December 2016

Volatile compounds and microbiota: Antibacterial properties in traditional preparation methods of medicinal herb Neurolaena lobata (Asteraceae)

Nina Djukic

Volatile compounds and microbiota: Antibacterial properties in traditional preparation methods of medicinal herb *Neurolaena lobata* (Asteraceae)

Nina Djukic

University of California, Berkeley

Department of Environmental Science, Policy, and Management

EAP Tropical Biology and Conservation Program, Fall 2016

16 December 2016

ABSTRACT

I assessed the differential effectiveness of traditional herbal preparation methods of medicinal herb gavilana (*Neurolaena lobata*) on an assay of four bacteria (*E. coli, S. aureus, Shigella* sp. and *Salmonella* sp.) known to cause food poisoning symptoms in humans. Medicinal plants are used both traditionally at home in many cultures and in the synthesis of pharmaceuticals. Their medicinal properties often come from their secondary compounds, chemicals that play a role in their ecophysiology and primarily defend them from herbivory. I prepared three types of *Neurolaena lobata* extracts - an oil, an alcohol, and a tea - and assessed the zones of inhibition of the bacteria around filter paper disks impregnated with each extract. These assessments were done "blindly" (without knowledge of the inoculum/extract combination on the Petri dish) and I compared the zones of inhibition of the treatments to those surrounding the control. Results showed no significant inhibition of any bacterial inoculum by any *Neurolaena lobata* treatment; the majority of disks showed no inhibition and did not differ from the control. However, oil seemed to be the least effective preparation method, *Shigella* sp. and *Salmonella* sp. appeared to be more resistant to antibacterial activity than *E. coli* and *S. aureus*, and *Neurolaena lobata* tea treatments appeared to increase in effectiveness at higher concentrations. There are still thousands of plants that have yet to be explored for antibacterial or other health-related properties, and diverse areas such as tropical forests must be protected in order for this potential to remain alive.

Compuestos volátiles y microbiota: Propiedades antibacterianas de métodos de preparación tradicionales de hierba medicinal *Neurolaena lobata* (Asteraceae)

RESUMEN

Evalué la eficacia de los métodos tradicionales de preparación de hierbas medicinales de gavilana (*Neurolaena lobata*) en un ensayo de cuatro bacterias (*E. coli, S. aureus, Shigella sp. y Salmonella sp*.). Es sabido que estas bacterias causan síntomas de envenenamiento de comida en humanos. Las plantas medicinales se usan tanto tradicionalmente en el hogar en muchas culturas como en la síntesis de fármacos. Sus propiedades medicinales a menudo provienen de compuestos secundarios, sustancias químicas que juegan un papel en su ecofisiología, principalmente como defensa contra herbivoría. Preparé tres tipos de extractos de *Neurolaena lobata* - aceite, alcohol y té - y evalué las zonas de inhibición de las bacterias alrededor de los discos de papel filtro impregnados con cada extracto. Estas evaluaciones se realizaron "ciegamente" (sin conocimiento de la combinación inóculo / extracto en la placa de Petri) y comparé las zonas de inhibición de los tratamientos con las que rodeaban el control. Los resultados no mostraron una inhibición significativa de ningún inóculo bacteriano por ningún tratamiento de *Neurolaena lobata*; La mayoría de los discos no mostraron inhibición y no difirieron del control. Sin

embargo, el aceite pareció ser el método de preparación menos eficaz. *Shigella sp*. y *Salmonella* sp. parecieron ser más resistentes a la actividad antibacteriana que *E. coli* y *S. aureus*, y los tratamientos con té *Neurolaena lobata* parecieron aumentar la eficacia en concentraciones más altas. Todavía hay miles de plantas en las que aún no se ha explorado sus propiedades antibacterianas u otras propiedades relacionadas con la salud, y áreas diversas como los bosques tropicales deben ser protegidas para que este potencial permanezca vivo.

Medicinal plants have been consumed by indigenous societies for thousands of years and continuing into the present. They are used as dietary supplements and spices, as cures for health maladies, and as tonics to relieve a variety of symptoms (Walls, 2009)**.** Beyond direct consumption of the plant, traditional preparation methods include teas and infusions, as well as decoctions, syrups, and tinctures. Western medicine's increasing interest in herbal medicines has also driven the creation of a variety of commercially available products including supplements, capsules, and essential oils (Walls, 2009).

The medicinal properties of plants often result from their secondary compounds, synthesized by plants for a variety of ecophysiological interactions including defending themselves against herbivory and attracting pollinators and other symbionts (Briskin, 2000). Chemicals used by plants that affect insect herbivore nervous systems may have similar effects on large mammals, due to the common ancestry of all multi-cellular organisms and their conserved cellular processes and pathways, which are affected by similar signaling molecules (Kennedy and Wightman, 2011). Research on pharmaceutical uses for compounds isolated from particular plants has in many cases scientifically affirmed their culturally acknowledged medicinal properties. It has also suggested that the preparation method of these plants may be important for bringing out these properties; recent studies on "novel drug delivery systems" (NDDS) for herbal drugs have demonstrated that many new methods (such as polymeric nanoparticles, nanocapsules, and liposomes) can more effectively harness the desirable compounds of the medicinal plants while protecting consumers from potential toxicity (Ajazuddin, 2010). However, the fact that many pharmaceuticals derived from plant compounds cannot be patented decreases the incentive for pharmaceutical companies to invest resources and make these products accessible. Thus, many people continue to use medicinal plants through traditional preparation methods, which may differ in effectiveness as well.

