Antimicrobial Assay of Sagebrush Roots

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ANTIMICROBIAL ASSAY OF SAGEBRUSH ROOTS

by

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Capstone submitted in partial fulfillment of the requirements for graduation with

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with a major in
Plant Science Research

in the Department of Plants, Soils, and Climates

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Abstract

Plants produce secondary metabolites for various functions, one of which is antibacterial activities. Sagebrush has historically been used by Native Americans for different medicinal purposes, suggesting that it may have secondary metabolites that would have medicinal values, including antimicrobial activities, and can be a natural source for antibiotics. This study aims to carry out the antimicrobial activity of Sagebrush root extract against a handful of bacteria. We tested the antimicrobial activity of root extract of Sagebrush against six bacteria such as Bacillus subtilis, Bacillus cereus, E. coli DHSα (Lab cloning strain), Agrobacterium tumefaciens GV3101 (Lab cloning strain), Pseudomonas syringae pv. tabaci, and P. syringae pv. tomato DC3000 (both plant pathogens). All the bacteria tested had some level of growth inhibition shown by the root extract. P. syringae pv. tabaci, P. syringa pv. tomato DC3000, A. tumefaciens, and B. subtilis all had decent zones of inhibition. However, P. syringae pv. tabaci showed the largest zone of inhibition. The root extract was least effective against E. coli which only had a diameter of the zone of inhibition of 0.25mm in response to 20 mg of crude root extract. Four biosafety level 2 human pathogens, Staphylococcus aureus, Staphylococcus epidermidis, Proteus vulgaris, and Micrococcus luteus, were also preliminarily tested. M. luteus and S. epidermidis had some growth inhibition shown by root extract. However, M. luteus produced the largest zone of inhibition of 13 mm with a 30 mg disk. Ultimately, metabolite extract of sagebrush roots does show inhibition of bacterial growth in the preliminary study, which suggests it does have the potential for use as an antibiotic.
Acknowledgments

I would like to thank Dr. Amita Kaundal for all the help and guidance she has given me through this project, and I would like to thank Dr. Jeanette Norton for her assistance. I would also like to thank the University Honors Program and USU’s Plant, Soils, and Climate Department for the opportunities they have given me. Finally, I’d like to give a special mention to David and Kayla Suisse for all the help they gave me on this project, and then to my husband, Erik McFadden, for his patience.
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Introduction

Plants contain various secondary metabolites. Secondary metabolites are organic compounds produced by the plants despite not being directly involved in primary growth and development. Plants often produce them to help protect them against abiotic and biotic stresses. Some secondary metabolites that plants produce are toxins, terpenes and phenolic compounds that act as antibiotics, and alkaloids (Gorlenko et al., 2020). That is why plants are successfully used as medicinal plants. Atropine is derived from many plants and is used to treat overdosing on cholinergic drugs or mushroom poisoning, lower heart rate, and reduce salivation and bronchial secretions (RxList, 2020). Quinines, an alkaloid, is derived from cinchona tree bark and have antimicrobial properties (Gorlenko et al., 2020). It can treat malaria, pneumonia, typhoid fever, and nasopharyngeal infections. As microbes are constantly evolving and new diseases are always coming out, people continue to turn to plants to search for new sources of antimicrobial agents. Specifically, plants that have had a history of being used as treatments.

Native Americans traditionally have used Sagebrush (*Artemisia tridentate*) for various purposes, including being used as a smudge, helping to stop internal bleeding, and getting rid of parasites in the digestive system (Tilford, 1999). Artemisinin is another secondary metabolite that has been extensively studied. It had been isolated from sweet wormwood (*Artemisia annua*) and is now used as an antimalarial drug (Krishna et al., 2008). Artemisinin also showed great potential for treating cancer (Krishna et al., 2008).
There are currently only a couple of studies about Sagebrush’s secondary metabolites. So far, they have been able to identify 220 phytochemicals. However, there is only a tiny amount of information available on the potential of using them for medicinal purposes (Turi et al., 2014). One study looked at how the volatile oils in Sagebrush may be helping control rumen bacteria in wild deer and how captive deer who didn’t eat Sagebrush would have to have adapted to make up for the loss of the antimicrobials. They ended up finding there was no adaptation between the rumen bacteria of the two deer types. However, they found that a specific concentration of Sagebrush’s volatile oils had a marked antibacterial effect on rumen bacteria (Nagy et al., 1968). They showed that Sagebrush makes a difference with its antimicrobial activity.

In ongoing research, efforts are on the antimicrobial properties of different above-ground parts of big Sagebrush, such as the flowers, leaves, seeds, and branches. The preliminary results revealed the antimicrobial activities of sagebrush extracts on *Bacillus subtilis* (Suisse et al., 2021). Since all the parts of Sagebrush, including root extracts, had been used for medicinal properties, likely, roots may also have antimicrobial properties. Therefore, my goal is to investigate sagebrush root extracts for antimicrobial activities. We hypothesize that the root extract of Sagebrush has antimicrobial activity and inhibits the growth of various pathogenic bacteria.

