Myrica cerifera, a Medicinal Plant of the Lumbee Tribe, has Antibacterial and Nematicidal Properties

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ABSTRACT
Currently threatening the world of medicine is a growing number of antibiotic-resistant diseases. More specifically, bacteria and nematodes have gained resistance to many of the world's leading antibiotics and nematicides, respectively, making infections more difficult to treat. Subsequently, these parasitic organisms are able to continue damaging crops and other living organisms like humans without strong interference. To help people and the environment, the development of new and novel antibiotics is vital. Previous research suggests that phytochemicals are a potential solution that will not only help inhibit bacterial growth but also reduce nematode survival. We hypothesized that *Myrica cerifera*, a plant often used by the Lumbee tribe to treat illness, possesses antibacterial and nematicidal properties. To answer our hypothesis, we began by collecting plant specimens to extract material for biological assays and to subsequently isolate and elucidate the structures of active components. The extract was evaluated for antibacterial properties with an agar diffusion assay and then nematicidal properties using *Caenorhabditis elegans*. *M. cerifera* extract was added onto an agar lawn at various doses, and the nematodes' lifespans were scored. The findings of this study show that extracts of this plant, more commonly referred to as 'wax myrtle', do significantly decrease the lifespan of *C. elegans* and increase the zone of inhibition for *Staphylococcus epidermidis* and *Staphylococcus aureus*. In addition, two compounds were isolated and characterized through chemical extraction, chromatographic separation, and spectroscopic analysis. These compounds could potentially be used to treat bacterial and nematode infections.

KEYWORDS
Antibacterial; Antimicrobial; *Caenorhabditis elegans*; Plant extract; *Myrica cerifera*; Nematicidal; NMR; Phytochemical

INTRODUCTION

Bacteria are single-celled organisms found in every ecosystem on Earth. The human body itself is an ecosystem for bacteria. Remarkably, there are approximately thirty-nine trillion bacteria in the human body.[1] While many bacteria are non-pathogenic and even aid our digestive system in breaking down food, some are pathogenic. Pathogenic bacteria can cause crop damage, food spoilage, and a plethora of diseases.[2] In order to survive, however, bacteria have become resistant to extreme temperatures and UV radiation, which makes destroying them exceptionally challenging.[2]

Similarly, nematodes, also called roundworms, are mainly free-living organisms, but the few parasitic nematodes survive at the expense of the host.[3] Humans are hosts for approximately 300 species of parasitic worms.[4] According to the CDC, an upwards of 1.2 billion people are infected with *Ascaris* globally, which is only one genus of nematode.[5] While there are drugs to treat these infections, a lack of healthcare, sanitation, and growing resistance to the small number of drugs currently available, causes the proportion of the population infected to proliferate.[6] Relatedly, parasitic nematodes are also responsible for destroying crops, collectively costing approximately $80-$118 billion in crop damage.[7] The range of plants damaged by parasitic nematodes include, but are not limited to, agronomic and vegetable crops, foliage plants, and fruit and nut trees.[8] This damage is jointly caused by the 4100 species of nematodes that have been identified as plant parasites.[9] The main nematodes responsible for plant damage typically stem from order Tylenchida, and while *C. elegans* fall under order Rhabditida; both orders are classified as family

Rhabditidae.[7, 10] As such, chemicals that reduce *C. elegans* survival are likely to have similar effects on nematodes within that family, given that organisms within a family often share similar gene sequences and functional domains.

To protect our health and food crops, compounds that kill either bacteria or nematodes are invaluable. However, microbial resistance to classic antibiotics is an increasing dilemma. Resistance to antibiotics occurs as a result of natural selection, which is when bacteria with an adaptive mutation survive and continue to reproduce, whereas the bacteria without the adaptive mutation are selected against.[11] Many treatments for life-threatening illnesses and operations rely on antibiotics, such as cancer therapy and amputations.[12] According to a survey of oncologists in the United Kingdom, nearly 50% claim chemotherapy will soon be unviable due to antibiotic resistance.[13] For example, when the immune system is already compromised from chemotherapy, an antibiotic resistant disease like methicillin-resistant *Staphylococcus aureus* (MRSA) can be especially fatal.[14] An additional major cause of infection in the hospital is *Staphylococcus epidermidis*, with one study showing that 46% of hospital infectious samples tested positive for *S. epidermidis*.[15] Unlike MRSA, which has resistance against primarily methicillin, all *S. epidermidis* strains in the previously mentioned study were resistant to three or more different kinds of antibiotics.[15]