A medicinal plant used frequently (and without Western pharmaceutical influence) in traditional Central American medicine is gavilana or Jackass bitters (*Neurolaena lobata*). While digestive problems are one of its primary targets, it is used for everything from fevers and diarrhea to intestinal parasites and skin infections (Bernhardt, 2008). Most scientific studies that have been performed regarding the medicinal properties of *Neurolaena lobata* have simply included it in a list of basic tests of medicinal plants rather than studying it in particular (Chariandy *et. al.*, 1999; Cáceres *et al.*, 1998; Lentz, 1998). However, studies have shown that it is an "antifeedant" against caterpillars, that it contains sesquiterpene lactones effective against *Plasmodium falciparum*(a parasite that carries malaria to humans), as well as other protozoan parasites ((Passreitr, 1997; Francois *et al.* 1996; Berger *et al.*, 2001). Studies done by previous students (Chin, 2015; Burke and Sullivan, 2015; Schaller, 2008) have also pointed to differential effectiveness based on concentration and preparation method of *Neurolaena lobata*, and varying methods of other traditional preparations such as drying have been shown to have differential effects on the retention of phytochemicals in these plants (Mahanom *et al.*, 1999). There is also data, including from previous UCEAP students' studies (Chin, 2015; Burke and Sullivan, 2015; Schaller, 2008) that suggests this plant may have antibacterial properties.

The antibacterial potential of *Neurolaena lobata* is particularly interesting, as bacteria are responsible for a variety of infections, both external and internal, and for much digestive discomfort

(Dyer 2003). A few are known to be particularly pathogenic to the human gut, producing, depending on their strain, symptoms from food poisoning to dysentery ("Shigella", CDC, 2016). These include *Escherichia coli,* a gram-negative bacillus; *Staphylococcus aureus*, a Gram-positive cocci; *Shigella* sp., a Gram-negative, rod-shaped bacteria; and *Salmonella* sp., a Gram-negative, rod-shaped bacteria.

Traditional remedies from medicinal plants and prepared with traditional, non-laboratory methods are still used by many people to treat bacterial infections. Additionally, they are often used for minor ailments and preventative healthcare, especially where access to clinics and expensive pharmaceutical products is limited. For this reason, it is important in both a social and scientific context to understand how different methods of consuming herbal remedies affect the effectiveness of the secondary compounds that give them their medical properties, and whether these medical properties are effective due to antibacterial properties or for other physiological reasons.

In this study, I asked the question: how do three different traditional preparation methods of *Neurolaena lobata* affect its antibacterial properties when tested on an assay of bacteria known to cause food poisoning and stomach ailments in humans?

MATERIALS AND METHODS

Study Site

I conducted research from 20 November 2016 to 2 December 2016. I performed the bacterial experiments and prepared the *Neurolaena lobata* extracts in the laboratory at the Monteverde Institute. I collected *Neurolaena lobata* from a single ~4m tree behind the Monteverde Institute between 22 and 23 November 2016.

Lab Methods

I prepared *Neurolaena lobata* extracts using three methods that are traditionally used and require materials that can be found in any home. I collected all *Neurolaena lobata* leaves from the same plant and only collected them if they were lacking herbivore damage. I used only adult (full-size) leaves that were within my reach (~2m)**.** While I used a whole leaf for the preparation of the tea, as is the traditional method, I finely chopped leaves with scissors and then ground them with a mortar and pestle for five minutes for the tincture and the infused oil.

I made a tea or "infusion" (extracts in water) by steeping one fresh adult leaf (about 1.2g) of *Neurolaena lobata* in 235mL of boiling water just removed from the stove for ten minutes, using methods similar to those used by Chin (2015). This is the most frequent and traditional way the herb treatment is prepared (Balick and Arvigo, 1993). Due to lack of observed antibacterial activity after the second trial, I increased the tea concentration for trial three by boiling two leaves (~2g) in 125mL of water for 20 minutes, a method more similar to that used by Schallert (2008). I made a tincture (extract in alcohol), which is traditionally made by soaking leaves for two weeks in one part distilled water and three parts 80 proof vodka or other strong alcohol (Walls, 2009). I made my extract, using lab materials, by soaking 5g of fresh *Neurolaena lobata* leaf in a 50mL equal-parts mixture of 80% methanol and 1% HCL for 48 hours, similar to methods used by Chin (2015). A third herbal preparation method I made for this experiment was an oil infusion. I packed lightly dried and ground plant material, either left out for 12 hours or microwaved between paper towels for one minute and 30 seconds, in with a carrier oil. Traditionally, this would sit in the sun for two weeks, but in the interests of time, I heated 50mL of olive oil with 5g of *Neurolaena lobata* leaves dried overnight for two hours on low heat (Walls, 2009). I strained the oil and alcohol extractions to remove plant matter after 48 hours. I made the tea fresh the day of the experiment, and stored the other extracts in a cool, dark room in sterilized containers. For controls,

I used boiled tap water, a 1:1 ratio of methanol and HCl with no additives, and olive oil (from the same bottle as used for the infused oil) with no additives.

I plated all bacteria on Mueller-Hinton agar, which I prepared and sterilized. As an Agar-pouring guideline, I marked all of the plates at approximately 1/4cm from the bottom of the dish. Once cooled, I flipped and refrigerated the plates. Bacterial cultures were obtained from the company Pre-Lab and the Instituto Nacional de Aprendizaje in Costa Rica. Due to the high concentration of bacteria upon arrival, I had to isolate individual colonies before using. I diluted bacteria from the original plates received from the lab by using a sterile syringe to put 6mL of saline solution in a sterilized test tube. I then swabbed a colony from the original plate and onto a new plate of Mueller-Hinton agar. I used another sterile loop to transfer it to another part of the plate, and used a third sterile loop to spread it over the rest of the plate. I then swirled this loop in the saline solution and used a new sterile swab to plate its contents on a new Petri dish. I incubated these new plates at 37 degrees C for 24 hours and placed them in the fridge to halt growth once I observed the presence of individual colonies.