**Methods**

**Crude Extract Preparation**
The sagebrush plants were dug up from the roadside, and the roots were removed. The roots were washed in tap water to remove the dirt and then patted dry with a paper towel. The lateral roots were removed from the main taproot. The cut roots were placed in a cardboard box on a paper towel, and 1 cm holes were cut in a cardboard box on all sides but the bottom to let the airflow (Figure 1a). The box containing the roots was covered with paper towels with holes cut approximately 1 cm in diameter. The box was then placed in a chemical hood with the airflow at about 100, which ran for 35 days (Figure 1b). Once dried, the roots were then cut up into small pieces, and a mortar and pestle were used to grind up the roots. Most of the roots were ground into fine powder except the cambium, which could not be ground up completely. The ground-up roots were placed in scintillation vials in methanol and left for 3-4 days to extract the metabolites (Yu et al., 2003) (Figure 2a). The crude extract was separated from the solid roots by vacuum filtration (Figure 2b).
The filtrate was put into another scintillation vial and left in the fume hood to evaporate the methanol and leave the solid dry crude extract (Figure 3a). The dry crude extract was weighed and then resuspended in DMSO by w/v to get a 100 mg/mL to 1000 mg/mL of concentration (Figure 3b&c). The crude extract was stored at 4°C until use. The metabolite extraction was done twice.

**Disk Preparation**

Disks were prepared by hole punching filter paper to create small filter paper disks of 5 mm. The prepared 5mm disks were sterilized by autoclaving at 121°C for 15 minutes. The sterilized disks were soaked in the root extract with DMSO (from 10mg-100mg of crude extract), 50 µg ampicillin, 50 µg kanamycin, 20 µg gentamycin, or DMSO (Figure 4). 10mg were used in the
first trial, then 20 mg, 30 mg, and 50 mg. Ampicillin, kanamycin, and gentamycin were the positive controls, and the DMSO was the negative control. The disks were left to dry out in a laminar hood before being stored in the freezer.

**Bacteria Culture Preparation**

*Bacillus cereus, B. subtilis, E. coli DH5α*, and *Agrobacterium GV3101* were grown in Lysogeny broth. *Pseudomonas syringae* pv. tomato DC3000 and *P. syringae* pv. tabaci were grown in King’s broth. *B. cereus, B. subtilis, and E. coli* were grown at 37°C for 24 hours. *P. syringae* pv. DC3000, *P. syringa* pv. tabaci, and *Agrobacterium* were grown at 28°C for 48 hours. All the bacterial cultures were sub-cultured and grown until OD reached 0.05₆₀₀nm. For 10 mg and 20 mg the OD was 0.05₆₀₀nm. However, the bacterial lawn was too thick at this OD, so we reduced it to OD 0.025₆₀₀nm for 30 mg and 50 mg testing.

**Antibacterial Susceptibility Assay**

For the first trial (10 mg), *Pseudomonas* was plated onto King’s B agar, and the rest of the bacteria was spread plate onto Mueller Hinton agar (Doughari et al., 2007). In a biological safety cabinet, 100 μL of the bacteria were applied onto plated agar. Once the bacteria were spread out, one of each type of disk was placed on top of the bacteria culture lawn. For the 10 mg trial, the positive and negative control disks were spread out in a circle with the root extract disk in the center (Figure 5a). In later tests, the root extract, DMSO as a negative control, and Kanamycin as a positive control were only used and were laid out in a triangle pattern (Figure 5b).
B. cereus, B. subtilis, and E. coli were grown in a 37°C incubator for 24 hours. P. syringae pv. DC3000, P. syringae pv. tabaci and Agrobacterium were grown in a 28°C incubator for 48 hours, after which the zones of inhibition were measured. Measurements were taken by measuring from the edge of the zone of inhibition to the other edge to measure the diameter (NCCLS, 2000) (Figure 6).