Promisingly, plants have a fantastic diversity of phytochemicals, many of which differ from those present in existing antibiotics. New chemicals may be helpful in creating revolutionary antibiotics that bacteria won't be resistant to.[16] Using the same chemicals to create antibiotics increases the odds that bacteria will be resistant to the medication. Phytochemicals are chemicals isolated from the plants they naturally originate in, and they perform in numerous ways to improve health.[17] A few plants that have these antibacterial properties include clove, roselle, rosemary, and thyme.[18] Their shared chemical classes include terpenes and flavonoids, which other investigators have shown have antimicrobial activity.[18,19] In this paper, we focus on *Myrica cerifera*, more commonly referred to as wax myrtle. Wax myrtle is an evergreen tree that some Native American tribes in North Carolina craft into teas to combat illness,[20,21] more specifically skin irritation and ailments of the digestive system.[22,23] It was hypothesized if wax myrtle was responsible for helping people recover, then compounds in the plant may possess antibacterial and nematocidal properties.

METHODS AND PROCEDURES

Plant harvesting and aqueous extraction

Myrica cerifera cuttings were collected in Spring 2019 in Fayetteville, NC. Leaves were removed from plants, dried, and stored at room temperature until use. To generate 20% aqueous extracts for biological assays, 1 g of ground leaves were combined with 5 mL ddH_2O, mixed by vortex, incubated for 20 minutes at 70ºC, and filter sterilized. The 20% solution was diluted with ddH_2O to generate less concentrated extracts.

Chemical extraction

For chemical extraction, the dried plant material was ground to a powder. The plant material was extracted with hot water, with 100 mg of plant material boiled in 500 mL of water for one hour, providing the aqueous extract. Volumes of 100 mL aqueous solutions were extracted with 3 x 20 mL of Chloroform ($CHCl_3$), then 3 x 20 mL of methylene chloride (CH_2Cl_2), and finally with 3 x 20 mL of ethyl acetate. The organic solvents were evaporated under a vacuum and analyzed by low-resolution NMR. This approach was not successful, and a different method was employed. The solid residue, recovered after filtration of the hot water solutions, was left to percolate overnight with 100 mL of $CHCl_3$ and filtered again. The $CHCl_3$ solution was evaporated under vacuum using a rotatory evaporator to generate a mixture that was purified by flash column chromatography on silica gel using a 5% gradient methylene chloride (CH_2Cl_2) in hexane as eluent.

NMR and LC-MC

All NMR data were recorded on a Bruker Avance 500 [500 MHz)] instrument equipped with a 1.7 mm TXI probe and using $CDCl_3$ as the solvent. Electrospray Ionization mass spectra (EIMS) (HRMS) data for accurate molecular weight and MS/MS fragmentation were measured using a Xevo G2-XS QTof spectrometer in positive ion resolution mode controlled by MassLynx v4.1 software (Waters Corporation). Key parameters were capillary voltage 2.5 kV, sampling cone 80 V, and source offset 80 V, source temperature 120, desolvation temperature 550, cone gas flow 100 L/hr, and desolvation gas flow 800 L/hr.

Agar diffusion assays

Glycerol stocks of *Micrococcus luteus, Bacillus subtilis, Corynebacterium xerosis, Proteus vulgaris, Neisseria sicca, Staphylococcus aureus, and Staphylococcus epidermidis* were streaked onto nutrient agar plates (Fisher Scientific, Pittsburg, PA, USA), grown overnight at 37 ºC and stored at 4 ºC until use. The day prior to agar diffusion assays,[23] 3 mL aliquots of nutrient broth (Fisher Scientific, Pittsburg, PA, USA) were inoculated from streak plates and grown overnight at 37 ºC. Overnight cultures were spread via a sterile L-spreader on Mueller-Hinton (MH) agar (Fisher Scientific, Pittsburgh, PA, USA) plates. Filter discs were soaked in 20% aqueous extract or ddH_20 (vehicle). The disc was shaken gently to remove excess extract and placed on MH agar plates. After overnight

incubation at 37 ºC, zones of inhibition (region of no growth around each filter disc) were measured by taking the diameter of each zone and normalized by subtracting the 6 mm disc size from each measured diameter. All agar diffusion assays were repeated three times.

