The Effect of Drought Stress on Plants' Antibacterial Activity: An Exploratory Study

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Abstract

An exploratory study was conducted on the antibacterial properties of plants and how they are affected by changes in drought conditions. Leaves were collected from four medicinal coastal sage scrub plants and two herbs, then the disk diffusion method was utilized to assess antibacterial activity of the extracts against two gram-positive bacteria (*Staphylococcus epidermidis* and *Bacillus subtilis*) in response to drought stress. The coastal sage scrub plants were studied over a four-month period from the end of the dry season to the beginning of the wet season, while the two herbs were conditioned to three levels of drought stress in a controlled environment.

In some cases for the coastal plants (white sage against *S. epidermidis* and *B. subtilis*, and yerba santa against *S. epidermidis*), the antibacterial activity was highest in the month with the lowest cumulative rainfall and decreased in the following month as rainfall increased. In contrast, black sage and jimson weed showed no clear relationship to rainfall. In the controlled study, oregano grown in the most severe drought stress conditions had the greatest level of antibacterial activity. This suggests that increasing drought stress in turn increases antibacterial activity. The significance of this research was to understand plants' responses to drought stress. Plants are known to make physiological changes to adjust to their environment, including defensive responses to prevent infection. In regards to human health, understanding plants' level of antibacterial activity in response to seasonal change could indicate the optimal time to harvest plants for medicinal purposes and compound discovery.

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Introduction

Secondary metabolites are molecules produced by plants, bacteria, and fungi that are not required for survival, but could potentially benefit the organism. While primary metabolites are produced from primary metabolic pathways such as glycolysis, Krebs cycle, and the Calvin cycle

(LibreTexts, 2021), secondary metabolites are produced from the surplus intermediates of these processes via processes such as the shikimic acid pathway (Hussein & El-Anssary, 2018). A main difference between primary and secondary metabolites is their ability to handle mutations (Erb & Kliebenstein, 2020). If the gene transcribing a primary metabolite were mutated, the result would be fatal for the plant since primary metabolites are essential molecules for the plant's survival. On the other hand, if the gene for an enzyme able to produce a secondary metabolite was mutated, the result would not hinder the survival of the plant much at all since the molecule is not necessary for survival. These mutations in secondary metabolites would result in neutral changes or could even be beneficial. In those cases, the beneficial mutations would be selected for and maintained in a plant population.

In many cases, secondary metabolite production is connected to a plant's immune response via the signal transduction pathway. The first step of this process is the stress detection mechanism. Here, transmembrane proteins in plant cells recognize pathogens via pathogen-associated molecular patterns (PAMPs). Following this recognition, enzymes are activated that either counteract the stress, or act as a messenger for further action. Once the message gets to the nucleus, certain genes are activated via transcription factors to produce proteins, often enzymes, to counteract the stressors by catalyzing the production of secondary metabolites from primary metabolites as precursors (Morris, 2001).

While they are not required by an organism to survive, secondary metabolites are essential to enhancing their chances of survival. Examples of secondary metabolites include phenols, alkaloids, terpenes, and saponins. Phenols are the largest group of secondary metabolites that are responsible for many functions including pigment, aroma, and antimicrobial properties (Hussein & El-Anssary, 2018). These compounds supplement the plants' survival in a variety of ways from deterring herbivores, to attracting pollinators for reproduction, to preventing infection from pests. Strong tastes and smells that tend to keep herbivores from grazing on plants' leaves and branches is what the genus *Salvia* and other genera are known for. Distinct aromas, in addition to pigments and visual patterns, may attract pollinators such as bees and bats. Some plants also produce antimicrobial compounds that protect them from bacteria, fungi, and viruses that attempt to invade via breaches in the epidermis or bark.

Many of the secondary metabolites that benefit the survival of plants are used by humans for several purposes. For example, the secondary metabolites that give certain plants and fruits distinct flavors and smells are farmed and then distributed across the globe. These include coffee, cacao, and vanilla which are popular food ingredients and flavors. Another important type of secondary metabolite is pigments which are then often used as dyes for clothing and art materials. Other plants are harvested for their medicinal value. These plants are used to treat ailments from external wounds to cough and cold symptoms.