To test antibacterial activity of the *Neurolaena lobata* extracts, I performed a bioassay using a process similar to Chin (2015), Burke and Sullivan (2015) and the Bauer-Kirby disk susceptibility test (Bauer et al., 1966). I plated diluted cultures of *Staphylococcus aureus*, *Escherichia coli*, *Shigella* sp., and *Salmonella* sp. using modified Bauer method. First, I withdrew 3mL of saline solution with a sterilized syringe and filled a small test tube. Then, I used sterile loops to transfer isolated colonies of the bacteria being tested into this solution. Standard Bauer method calls for this suspension of bacterial colonies to be compared with a 0.5 McFarland turbidity standard; as I did not have access to materials for this mixture, I instead transferred a standard amount of colonies into the solution. A sterile swab was then used to plate the bacterial suspension. After dipping the swab into the solution and removing excess liquid on the wall of the tube, I streaked all over the surface of the agar with standard side-to-side motion, and repeated this twice, each after a 60 degree turn of the Petri dish, for a total of three times.

The number of colonies suspended in solution differed slightly by trial. For the main trial of my experiment, two colonies of *E. coli* (which had the largest colonies) and three each of *S. aureus, Shigella* sp., and *Salmonella* sp. were suspended in solution. For the follow-up trial, only *E. coli* and *S. aureus* were suspended in solution, with one and two colonies, respectively. In the first two trials, nine Petri dishes of each bacteria were plated, and three plates of each inoculum were used for treatments of each extract - oil, alcohol, or tea. I put three treated disks and one control in each plate, resulting in nine repetitions for every combination of treatment and bacteria. For the follow-up trial, I plated six Petri dishes of each bacteria, and used three dishes of each inoculum for alcohol treatments and three for tea. There were still nine repetitions for each combination. This resulted in 36 Petri dishes for the main trial and 12 for the follow-up.

To plate the extracts on the bacterial inoculum, I dipped a hole-punched piece of filter paper into a test tube of each extract. I removed the excess liquid by tapping on the side of the test tube, and I then placed the treated disk in a Petri dish that had been inoculated within the last 30 minutes. I sterilized the tweezers used to plate the disks with a burner between uses. I placed each disk in a separate quadrant at least 20mm from other disks; for each Petri dish, there were three disks of a given treatment as well as a control of the same medium the extract was made from without *Neurolaena lobata*. After I plated the discs, my advisor labeled the plates with a symbolic code unknown to me so that measurement could be done "blindly". I allowed the plates to sit in the incubator at 37 degrees C for 18 hours.

In addition to the negative controls included on each Petri dish, I also plated four positive control plates using a main-trial standard inoculum of each bacteria (*E. coli, S. aureus, Shigella* sp., and *Salmonella* sp.) and a suspension in solution of Amoxicillin, an antibiotic effective against a wide range of bacteria. I placed one disc in the center of each plate of bacterial inoculum using standard plating method. I incubated the plates at 37 degrees C for 18 hours.

Once I removed the Petri dishes from the incubator, I measured their inhibition zones with calipers. Due to small and patchy inhibition zones, this data was classified numerically and visually into three classes of inhibition: 0 (no inhibition), 1 (inhibition of 1mm around disk, patchy inhibition, or large (3-4mm)but incomplete inhibition), or 2 (3-4mm of partial inhibition or extremely clear 2mm inhibition).If I was uncertain about the inhibition zone around a disk, I looked at it with light under a dissecting scope. After completion of the experiment, I sterilized my workspace with 10% bleach and passed all contaminated tools through an ethanol-burning flame to sterilize them. Bacterial cultures and tools contaminated with inoculum were autoclaved at a the Clínica de Santa Elena with permission from microbiologist Gloriana Barrantes.

RESULTS

Overall, it did not appear that the *Neurolaena lobata* extracts had a predictable antibacterial effect. The majority of all treatments (67/90) resulted in a degree of inhibition of 0 (no inhibition -Figure 1). Due to contamination, some repetitions of the tests were not included for data analysis (nine tests of *S. aureus* and oil, three of *S. aureus* and tea, and three of *E. coli* and tea were contaminated). There was a statistical difference between the amount of repetitions that showed degrees 0 (none), 1 (slight), and 2 (significant) of inhibition (n=90, X^2= 17.69, df=6, $p<0.05$) across bacterial inoculums. From the graph, it would appear this is due to the large number of 0's relative to 1's or 2's (Figure 1). There was no statistical difference between the amount of tests with inhibition by inoculum.

