of the inhibiting compound may be in higher concentration.

Past studies on other plant species have also yielded positive results for antibiotic properties. *Verbascum thapsus*, or common mullein, is an ancient medicinal herb that helps treat respiratory problems and urinary tract infections. Dulger, G; Tutenocakli & Dulger, B utilized a similar method as us to test *V. thapsus* for antibiotic inhibition of bacteria commonly found in urinary tract infections. They performed their extract with 95% ethanol, and found that their extracts compared well with standard antibiotics on Mueller-Hinton plates (Dulger, Tutenocakli, Dulger 2015). Their study provides a possible outline regarding how future experiments could be carried out with *H. hypericoides*.

## Conclusions

*Hypericum hypericoides* could potentially be utilized as a way to restore the natural gut microbiota in individuals with Type II diabetes. It has been used by the Lumbee people to treat metabolic and nutritional disoders like Type II diabetes, hinting that it may have healing properties like other plants seen within the Hypericaceae family. Our experiments confirm that *Hypericum hypericoides* does exhibit antibiotic properties against some commensal bacteria commonly found on and in the human body, although future experiments with more pure extracts from the plant would be ideal. For these experiments, the goal was to utilize similar methods that someone making the tea to drink would have used, thus illustrating that even at the level of an aqueous extract the plant could exhibit antibiotic properties.

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