Several of these plants used for medicinal purposes have secondary metabolites with antibacterial properties. In oregano, the secondary metabolite responsible for antibacterial activity is a type of phenol called carvacrol (Nostro et al., 2007). In onions and garlic, the secondary metabolite responsible for antibacterial activity is a thiosulfinate called allicin (Borlinghaus et al., 2014).

Between garlic and onion varieties, there are different levels of allicin depending on the species (Benkeblia, 2004). While the responsible compound for antibacterial activity is known for many plants, in others, it is yet to be identified. For example, with white sage, components were isolated from its extracts, however the molecule responsible is not known (Srivedavyasasri et al., 2016).

Because secondary metabolites are produced to aid plant survival, there are many environmental factors that influence the production of these molecules. These include water level, temperature, salinity, metal composition in soil, and light. The intensity of these conditions causes either the decrease or increase in certain groups of secondary metabolites in certain plants. For example, with low light intensity, lower amounts of pigments are produced. With increased salinity in soil, polyphenol production increases (Ramakrishna & Ravishankar, 2011).

As with other secondary metabolites, the environment has an impact on the antibacterial properties of plants. A study demonstrating this was conducted on the model species, *Trachyspermum ammi*, also known as ajwain - a commonly used Indian herb (Azhar et al. 2011). The research team planted ajwain seeds, and when germinated, separated the seedlings into three watering groups: 100%, 80%, and 60%, with the percentages referring to the water capacity. Here, 100% water capacity refers to complete saturation of soil as calculated by the weight of soil before and after drying (Allison et al., 1954). 80% and 60% watering capacities were maintained by the same methods and calculations. Different aspects of the plants were recorded after 67 days of treatment including photosynthesis rate, plant size, and phenol levels (antibacterial metabolites). The plants watered at 60% capacity were found to have the highest levels of phenols out of the three groups while also being the smallest in size. These conclusions showed that ajwain should be harvested in the dry season for optimal benefits from their secondary metabolites.

Another study was done by Ataie Kachoie et al. (2012) on the impact of drought stress on the antibacterial activity of *Thymus daenensis,* thyme. Thyme plants were grown from seed in the field and exposed to four drought treatments ranging from well-watered to severe drought stress at 100%, 60%, 40%, and 20% watering capacity for 45 days. The antibacterial activity was measured using the agar disk diffusion method against four types of bacteria (gram-positive and gram-negative). The results showed that antibacterial activity against various bacteria was not consistent. For one bacteria, *Bacillus cereus*, there was no significant difference between the control (100%) and the severe drought stress (20%). For *Streptococcus agalactiae* and *Salmonella typhimurium*, the severe drought stress resulted in the highest antibacterial activity. With *Staphylococcus aureus*, the 60% water treatment resulted in the highest antibacterial activity. Overall, no uniform trends were observed among the drought treatments and bacterial inhibition.

With these studies done on the impact of drought on plant models such as ajwain and thyme, I wanted to apply this research to four coastal sage scrub plants native to the San Diego area as well as on two commonly used herbs that exhibit antibacterial properties. The purpose of this exploratory study was to identify any patterns in the antibacterial properties due to drought stress in these plants. With that, the research question was whether drought stress affects the production of antibacterial molecules in plants. From what is known about plants' response to stress, it was hypothesized that plants under drought stress would produce more antibacterial compounds due to stress response. The

prediction tested was that the extracts made from plants experiencing the most drought stress would have the highest level of antibacterial activity.

Methods

Experimental Design

For this study, there were two main parts to test antibacterial activity in both an outdoor environment and in a controlled environment. The outdoor environment was focused on the coastal sage habitat at Sunset Cliffs, San Diego, CA. The species of interest in that habitat were *Salvia apiana* (white sage), *Salvia mellifera* (black sage), *Eriodictyon californicum* (yerba santa), and *Datura wrightii* (jimson weed) (Las Pilitas Nursery, 2012). The second part of the study was for plants grown in a controlled environment (from now on referred to as "indoor plants") to isolate the effect of drought stress without possible complications of temperature fluctuation and wind stress. For the indoor study, three groups of *Origanum heracleoticum* (oregano) and *Allium cepa* (onion) plants were separated by watering treatment. The significance of these two parts was to compare the impact of drought stress in a field environment to that in a controlled environment. Following leaf collection for each part, ethanol extracts were made of each plant sample to test for the antibacterial activity of each sample via the disk diffusion method.