C. elegans husbandry
Initial *C. elegans* stocks are ordered from the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota. Once acquired, the stocks were grown and maintained using general handling methods.[25] The nematodes (wild-type, N2) shipped from the CGC were backcrossed four times before any experiments.

Survival Assay

Plates were prepared with standard normal growth agar, and wax myrtle was added to some of the agar at varying doses (0.1 mg/mL, 0.01 mg/mL, or 0.001 mg/mL) following autoclave. We added OP50-1 to the plates and allowed them to dry before killing the bacteria with UV exposure. Worms were then age-synchronized by bleaching following standard protocols.[26]. Eggs were placed on the NGA plates +/- wax myrtle. Unhatched eggs were counted at 24 hours following bleaching. Once the nematodes reached L4 maturity, they were plated onto corresponding wax myrtle, and fluorodeoxyuridine (50 μM FUdR) treated NGM plates. Worms were housed at 20 ºC.

Scoring lifespan

If the platinum tip of a worm pick did not trigger a touch response in a *C. elegans*, then the worm was scored as dead and removed from the plate. Worms were transferred to plates with fresh *E. coli* when their food source started to run low or approximately three days after they were last moved. Any worms missing or damaged from crawling on the plastic side of the petri dish were not scored and marked as 'lost.' The damaged worms were then removed from the plate. All 'lost' worms were censored from data analysis to avoid confounding variables affecting the results.

Statistical analysis
Growth inhibition by aqueous extracts was compared to a negative control using Dunnet's multiple comparison test, and p-values below 0.05 were considered statistically significant. Calculations were performed using GraphPad Prism.
For the lifespan analysis, the data gathered was compiled on GraphPad Prism. Then, the Log-rank test was used to determine significance, comparing the extract versus vehicle treatment. A p-value below 0.05 was considered statistically significant.

RESULTS
Chemical extraction
Following a series of unsuccessful attempts to separate the components in the aqueous solution, promising results were acquired from the organic extracts obtained from the solid residue that was isolated by filtration of the mixtures resulting from the boiling of the plant material. Two extracts were isolated: one in $CHCl_3$ (4.67 g) and one in CH_2Cl_2 (3.58 g). These extracts were purified by flash column chromatography on silica gel using a 5% gradient methylene chloride (CH_2Cl_2) in hexane as eluent. At the end of both chromatography columns, two compounds were isolated, and the TLC and low-resolution NMR showed that two different compounds were present. Of the two compounds discovered, one was named tcpl1, a yellow solid, 1.03 g, and the second was named tcpl2, a green-brown solid, 0.464 g were isolated and further purified.

Isolation
The waxy material (sample TCPL1, 0.1952 g) was dissolved in 15% ethyl acetate/85% hexanes (400 μL). The mixture was sonicated for 5-10 minutes at room temperature until a homogenous solution was obtained. Approximately 50 μL of the solution was charged across a preparative thin-layer chromatography plate (EMD plates, Silica Gel 60 F254, size 10x20 cm, layer thickness 250 μm), and the mixture was eluted with 15% ethyl acetate/85% hexanes at room temperature. The component at $R_f = 0.33$, which eluted as a yellow band, was scraped off the plate, and the recovered light-yellow solid was treated with 5 mL of ethyl acetate in a 20 mL scintillation vial. The insoluble silica gel was allowed to settle, and the supernatant was separated and passed through a glass pipette packed with glass wool. The process was repeated three times, and the combined supernatants were concentrated at room temperature under nitrogen to provide a light-yellow residue which was eluted from a C_{18} solid-phase extraction cartridge with 80% MeOH in H_20 and evaporated to dryness under nitrogen.

NMR structure elucidation
The structures of the two components of the mixture, present in a 4:1 ratio of Compound 1 to Compound 2, were identified using 1D ^1H, 1H-^1H COSY, ^1H-^{13}C HSQC, and ^1H-^{13}C HMBC experiments (**Figure 1**).