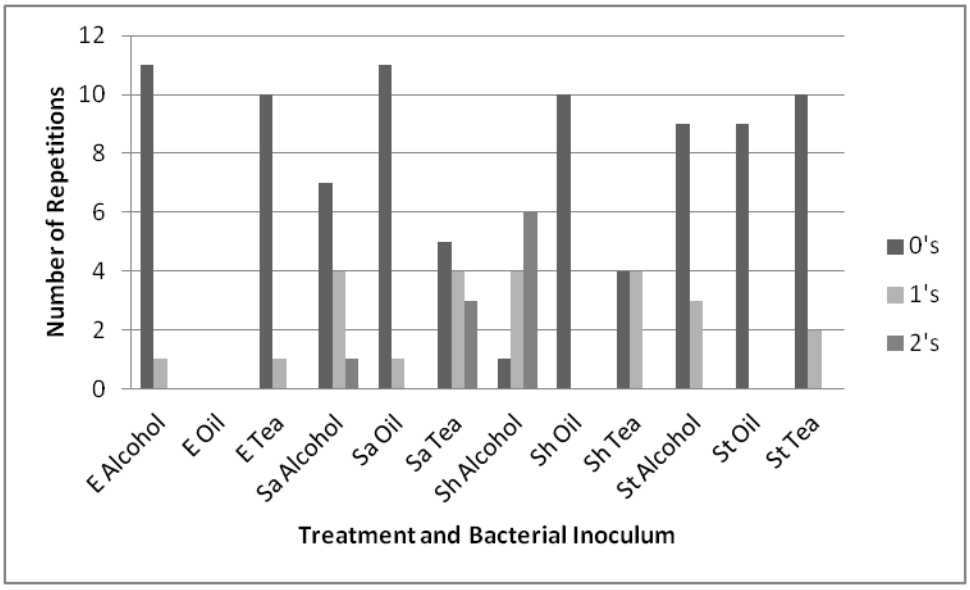


Figure 1. Main trial: Totals for each degree of inhibition per treatment and bacterial inoculum. Inhibition was either 0 (no inhibition), 1 (slight inhibition) or 2 (total inhibion. *E. coli* is represented as "E", *S. aureus* as "St", *Shigella* sp. as "Sh" and *Salmonella* sp. as "Sa". Number of repetitions with 0 is majority (67/90) of total.

However, a simple totaling method of data representation does not indicate the results of the tests done with controls included in each Petri dish. I determined that a more accurate way to assess inhibition by a treatment was to compare the percent of tests inhibited by the treatment to the percent of tests inhibited by the control. This is because any inhibition that was observed in both the treatment (with

Neurolaena lobata) and the control (without *Neurolaena lobata*) is not truly inhibition caused by *Neurolaena lobata.* Data analyzed by this method still showed no predictable antibacterial effect of *Neurolaena lobata*. The results of most repetitions (63/90) were inconclusive, as inhibition from *Neurolaena lobata* treatment did not differ from the control.

Table 1. Main trial: Total inhibitions as compared to control per bacterial inoculum and *Neurolaena lobata* treatment. Diminished repetitions due to contamination.

Bacterial Inoculum	*Neurolaena lobata* Extraction Method	Total Experimental Repetitions	# Tests Inhibition of Treatment Greater than Control	# Tests Treatment same as Control	# Tests Inhibition of Control Greater than of Treatment
E. coli	Oil	9	0	9	0
	Alcohol	9	1	8	0
	Tea	6	1	5	0
S. aureus	Oil	0	N/A	N/A	N/A
	Alcohol	9	1	8	0
	Tea	6	0	6	0
Shigella sp.	Oil	9	0	9	0
	Alcohol	9	0	8	1
	Tea	9	0	8	1
Salmonella sp.	Oil	9	0	8	1
	Alcohol	9	0	7	2
	Tea	9	0	8	1

Although there was no significant difference between inoculums, trends in differential inhibition were observed. These results are based on 24 total repetitions of *E. coli*, 15 total repetitions of *S. aureus*, and 27 total repetitions each of *Shigella* sp. and *Salmonella* sp. *E. coli* showed that treatments were more inhibitory than the control in 2/24 tests; *S. aureus* showed that treatments were more inhibitory than controls in 1/15 tests; and *Salmonella* sp. and *Shigella* sp. each showed this in 1/27 tests. Additionally, while *E. coli* and *S. aureus* did not show that the control was more inhibitory than the treatment in any tests, *Shigella* sp. showed that the controls were more effective than the treatments in 2/27 tests, while for *Salmonella* sp. this was true in 5/27 tests. According to this experiment, there appears to be a trend that *Shigella* sp. and *Salmonella* sp. are more resistant to *Neurolaena lobata* treatments than *E. coli* and *S. aureus*.

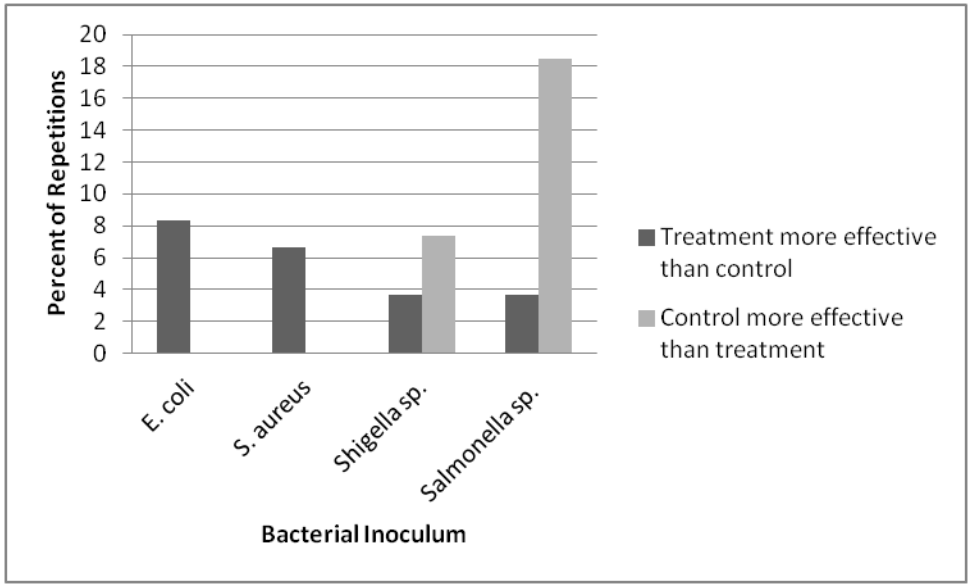


Figure 2. Main trial: Percentage of tests with inhibition of treatment greater than control and inhibition of control greater than treatment, by bacterial inoculum - n=24 (*E. coli*), 15 (S. *aureus*), 27 (*Shigella* sp.), 27 (*Salmonella* sp.).