Disk diffusion experiments were conducted in order to collect quantitative data of antibacterial activity by measuring the diameter of inhibition zones for each sample. In addition to the zones of inhibition, observation of biomass of the plants grown in a controlled environment was an aspect of qualitative data. This aided in understanding the impact of drought stress on the morphology of the plants. Graphs of demonstrated bacterial inhibition of each sample along with water level (either from cumulative rainfall or watering in the lab) were used to explore the effects of drought stress on the antibacterial activity of these plants.

Data Collection of Outdoor Plants

Mature leaf samples for jimson weed, yerba santa, white sage, and black sage were collected from the same plants during the first week of each month for four months starting in October 2021 and ending in January of the following year. This time period was chosen to observe changes in antibacterial activity over the course of the months with the corresponding changes in rainfall. Mature leaves were sampled for consistency and sufficient biomass. As an exception, younger leaves were collected for jimson weed in the month of October because jimson weed had just started producing leaves that month after a massive caterpillar attack had stripped the plant. For each sample collection, 20 leaves of white sage, 50 leaves of black sage, four leaves of yerba santa, and 15 leaves of jimson weed were harvested in order to obtain a dry weight of at least 0.5 grams to use for making ethanol extracts.

In addition to leaf collection of these plants, data for average monthly rainfall was recorded. This information was obtained from the *National Weather Service* website via the "past weather" tab.

From there, average monthly rainfall was searched for based on geographic location - in this case, San Diego, CA (National Weather Service, 2022).

Data Collection of Indoor Plants

Two trials of the indoor-plant experiment were run. In both cases, the groups labeled "100%" were watered regularly every other day; the groups labeled "50%" were watered every three days; the groups "25%" were watered every four days. For each group, the plants were watered to level six on the moisture meter (iPower 3-in-1 Soil Moisture/Light/pH Meter for Plant Care Sensor) for consistency. For the first trial, the three groups were stressed according to their assigned conditions for 1.5 months before being tested. The oregano variety used was oregano Greek purchased from Andersen Nursery (in San Diego) as seedlings. The onion varieties used for the first trial were red and white onions also purchased from Andersen Nursery as seedlings. For the second trial, a batch of ambition shallots purchased at the same nursery was used following three weeks of stress conditioning due to the first batch having insufficient biomass. It should be noted that all these onions are of the same genus and species, but are different varieties since red and white onions were not available. The oregano plants from the first trial were consistently stressed throughout this entire period. When the time came for sample collection, 50 oregano leaves were harvested while for the onion plants, entire bulbs were extracted and cut into thin slices. For the onions, the bulbs were used rather than the leaves because in culinary and medicinal uses, the bulbs contain the compounds responsible for the health benefits.

Disk Diffusion Experiment

Two weeks prior to the experiment, four to eight 25-milliliter Luria-Bertani (LB) agar plates were prepared in 4 inch-diameter Petri dishes. The day prior to running this experiment, two gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus epidermidis,* were inoculated into 500 microliters of LB broth and incubated at 37C°C with continuous shaking for a 24-hour period. On the day of the experiment, each plate was labeled according to the designated bacteria, and divided into six sections to create a space for each extract. For the Sunset Cliffs plants, each plate was labeled with sections for yerba santa, white sage, black sage, jimson weed, and a control. For the indoor plants, each plate was labeled with sections for 100% onion, 50% onion, 25% onion, 100% oregano, 50% oregano, and 25% oregano extracts. For both parts, two replicate plates were prepared. Refer to *Figure 1* for the diagrams of plating for the outdoor and indoor plants, respectively.