**Results & Discussion**

The antibiotic susceptibility assay revealed that the root crude extract of Sagebrush has some level of antimicrobial activity against all tested bacteria. The highest inhibition was against the two Pseudomonas syringae and Agrobacterium tumefaciens. Pseudomonas syringae pv. tabaci at 50 mg had the highest with a zone of inhibition at 12 mm (Figure 7a &d and Table 1). P. syringae pv. tomato DC3000 and Agrobacterium tumefaciens both had 10 mm zones of inhibition at 50 mg. Bacillus subtilis also had a high level of resistance at 8 mm at 50 mg (Figure

![Figure 5- (a) 5 Disk Arrangement (left). (b) 3 Disk Arrangement (right)](image)

![Figure 6- Red Lines Across the Diameter Show Where Measurement Was Taken for Zones of Inhibition.](image)
7c&b and Table 1). B. cereus and E. coli had a much smaller zone of inhibition (Table 1). Three known antibiotics, kanamycin (50 μg), ampicillin (50 μg), and gentamycin (20 μg), were also tested as positive controls against these bacteria in the study (Figure 7 and Table 1). The antibiotics are pure and showed a very large zone of inhibition compared to crude extract disks. The secondary metabolites in the crude extract of the root extract are not pure and in smaller amounts; that is why the zone of inhibition is much smaller. However, the antibacterial components in the crude extract are still potent enough to inhibit bacterial growth. This preliminary study is helpful to study further the effect of root extract on bacterial growth.

![Figure 7- Plates with 50mg Root Extract Disks. (a) P. syringae pv. Tomato DC3000 (b) B. subtilis (c) Agrobacterium (d) P. syringae pv tabaci](image-url)
The first trial that we did was with 10 mg disks. D. Suisse et al. (2021), in their study, found that the above-ground parts of Sagebrush were very potent at 10 mg, that is why we chose 10 mg as a starting point. After getting small diameter of the zones of inhibition, we doubled the extract amount. That helped a lot, and for most bacteria, we saw an increase in the diameter of the zone of inhibition, except for B. subtilis and P. syringae pv. tomato DC3000. While we did see an increase in the diameter of the zone of inhibition, they were still relatively small, and we wanted to see if we could get even larger ones. Two more extract disk amounts were prepared: 30 mg and 50 mg, which were tested simultaneously. Due to limited amounts of extract left and unable to get more sagebrush roots since the ground was frozen, we decided to only test the four bacteria with the highest amounts of growth inhibition. Those four were the two plant pathogens *Pseudomonas syringae*, *Agrobacterium* and *Bacillus subtilis*. For all
but *B. subtilis*, we saw a much larger diameter of the zones of inhibition at 50 mg (Table 1). *B. subtilis* was essentially constant at every amount of extract, with a slight dip at 20 mg. *P. syringae pv. tomato* DC3000 also had a dip in the diameter of the zone of inhibition size at 30 mg (Table 1). We had only one replicate for 30mg and can have a trial error. All the other amounts had two replicates, which could also have trial errors, but is more reliable. These results follow what Sini and Malathy suggest, saying that the roots of many medicinal plants are known to have antimicrobial activities (Sini & Malathy, 2005).

We also tested the crude extract against some biosafety level 2 (BSL2) pathogens. David Suisse and Dr. Amita Kaundal tested the above-ground crude extract of sagebrush against four BSL 2 bacteria and included some of my root extract disks in their experiment. The four bacteria tested were *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Proteus vulgaris*, and *Micrococcus luteus* (Table 2). We used two extract concentrations: 30 mg and 100 mg disks. There was no bacterial growth inhibition seen at 100 mg disks for *S. aureus* and *P. vulgaris*, but *S. epidermidis* had a diameter of the zone of inhibition of 7 mm (Figure 8a and Table 2). The 100 mg *M. luteus*, unfortunately, got contaminated. As for the 30 mg disk, none of the bacteria except *Micrococcus luteus* showed growth inhibition which had a diameter of the zone of inhibition of 13 mm (Figure 8b and Table 2).
### Table 2: Average diameter of the zones of inhibition (mm) for different amounts of root extracts as well as Ampicillin, Chloramphenicol, and Vancomycin against BSL2 pathogen.

<table>
<thead>
<tr>
<th></th>
<th>Roots (30mg) mm</th>
<th>Roots (100mg) mm</th>
<th>Ampicillin (30μg) mm</th>
<th>Chloramphenicol (30μg) mm</th>
<th>Vancomycin (30μg) mm</th>
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</thead>
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<tr>
<td><strong>Proteus vulgaris</strong></td>
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<td>0</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Micrococcus luteus</strong></td>
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<td>Contaminated</td>
<td>ND</td>
<td>ND</td>
<td>26</td>
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<td>0</td>
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<tr>
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<td>7</td>
<td>ND</td>
<td>14</td>
<td>ND</td>
</tr>
</tbody>
</table>

Figure 8 - BSL 2 pathogen. (a) 100mg disk on *S. epidermidis*. (b) 30mg disk on *M. luteus*. 
Conclusion

As the root extract had some bacterial growth inhibition against many bacteria, the sagebrush roots crude extract may contain some compounds with strong antimicrobial activities. However, the root crude extract showed a larger diameter of the zone of inhibition against BSL 2 pathogens, especially against *M. luteus*, compared to normal bacteria. It is a preliminary study and needs to be verified a couple of times. We would also need to test a variety of bacteria, as well as potentially expand into fungi as well. Another area that would require further study is separating and identifying the components of secondary metabolites in the root extract responsible for antimicrobial activity. By separating the extract by High-Performance Liquid Chromatography (HPLC) into different fractions of secondary metabolites and testing each fraction, we would narrow down which part is responsible for the antibiotic reaction (Salem et al., 2020).
Reflective Writing