Compound 1. 2',4'-dihydroxy-3'-methyl-6'-methoxychalcone - ^{1}H NMR (500 MHz, $CDCl_3$) δ 13.61 (s, 1H), 7.99 (d, J = 15.7 Hz, 1H), 7.84 (d, J = 15.7 Hz, 1H), 7.68 – 7.62 (m, 1H), 7.50 – 7.35 (m, 4H), 3.66 (s, 3H), 2.16 (s, 3H), 2.13 (s, 3H). HR-ESI-MS (calculated 299.1283, found 299.1288, 1.7 ppm). The spectral data matched that given in previous reports.[27–29]

Compound 2. 5,7-dihydroxy-6,8-dimethylflavanone (demethoxymatteucinol) ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.27 (s, 1H), 7.70 – 7.61 (m, 1H), 7.50 – 7.35 (m, 5H), 5.41 (dd, J = 12.8, 3.2 Hz, 1H), 3.04 (dd, J = 17.1, 12.9 Hz, 1H), 2.85 (dd, J = 17.1, 3.1 Hz, 1H), 2.08 (s, 3H), 2.08 (s, 3H). HR-ESI-MS (calculated 285.1127, found 285.1127, 0.0 ppm) The spectral data matched that given in previous reports.[28-31]

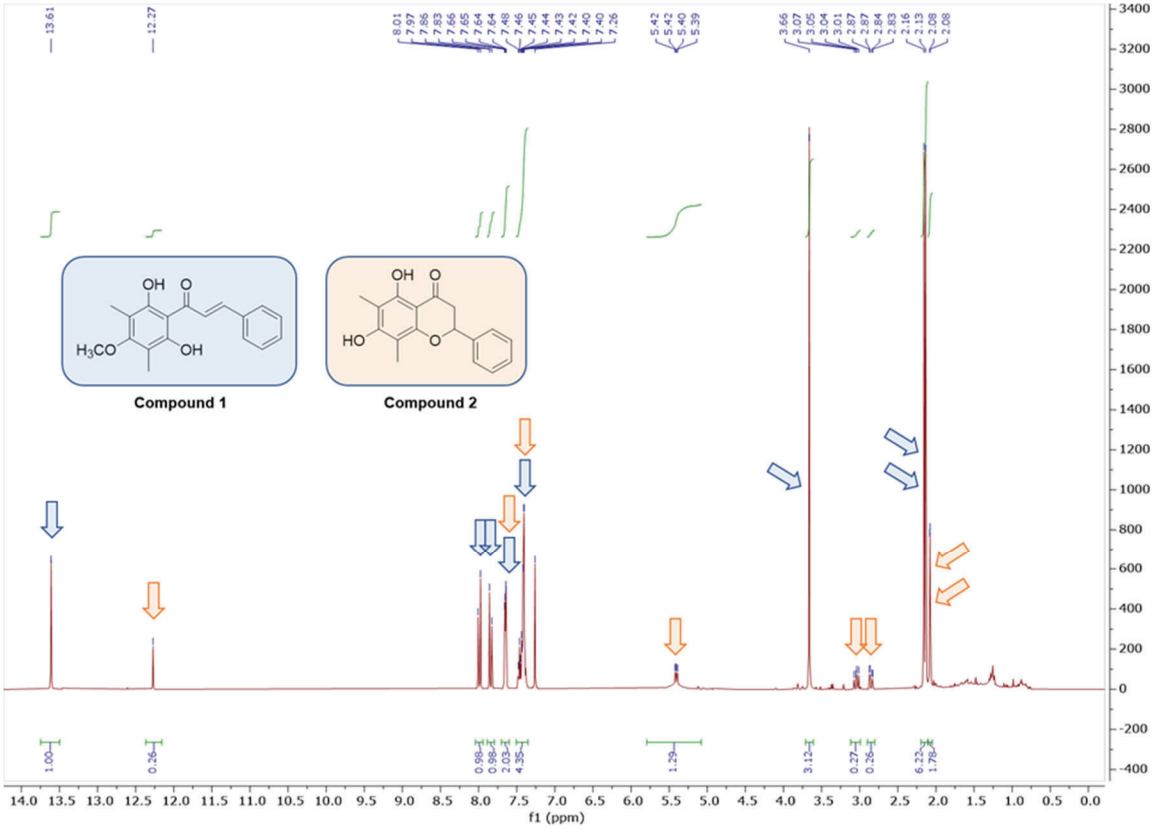

Figure 1. ^{1}H NMR of compounds 1 (blue) and 2 (orange).