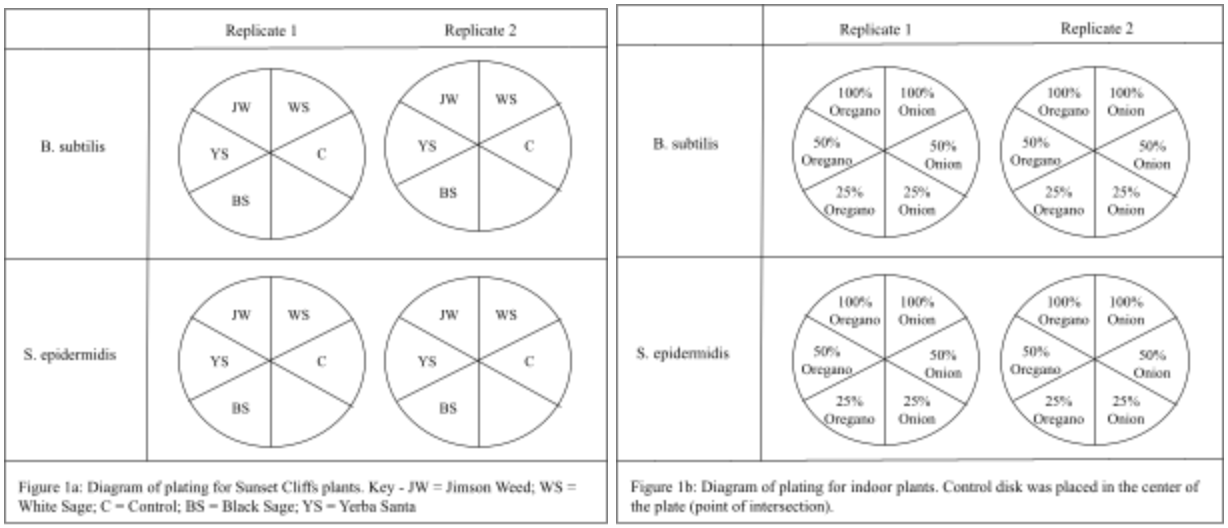


Figure 1: Diagram of plating for (a) Sunset Cliffs plants and (b) indoor plants.

Following collection of leaves from both the Sunset Cliffs and indoor plants, the leaves (and onion slices) were dried in a fruit dehydrator for 12 to 24 hours depending on the leaf density to produce dry/brittle leaves. When the leaves were dried to a brittle texture, each sample was ground using a mortar and pestle until it became a fine powder. Following this, 0.5 grams of each sample was weighed out then transferred into the appropriately labeled 25-milliliter test tube with 1.25 milliliters of ethanol for the Sunset Cliffs plants (with the exception of 1.75 mL of ethanol for yerba santa) and 1.0 milliliters for the indoor plants. Ethanol was used for extraction due to its relatively low toxicity and the fact that both polar and nonpolar molecules dissolve in it. After one extract for each plant had been prepared, they were sonicated for 2.5 minutes using an ultrasonic bath sonicator. Without disturbing the solution, 15 microliters of supernatant from each extract was pipetted onto a plain 6 millimeter-diameter filter disk (Becton, Dickinson and Company). This was done for each extract to prepare two replicates for two different species of bacteria - resulting in a total of four filter disks prepped for each extract. While the ethanol evaporated off the disks, two LB agar dishes were plated with 100 microliters of *B. subtilis* culture and two were plated with 100 microliters of *S. epidermidis* culture *(Figure 1)*. When both the outdoor and indoor plants were being tested simultaneously, four LB dishes were plated for each bacteria instead of two.

After the appropriate bacteria were plated on each petri dish, the filter disks of the extracts were arranged according to their labeled section on the plate, and gently pressed onto the agar (*Figure 1*). Once all the disks were arranged, the plates were placed in an incubator set at 37°C for a 48-hour period. Following the incubation period, the plates were removed to measure the diameters of inhibition around the filter disks in centimeters. Measurements were taken with a plastic ruler.

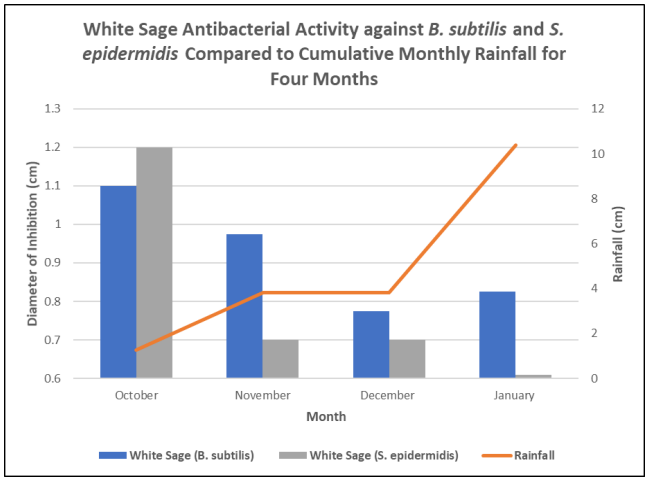
Results

All experimental results are shown in *Figures 2* and *3*. In all the graphs, the primary vertical axis begins at 0.6 cm because that signifies the minimum diameter of inhibition given the diameter of