Word Count: 1032

I have loved science and always knew I wanted to get into research all my life. Once I got into college, I started looking for opportunities to get involved. In my first semester, I ended up having Dr. Steve Young reach out to me with an offer to join his Invasive Plant Lab as part of an honors exclusive program. It was a year-long program that allowed undergraduates to develop and carry out their experiments. The program would wrap up in May with a trip to Taiwan, where we would share our research and get to participate in hands-on learning with what research they are doing there regarding invasive plants. I accepted Dr. Young’s invitation and was soon hands deep in my first undergraduate research project. Unfortunately, the program never adequately wrapped up due to Coronavirus hitting that spring.

Good things did end up coming out of it, though. Since I told Dr. Young I was interested in doing more with microbiology and genetics of plants, he referred me over to Dr. Amita Kaundal and her lab. Dr. Kaundal had me jump in right away and help another of her undergraduates, David Suisse, who was just starting to do the first trials for testing the above-ground parts of Sagebrush. I was able to help as they went through the experiment process and as they started standardizing the methodology. As Covid came through, I took a break from undergraduate research for a year and a half. I then, once again, got involved in Dr. Kaundal’s lab. I helped with many more students’ projects, but the sagebrush experiment still stuck with me. When it came time to choose my capstone project, and Dr. Kaundal offered to let me test out the sagebrush roots, I was very excited about it and didn’t hesitate to accept. By getting to do my capstone project, I achieved one of the goals I had for myself in college, which was to do my research.
Not just that, but I wouldn’t know what direction I’d want to take after graduation without getting the opportunity to do my capstone project. After a break, I would love to go to grad school. Doing my own research will be a great thing that will hopefully help influence my chances of getting in for the better.

While I may have been involved in research for a couple of years at that point, it wasn’t until I had Dr. Kaundal as my mentor that I learned what it means to be genuinely engaged in research. The relationship I’ve built with Dr. Kaundal has been the most effective relationship I’ve made with faculty here at USU. While she pushed me to learn things myself and to take responsibility, she also showed me that professors are people who can also become friends as they are, in many ways, just like us. By having this relationship with Dr. Kaundal, I feel comfortable knowing I can reach out to her in the future for help with any future research I may do or even as a reference for a future career or for applying to grad school.

My research on sagebrush roots required me to use a lot of the knowledge I have gained with my major. I have had to apply many of the lab skills and techniques I learned in my labs – especially organic chemistry and my biology lab– through the methodology of my experiment. Especially as Sagebrush is a plant and my major is plant science, I’ve been able to take into account things, such as how secondary metabolites work within the plant, into account and look at the experiment from a plant scientist perspective. Long before I had any idea what my capstone project would be, I had an interest in secondary metabolites and what they could do for the plant. I learned a lot about them, and that knowledge helps me now with my research project.
Another thing I was able to appreciate more about my project because of the knowledge I had gained in my major was the bacteria we were testing against. When I started my capstone project and had a list of bacteria to test against, I didn’t know what any of them were. I kind of just accepted it and figured that was that. It wasn’t until the plant pathology class I took in the fall of 2021 that we talked about several of them. It was really amazing to get to learn in-depth more about what these bacteria were, how they operated, and how they affected the plants. By understanding that, I could more fully understand and appreciate why plants would need to have antibiotics made against them.

While I may have had a lot of background knowledge on many of my projects, it required me to learn more about microbes and antibiotics, which are subjects I am not quite as familiar with. Plants are easily locked hand in hand with these subjects and many more in the web of knowledge. By knowing how these work, I can better help people understand how to take care of their plants and protect against harmful things, and even a bit into what plants can be used medicinally for different things, which is a subject that fascinates me.

What really makes me thrilled and proud of my capstone project is knowing that what I found can have enormous implications. Since sagebrush roots do have antibiotic abilities, saying someone can narrow down precisely what compound has that antibiotic ability, it can be something that helps with a problem that people need help with. When more pathogens are tested, we might find that it is a really beneficial tool that had previously been missing in our world. As such, it could completely change people’s lives for the better. It means that this relatively simple capstone project I am doing now has the potential to have a massive impact on many people’s lives.
Bibliography


Professional Author Bio

Lauren McFadden is graduating spring 2022 from Utah State University with a Bachelor of Science degree in plant science with a research emphasis. She will also be graduating in the honors program with an undergraduate research transcript designation. After graduation, she will be searching for a job where she can work with plants and help other people better understand how to care for their own plants.