Agar diffusion assay

To assess wax myrtle for antibacterial activity, we performed agar diffusion assays using extract against 13 common laboratory strains. Measured zones of inhibition were tested for statistical significance using GraphPad Prism. Aqueous-derived extract from wax myrtle significantly inhibited the growth of two of the 13 strains: *S. aureus* and *S. epidermidis* (**Figure 2**).

Nematicidal activity

To determine how wax myrtle affects *C. elegans* survival, we monitored plates of synchronous worms that were exposed to varying doses of wax myrtle extract and then compared them to vehicle-treated animals. The data gathered was compiled on GraphPad Prism. Then, a p-value of 0.006 was obtained from a Log-rank test. These results (**Figure 3**) reveal wax myrtle extract significantly decreased the survival of *C. elegans*, which was a trend observed in all three biological replicates. We also noted the percent of eggs unhatched compared to hatched nematodes on day one following treatment with wax myrtle. The results were compiled on GraphPad Prism, and a p-value of 0.0105 was obtained from a one-way repeated measures ANOVA test with Geisser-Greenhouse correction. The results (**Figure 4**) reveal wax myrtle significantly reduces the number of eggs that hatch at higher doses.

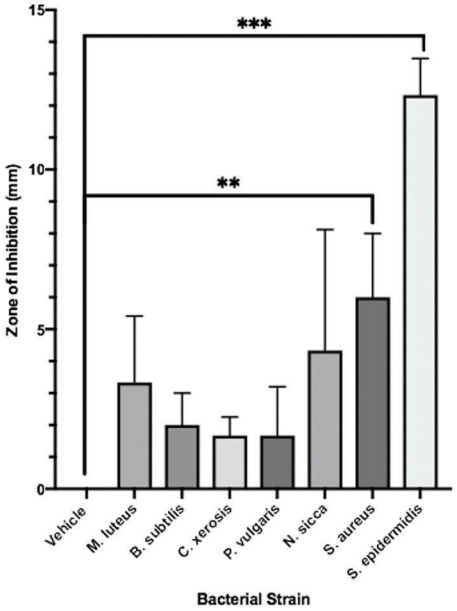

Figure 2. Agar diffusion assay showing bacterial inhibition by wax myrtle. Zone of inhibition (mm) of various bacterial strains by wax myrtle aqueous extract. The bacteria used in this assay include *Micrococcus luteus, Bacillus subtilis, Corynebacterium xerosis, Proteus vulgaris, Neisseria sicca, Staphylococcus aureus, and Staphylococcus epidermidis*. Bars represent the mean of three trials +/- SD. Statistical significance was calculated using a Dunnett's multiple comparison test against the vehicle treatment on Graph Pad Prism 8.0. ** $p<0.01$, *** $p< 0.001$, observations (n= 3).