the filter disks used. The diameter of inhibition positively correlates with the level of antibacterial activity - larger diameters indicate higher antibacterial activity. *Figure 2* shows the results of the Sunset Cliffs plants and their antibacterial activity over the span of four months. Some trends can be seen when taking the monthly rainfall into consideration. For rainfall, cumulative average rainfall was counted since September 2021 because in previous months, there had been as low as 0.07 cm average rainfall since April of that year, so the plants were assumed to be most drought-stressed in October. Generally, a large zone of inhibition can be seen in the month of October where there was very little rainfall compared to the following months. In the case of white sage, a clear inverse relationship can be seen between the amount of cumulative rainfall and the antibacterial activity against *S. epidermidis* (*Figure 2a*). In contrast, the antibacterial activity of black sage did not fluctuate as much despite the change in rainfall over the months as can be seen in *Figure 2b*. As for yerba santa, a significant drop in antibacterial activity can be seen between October to November as the rain increased. Following November, the activity against the two bacteria varied with less dramatic fluctuations against *S. epidermidis* and more dramatic ones against *B. subtilis* (*Figure 2c*). Jimson weed had the most irregular patterns of antibacterial activity while only exhibiting inhibition in the months of November and December with 3.83 cm of cumulative rainfall (*Figure 2d*). In regards to biomass, that of black sage, white sage, and jimson weed increased dramatically with the increase of rainfall. On the other hand, yerba santa did not display visual changes with the change in rainfall.

Figure 3 shows how drought stress in a controlled environment affects the level of bacterial inhibitory activity of commonly eaten plants. In this case, two trials were conducted on the same oregano plant, meanwhile, new onion plants were acquired for the second trial due to inadequate biomass available from the first trial. In both trials, onion did not show inhibitory action against either bacteria - thus only results for oregano against *B. subtilis* and *S. epidermidis* are shown. From this, an inverse relationship can be seen between watering capacity and level of antibacterial activity. Oregano watered at 25% capacity (every four days) exhibited the highest antibacterial activity compared to oregano watered at 50% capacity and 100% capacity. The morphology of the plants showed distinct differences between the three groups. Oregano watered at 100% capacity (not stressed) had the largest biomass, while oregano watered at 50% capacity then 25% capacity had successively smaller biomasses.

a



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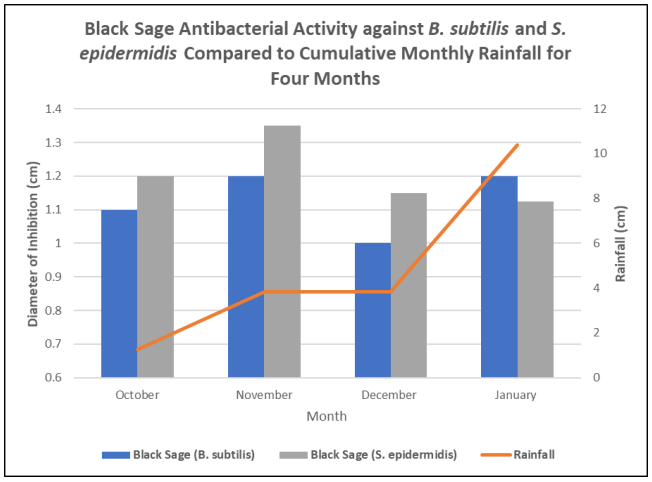

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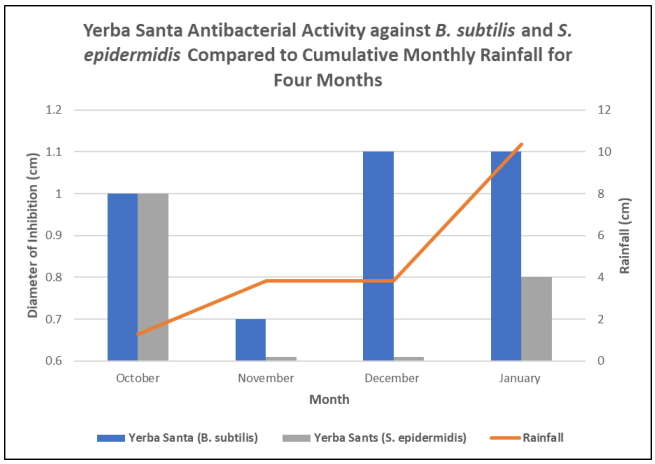