I also analyzed data from the main trial by *Neurolaena lobata* extraction method. This was based on 27 repetitions with oil, 36 repetitions with alcohol, and 30 repetitions with tea. In tests done with oil treatments, 0/27 of the tests showed *Neurolaena lobata* oil to be more effective than pure olive oil; however, pure olive oil was more inhibitory than *Neurolaena lobata* in olive oil in 1/27 tests. Out of repetitions with alcohol extract (across all bacterial inoculums), 2/36 tests showed that the treatment was more inhibitory than the control, while 3/36 tests showed that the control was more inhibitory than the treatment. Tea as an extraction method produced similar results; tea was more inhibitory than pure water in 3/36 tests, while pure water was more inhibitory than *Neurolaena lobata* tea in 3/30 tests. Tea and alcohol extractions were the only *Neurolaena lobata* treatments that resulted in bacterial inhibition.

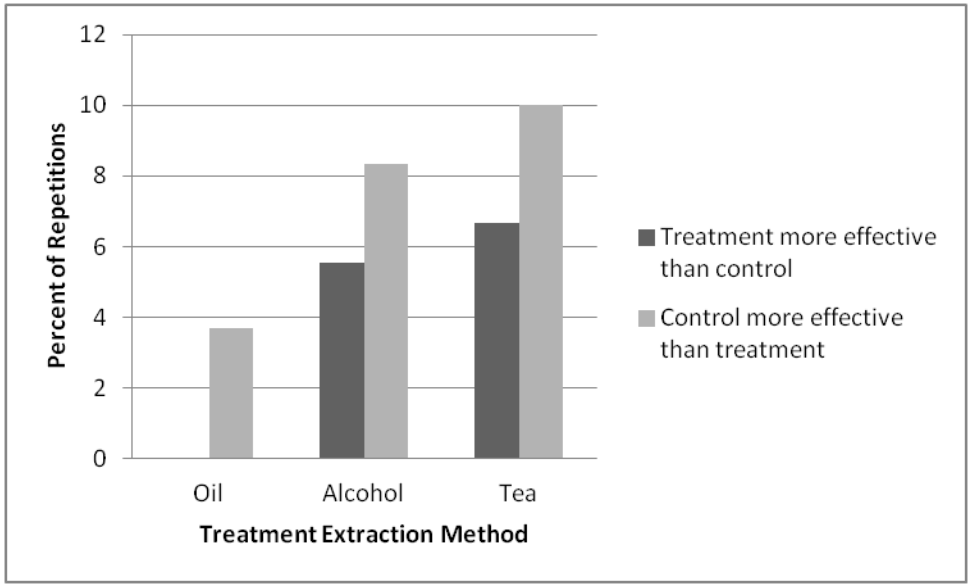
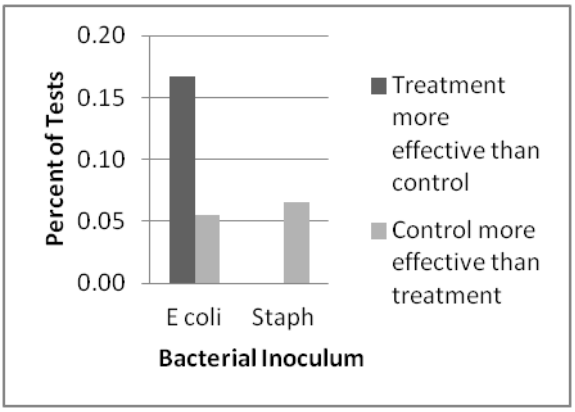
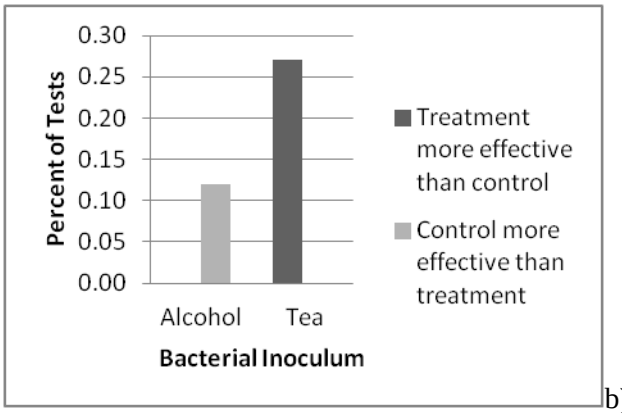


Figure 3. Main trial: Percent of tests where treatment was more effective than control and where control more effective than treatment per extraction method - n= 27 (oil), 36 (alcohol), 30 (tea).

Based on which treatments and inoculums resulted in bacterial inhibition in the main trial, I did a follow-up trial with *E. coli* and *S. aureus* and only tea and alcohol treatments. The results of this trial were also largely inconclusive, with 8/28 tests showing degree 0 inhibition and the majority (18/28) of tests showing treatments to have the same degree of inhibition as the control. The sample size was smaller, with 18 tests performed with *E.* coli, 10 with *S. aureus*, 17 with alcohol treatments, and 11 with tea treatments (these irregularities due to the exclusion of some tests due to contamination). I observed a similar trend as in the main trial in that pure HCl and methanol was more effective than the related *Neurolaena lobata* treatment in 2/17 of these tests; a treatment of alcohol and *Neurolaena lobata* was not more effective than the control in any tests. Tea showed the opposite trend, with more inhibition by treatment than control in 3/11 tests and control not more effective than the treatment in any tests (0/10).By inoculum, *E. coli* was more inhibited by the *Neurolaena lobata* treatment than the control in 3/18 tests, while *S. aureus* was not inhibited by *Neurolaena lobata* in any trials (0/10). *E. coli* was more inhibited by the control than the treatment in 1/18 tests, while *S. aureus* was more inhibited by the control than the treatment in 1/10 tests. While some of this difference may be explained by sample size, these results may suggest that *E. coli* was most sensitive to antibacterial effects from *Neurolaena lobata* overall, as it also showed the greatest amount of antibacterial effective treatments (2/24) in the main trial.

a) 



b)

Figure 4. Follow-up trial. Percent of tests treatment more effective than control and control more effective than treatment. a) *E. coli* and *S. aureus* inoculums. b) Alcohol and tea treatments.