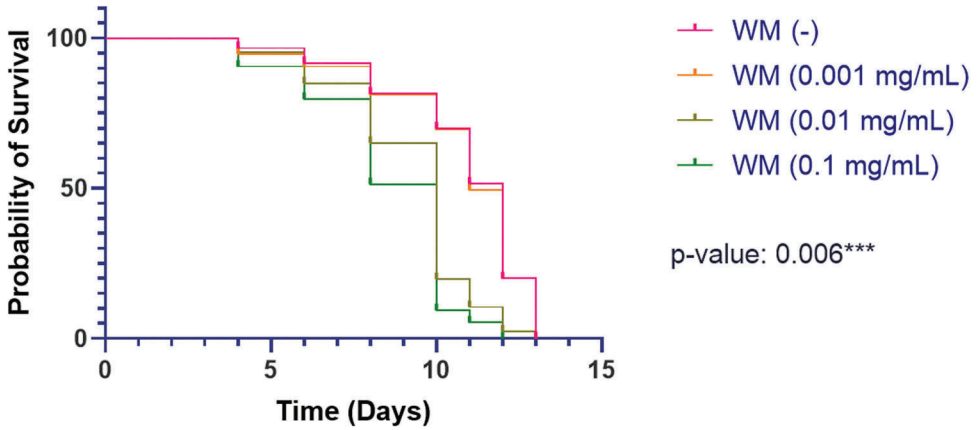

Figure 3. The effects of wax myrtle on *C. elegans* survival. The lifespan of *C. elegans* relative to time (days) in L4's maintained at 20 ºC. Values plotted are the number of dead worms per day after the initial treatment. Statistical significance was calculated with a Log-rank test on GraphPad Prism 8.0.0. *p=0.006, observations (n = at least 50 nematodes). The lifespan analysis was repeated two additional times for a total of 3 experimental replicates. The same trend was observed (see also Figures S1 and S2).

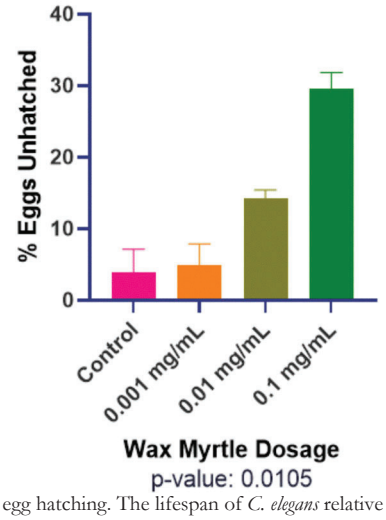

Figure 4. The effect of wax myrtle dosage on *C. elegans* egg hatching. The lifespan of *C. elegans* relative to time (days) in L4's maintained at 20 ºC. Values plotted are the number of eggs unhatched on day one following treatment. Statistical significance was calculated with a one-way repeated measure ANOVA test, with Giessen-Greenhouse correction on GraphPad Prism 8.0.0. *p=0.0105, observations (n = at least 50 nematodes). The bar graph is compiled from data gathered from three biological replicates.

DISCUSSION

This study began by examining the chemical properties of an herbal plant native to North Carolina, *Myrica cerifera.* Lumbee Indians, a Native American nation with homelands in southeast North Carolina, historically used and continue to use *M. cerifera*, also known as wax myrtle, as a medicinal plant to address digestive problems and skin irritation.[23] Several digestive issues (*e.g.,* inflammatory bowel disease, gastroesophageal reflux disease, and intestinal worms) are associated with microbial imbalance.[32,33] Additionally, bacteria, particularly *S. aureus,* underlie the majority of skin infections.[34] Therefore, we hypothesized that a plant used for generations to treat these types of digestive issues might possess antimicrobial properties.

Initially, we predicted there would be traces of terpenes and flavonoids in the chemical extraction, and the compounds we isolated confirmed our theory. The second compound was indeed a flavonoid. What we did not anticipate finding was a chalcone in the first compound, but chalcones are known to have antimicrobial activity as well as other beneficial biological activities.[35]

Our results confirm that the phytochemicals present in wax myrtle offer new antibacterial alternatives. In this experiment, we discovered wax myrtle extract significantly decreases the zone of inhibition for *S. epidermidis* and *S. aureus*. *S. epidermidis* is known for creating biofilms on catheters, and surgical implants,[36] whereas *S. aureus* is a common cause of skin and respiratory infections.[37] Furthermore, the emergence of methicillin-resistant *S. aureus* (MRSA) is an ongoing crisis in the medical community.[38] The development of new antibiotics is vital in decreasing the severity and number of cases for both species of staphylococcus bacteria.