Differences in inhibition and no inhibition between bacterial inoculums were not statistically significant (Figure 4a). There was, however, a statistical difference between treatments by extraction type (Figure 4b; n=28, X^2=4.94, df=1, p<0.05).

Table 2. Follow-up trial: There was a significant difference in bacterial inhibition between different *Neurolaena lobata* treatments (alcohol and tea); (n=28, X^2=4.94, df=1, p<0.05).

	Yes	No
Alcohol	0	17
Tea	3	8

The results of the positive control performed with Amoxicillin showed inhibition zones of 2-3mm around *Shigella* sp. and *Salmonella* sp., while the inhibition zone for *E. coli* was 5-7mm and that for *S. aureus* was 10mm.

DISCUSSION

While the majority of tests (main trial: 67/90, follow-up trial: 18/28) did not show that *Neurolaena lobata* treatments produced bacterial inhibition that differed from that of the control, the fact that an antibacterial effect of *Neurolaena lobata* was observed at all means that the potential for continued investigation of the secondary compounds and antibacterial properties of this plant is high. Some treatments used in this experiment appeared to be more effective than others, and some bacteria appeared more susceptible; both observations that would benefit from future study.

By inoculum, *Salmonella* sp. and *Shigella* sp. appeared less vulnerable to *Neurolaena lobata* treatments overall than *E. coli* and *S. aureus* (Figure 2), although there was no statistical difference. Both *Shigella* sp. and *Salmonella* sp. had fewer total tests in the main trial where treatments were more effective than controls than *S. aureus* and *E. coli*. Additionally, *Shigella* sp. and *Salmonella* sp. were the only bacteria in the main trial to show that the controls were more effective than the treatments, and the only tests in which inhibition was greater in the treatment than the control were those tested on inoculums of *E. coli* and tea *S. aureus* (Table 1). *E. coli* had more tests where the inhibition by a *Neurolaena lobata* treatment was greater than the control than any other bacteria, especially looking at results from the follow-up trial (Figure 4b). The follow-up trial also showed greater inhibition by treatment overall and did

not include either *Shigella* sp. or *Salmonella* sp. (Figure 4b). All of this suggests that *Shigella* sp. and *Salmonella* sp. are more resistant to antibacterial properties than *E. coli* and *S. aureus.*

This hypothesis is also supported by the results of the positive control, which showed that the inhibition zones for the antibiotic Amoxicillin with *Shigella* sp. and *Salmonella* sp. were much smaller than those for *E. coli* and *S. aureus*. Knowledge of the human health impacts of these bacteria corroborates a hypothesis of differential aggression as well. While *E. coli* and *S. aureus* both have strains that can be harmful to humans, they also represent large groups of bacteria of which many strains are inert (E. Coli General Information, CDC, 2015). *S. aureus*, for example, is found on the skin and in the nose of about 25% of healthy people and animals (Foster, 1996). *Shigella* strains, on the other hand, are important causes of disease in the developing world and are capable of causing deadly epidemics (Shigella, CDC, 2016), while *Salmonella* strains cause one million foodborne illnesses and 380 deaths annually in the United States alone (Salmonella, CDC, 2016). However, the strains I worked with, as they came from a teaching laboratory, were not pathogenic.

Extraction methods also showed differential trends in antibacterial effectiveness. For example, oil did not produce greater inhibition from the treatment than the control in any of the repetitions where it was used. This supports the hypothesis that antibacterial compounds in *Neurolaena lobata* are not oil-soluble. In the main trial, alcohol and *Neurolaena lobata* appeared to be more effective in inhibiting bacterial growth, producing greater inhibition from the treatment than the control in more tests than tea did. However, I observed the opposite trend in the follow-up trial, where the difference in inhibition between extraction methods was statistically significant (Table 2; Figure 4a). These results suggest that *Neurolaena lobata* tea may be more effective at higher concentrations (even higher than those it is traditionally), as I steeped a larger amount of leaves, for longer, in a smaller quantity of water to prepare the tea for the follow-up trial; however, these results may also be skewed by a smaller sample size. Additionally, the main trial showed that pure alcohol (the control) produced greater inhibition than the treatment more often than the treatment produced greater inhibition than the control. This leads me to suggest that alcohol may be more inhibitory to bacteria at higher concentrations, and diminishes in antibacterial effectiveness when mixed with *Neurolaena lobata*. However, further study is necessary to determine the relative effectiveness of these herbal preparations, and analysis of compounds through a method like gas chromatogrpahy would be beneficial to determine in which substances *Neurolaena lobata* may be soluble.

It is interesting to note that previous students' projects , such Burke and Sullivan's (2015), showed that *Neurolaena lobata* inhibited bacterial inoculums of *E. coli* and *S. aureus*, potentially to a greater extent than the results of my experiment showed. There are a number of possible interpretations to explain a lack of observed bacterial inhibition in this study. First of all, it is possible that the use of pure cultures ordered from labs meant that I was using bacteria that were either different strains than those used by previous students, more resistant to antibacterial effects, or simply in too high of a concentration. Additional factors, such as the seasonality, age of the leaves, and location of the plant, also could play into the antibacterial potency of *Neurolaena lobata* leaves. Factors like weather, soil, and environmental conditions (e.g. amount of light) could have affected the growth and resources available for secondary compound production (Coley and Barone, 1996) in the *Neurolaena lobata* plant I used. Additionally, I used exclusively adult (full-size) leaves to make my extractions; however, some research has shown that new leaves generally have higher concentrations of the secondary compounds that give them antibacterial properties (Coley and Barone, 1996).

While the effectiveness of Amoxicillin, the antibiotic used in the positive control, to cause bacterial inhibition should not be overlooked, there are still many reasons to continue investigating the potential of traditional medicinal plant remedies. Antibiotics like Amoxicillin have proven to be effective against a wide range of bacteria, including those that cause tonsillitis, bronchitis, pneumonia, and various infections of the ear, nose, throat, skin, or urinary tract ("Amoxicillin Uses, Side Effects & Dosage

Guide", Drugs.com, 2016); however, these can also be expensive, difficult to access, and ineffective or causing unpleasant side effects. Traditional medical remedies prepared with traditional, non-laboratory methods are still used by many people to treat bacterial infections, and continue to be used for used for minor ailments (such as digestive problems) and preventative healthcare, especially where access to clinics and expensive pharmaceutical products is limited. The continuing social and medical relevance of medicinal plants such as *Neurolaena lobata* means that continuing investigation of the mechanism for their medicinal properties (and potential future applications) is both an important and immediate goal.

Further study could investigate the secondary compounds present in *Neurolaena lobata* through methods like gas chromatography; this would help determine in which solvents these compounds are soluble to verify the ideal preparation method. Additionally, further research with methods of concentration, such as distillation, would be useful in determining the physiological impacts of *Neurolaena lobata.* Additional assays performed with more concentrated treatments would be helpful in assessing the antibacterial activity of its compounds and determining whether *Neurolaena lobata* is more inhibitive to certain kinds of bacteria than others. Finally, the conduction of clinical trials with safe and concentrated *Neurolaena lobata* treatments would be necessary to make conclusions about its effects on the human body.

Sources of Error

Working in a lab without certain tools or standard items available meant that many things used in my experiment I made myself, which may have reduced the accuracy of my experiments. Agar was boiled and "autoclaved" in a pressure cooker by me, and then plated using just my assessment and a line on the Petri dish to make a certain thickness, which was likely not standard. It was hard to grab exactly one colony to transfer into the suspension, and swabbing was difficult to standardize as well, as it was hard to make it a smooth "mat" of bacteria and not streaky. The sterilization methods I used for the tools in my experiment may not have removed all bacteria, or extracts may have become contaminated through storage, as some of the Petri dishes were contaminated. Finally, with the disk plating method, hot tweezers were sometimes used to plate filter disks, which could either promote or destroy bacterial growth, and it was hard to standardize the amount of extract on the filter disk.

Conclusion

The efficacy of medicinal plant preparations to bring about beneficial health impacts for the human body is a question of high relevance for a number of people in the world. Though there may be little data on the specific impacts of *Neurolaena lobata*, the study of chemical compounds synthesized by plants and their effects on human physiology is a growing field that is being actively contributed to, even in small ways such as this study. While plant phytochemicals affect plant physiology, the common ancestry of all multi-cellular organisms means that many have conserved cellular processes and pathways that are affected by similar chemicals (Kennedy and Wightman, 2011). Thus, the potential for compounds used by plants for their predators and symbionts to be harnessed to perform similar functions in humans, whether they be antibacterial or stimulative to the nervous system, is high (Briskin, 2000).

This study was conducted in Costa Rica, a place where only a quarter of the original forest cover is still standing (Blasak, 2011). Tropical forests such as those found in Costa Rica differ in a variety of ways from temperate forests, meaning that threats posed to them have different consequences. Although tropical forests such as these cover only about 7 percent of the land surface of the earth, they harbor close to half of all species (Lindsey 2007). Additionally, as a consequence of plant-herbivore interactions, tropical plants possess an assortment of protective measures including a wide variety of secondary metabolites, many of which are more common in tropical than temperate forests (Coley and Barone, 1996). As one of many examples, *Neurolaena lobata* is found only between southern Mexico and

northern South America; many species of tropical forests are so specialized to microhabitats that they can only be found in small areas, making them vulnerable to extinction ("Neurolaena lobata", Lindsey 2007).

As the beneficial medicinal effects of plants largely result from the combinations of their secondary compounds, and the diversity and abundance of plant secondary metabolites is greater in tropical than temperate forests, it follows logically that the conservation of tropical forests is the only way that these compound combinations and their potential for human health may be kept alive (Briskin, 2000; Coley and Barone, 1996). The potential benefits for human bodies are one of the anthropocentric reasons to protect tropical forests, extending beyond even the ecological impacts on other species or the atmospheric composition of the earth. Already, people rely on a range of products derived from plants for nutritive and medical needs, both in traditional and pharmaceutical settings; in recent years, compounds from plants have been shown to do everything from enhance brain function to attack cells that cause cancer (Briskin, 2000; Kennedy and Wightman, 2011). However, many traditional medicinal plants have not been well studied, nor have the majority of plants with secondary compounds been researched with regard to the composition of these compounds or their impacts on human physiology. The potential for discovery with these plants is enormous; and if the remaining tropical forest is cut down, they will never be found.

ACKNOWLEDGMENTS

First, I would like to thank my advisor, Sofía Arce Flores, who is intelligent, witty, and wonderful, for spending hours tampering with potentially pathogenic Petri dishes in the lab with me, helping me ascend a huge learning curve in microbiology, and encouraging me even when I was discouraged. I would also like to thank Andrés Camacho, for obtaining my long list of materials and providing comic relief; microbiologist Gloriana Barrantes, for taking time after work to inspect my ameteur plating techniques; and the staff at the Monteverde Institute and the Estación Biologica for their resources and their indefatigable helpfulness; and my host family, for giving me the opportunity to be a part of their family for a little while. As with most things in life, what has stood at the basis of all this learning has been my emotional well-being, and I would like to thank my peers (and all EAP staff), for their accumulated wisdom, knowledge, and curiosity, and for hiking 12 miles in rubber boots, swimming with sharks, eating rice and beans, and laughing, agonizing, and living with me. These memories will be cherished. Finally, I would like to thank Frank Joyce, who gave me the confidence to undertake this project, maintained the spirit of the course as one of humor, support, and freedom, is patient, thoughtful, and inspiring, and genuinely, actually, really good at his job. To be present in so many novel, challenging, and stimulating situations surrounded by mentors and friends has made this such an enjoyable and educational time in my life, and whichever path I go down I will trace some root of it to this. I could not have done any of this without you. Thanks to all.