Additionally, wax myrtle was found to contain compounds that reduce the lifespan of *C. elegans* as well as inhibit the growth of certain bacteria. More specifically, the plant extract significantly decreased longevity in wild-type (N2) *C. elegans*. Results shown in **Figure 3** have a p-value of 0.006%, denoting statistical significance. Wax myrtle's nematicidal properties are important because many current nematicides are toxic not only to nematodes but also to the plants and people they treat. A now banned nematicide called dibromochloropropane (DBCP) is hazardous to men because it's a testicular toxin. Another banned pesticide is aldicarb, which poisoned over 2,000 people in California in the early 1980s due to contamination.[39] More and more nematicides are getting banned due to unwanted toxicity at higher concentrations. Luckily, most phytochemicals are safer on the environment and humans than traditional chemical nematicides, so potential nematicides made from wax myrtle could provide a safer alternative for exterminating parasitic nematodes.[40] This can help cut down the billions of dollars wasted on crop damage and help combat the rising *Ascaris* infections. Our additional finding that wax myrtle significantly decreases fecundity, or the capability of *C. elegans* to produce offspring, further exemplifies why this evergreen shrub has potential as a nematicide. This capability to decrease fecundity is a trait shared by nematicide emamectin benzoate. Emamectin benzoate has been proven to be more effective than three other nematicides, which lack the ability to reduce the population by decreasing fecundity.[41]

In the future, we would want to determine what target(s) wax myrtle extracts attack the bacteria. *S. epidermidis* and *S. aureus* are gram-positive cocci arranged in grape-like clusters. These bacteria belong to Phylum Bacillales, which are correlated to the effectiveness of wax myrtle. That said, there was one more bacterium included in this assay belonging to Phylum Bacillales that was not significantly inhibited, *B. subtilis*. However, a critical difference is the genus of each bacterium. The two significantly inhibited bacteria are classified as genus *Staphylococcus,* whereas the bacteria that was not significantly inhibited is classified as genus *Bacillus*. The underlying molecular mechanism will be investigated.

Not only would we look into what targets the plant extract has, but we can also test structurally similar compounds to the extract. Moreover, we would like to continue testing survival, and other plant extracts on our extra wild-type (N2) nematodes and on mutant strains, particularly GC565 and BS3164. Both strains induce tumor formation in the germline at 25 ºC, which will be useful in evaluating the anti-cancer activity of the chalcones identified in the wax myrtle extract.[42,43] This will potentially provide us with knowledge on how the plant extracts shorten the lifespan of nematodes and what pathways the extract uses. In conclusion, wax myrtle's antibacterial and nematicidal properties will help shape future investigations in revolutionizing antibiotics and nematicides.

CONCLUSION

This study addresses the antibacterial and nematicidal effects of *Myrica cerifera*, more commonly known as wax myrtle. Our results show wax myrtle significantly decreases *C. elegans* lifespan and fecundity as well as the growth of *S. epidermidis* and *S. aureus*, indicating wax myrtle can alter how we develop antibiotics and nematicides in the future.

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ABOUT STUDENT AUTHORS

Ashley Edwards will graduate from the University of North Carolina at Pembroke in the Fall of 2022 with a BS in biology, a BS in chemistry, and a BA in music. She conducted this research to gain firsthand experience in a laboratory and learn about *C. elegans* husbandry. She feels this will better prepare her for a Ph.D. program following graduation. In addition to maintaining her passion for music, she hopes to work more with *C. elegans* in the future.

Kazhmiri Deberry will graduate from the University of North Carolina at Pembroke in the Spring of 2023 with an RN-BSN. She is enthusiastic about helping others and will use her science training in her future career.

Hannah Mariani graduated from the University of North Carolina at Pembroke in Fall 2020 with a BS in biology. She is currently applying to MPH programs and credits her work in the research lab with developing her scientific skills.

Darian Higgins is a senior at the University of North Carolina at Wilmington who is planning to graduate in May of 2021. Her major is Exercise Science, with a concentration in Allied Health. Darian is enthusiastic about becoming a pharmacist, and she is excited that the research work she completed through the collaboration with UNC Pembroke allowed her to integrate firsthand laboratory aspects of pharmaceutical science into her career path.

Nicholas Cochran will graduate from the University of Wilmington in the Summer of 2021 with a major in Biology and a minor in Chemistry. The work he completed through the collaboration with UNC Pembroke inspired him to continue doing research in Pharmaceutical Chemistry, and he now plans to pursue a career in this field.

PRESS SUMMARY

This study looks into the antibacterial and nematicidal effects of an evergreen tree, more specifically known as wax myrtle. Our results show wax myrtle significantly decreases lifespan in roundworms and the growth of certain bacteria. This suggests that wax myrtle can improve how scientists develop antibiotics and nematicides in the future.