LITERATURE CITED

Ajazuddin, S. S. 2010. Applications of novel drug delivery system for herbal formations. *Fitoterapia* 81(2010): 680-689.

Amoxicillin Uses, Side Effects & Dosage Guide. Uses, Side Effects & Dosage Guide - Drugs.com. *Drugs.com.*22 July 2016. Web. 05 Dec. 2016.

Balick, M. J., and Arvigo, R. 1993. *Messages from the gods: a guide to the useful plants of Belize*. Oxford University Press, USA.

Bauer, A. W., Kirby, W. M. M., Sherries, J. C., and Turck, M. 1966. Antibiotic Susceptibility Testing by a Standardized Single Disk Method. TURCK *American Journal of Clinical Pathology* 45: 493–496.

Berger, I., Passreiter, C. M., Cáceres, A. and Kubelka, W. 2001. Antiprotozoal activity of *Neurolaena lobata*. *Phytotherapy Research* 15: 327–330.

Bernhardt, E. 2008. Medicinal Plants of Costa Rica. *Distribuidores de la Zona Tropical*.

Blasak, R. 2011. Ethics and environmentalism: Costa Rica's lesson. *United Nations University.*

Briskin, D. P. 2000. Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. *Plant physiology*, 124(2), 507-514.

Burke, S. and Sullivan, K. 2015. Antimicrobial activity of three neotropical plants and diet effect on the secretions of Lithobates taylori (Ranidae: Anura). UCEAP 2015.

Cáceres, A., López, B., González, S., Berger, I., Tada, I., & Maki, J. 1998. Plants used in Guatemala for the treatment of protozoal infections. I. Screening of activity to bacteria, fungi and American trypanosomes of 13 native plants. *Journal of Ethnopharmacology*, *62*(3), 195-202.

Chariandy, C. M., Seaforth, C. E., Phelps, R. H., Pollard, G. V., & Khambay, B. P. S. 1999. Screening of medicinal plants from Trinidad and Tobago for antimicrobial and insecticidal properties. *Journal of Ethnopharmacology*, *64*(3), 265-270.

Chin, C. C. 2015. Antibacterial and antifungal properties of *Neurolaena lobata*. UCEAP 2015.

Coley, P. D., & Barone, J. A. (1996). Herbivory and plant defenses in tropical forests. *Annual review of ecology and systematics*, 305-335.

Dyer, B. D. 2003. A Field Guide to Bacteria. *Cornell University Press.* Ithaca, NY. Print.

E. Coli General Information. Centers for Disease Control and Prevention. Centers for Disease Control and Prevention, 06 Nov. 2015. Web. 26 Nov. 2016.

Foster, T. 1996. Chapter 12: Staphylococcus. In *Medical Microbiology*. Galveston, TX: University of Texas Medical Branch at Galveston. 4th Edition.

Francois, G., Passreitr, C. M., Woerdenbag, H. J., and Van Looveren, M. 1996. Antiplasmodial Activities and Cytotoxic Effects of Aqueous Extracts and Sesquiterpene Lactones from *Neurolaena lobata*. *Planta Med* 62(2): 126-129.

Goff, S. A., and Klee, H. J. 2006. Plant volatile compounds: sensory cues for health and nutritional value?. *Science*, 311(5762), 815-819.

Hale, T. and Keusch, G. 1996. Chapter 22: Shigella. In *Medical Microbiology*. Galveston, TX: University of Texas Medical Branch at Galveston. 4th Edition.

Kennedy, D. O., & Wightman, E. L. (2011). Herbal extracts and phytochemicals: plant secondary metabolites and the enhancement of human brain function. *Advances in Nutrition: An International Review Journal*, 2(1), 32-50.

Lentz, D. L., Clark, A. M., Hufford, C. D., Meurer-Grimes, B., Passreiter, C. M., Cordero, J., and Okunade, A. L. 1998. Antimicrobial properties of Honduran medicinal plants. *Journal of ethnopharmacology*, *63*(3), 253-263.

Lindsey, R. 2007. Tropical Deforestation. *Earth Observatory*. NASA. Web 06 Dec 2016.

Mahanom, H. Jr., Aziza, A., Dzulkifly, M. 1999. Effect of different drying methods on concentrations of several phytochemicals in herbal preparation of 8 medicinal plants leaves. *Malaysian Journal of Nutrition* 5(1): 47-54.

Passreitr, C. M. and Isman, M. B. 1997. Antifeedant bioactivity of sesquiterpene lactones from *neurolaena lobata* and their antagonism by γ-aminobutyric acid. *Biochemical Systematics and Ecology* 25(5): 371-377.

Salmonella. Centers for Disease Control and Prevention. *Centers for Disease Control and Prevention*, 05 Aug. 2016. Web. 28 Nov. 2016.

Schallert, E. 2008. Antibacterial Properties of Medicinal Plants. UCEAP 2008.

Shigella. Centers for Disease Control and Prevention. Centers for Disease Control and Prevention, 03 Aug. 2016. Web. 5 Dec. 2016.

Walls, D. 2009. Herbs and natural therapies for pregnancy, birth, and breastfeeding. *International Journal of Childbirth Education* 24(2): 29-37.