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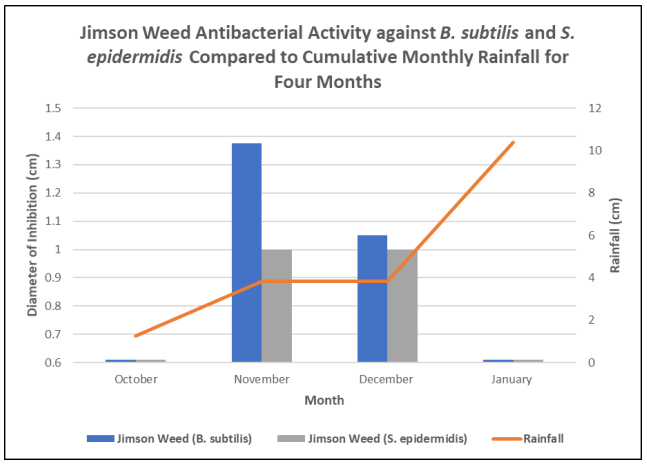
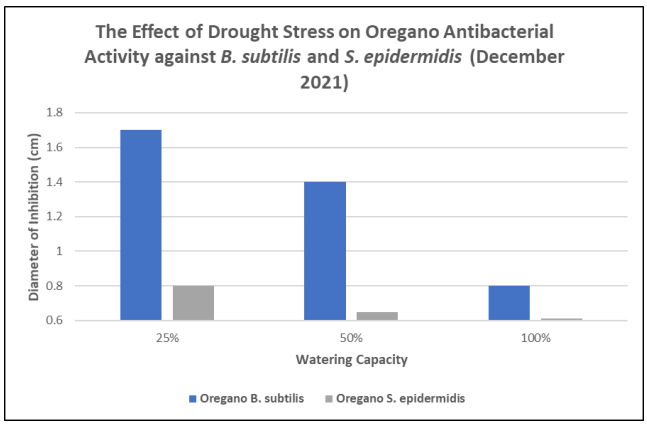


Figure 2: Antibacterial activity of Sunset Cliffs plants against *B. subtilis* and *S. epidermidis* compared to four months of cumulative rainfall: (a) white sage; (b) black sage; (c) yerba santa; (d) jimson weed.

a



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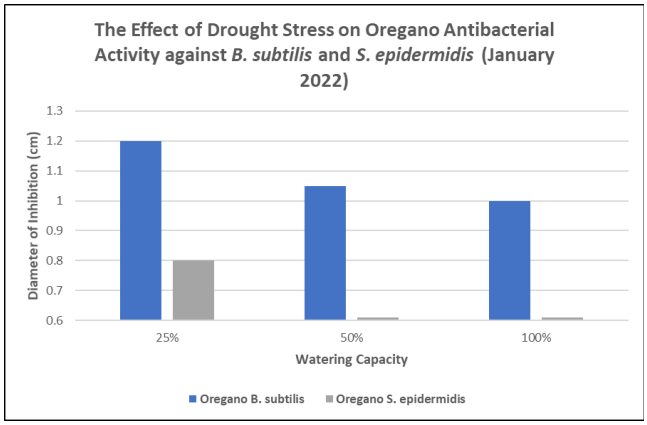

Figure 3: Antibacterial activity of oregano against *B. subtilis* and *S. epidermidis* when put under different levels of drought stress. Two trials were run - (a) in December 2021 and (b) in January 2022.

Discussion

This study explored how drought stress affects the production of antibacterial molecules in plants. Due to plants' mechanisms to stress response, the plants under the most drought treatment were expected to have the highest antibacterial activity.

When comparing the level of antibacterial activity of the Sunset Cliffs plants to the amount of rainfall over four months, there were varying results among the four plants tested. In most cases, there was at least 1.0 cm of inhibition in the month of October when there was the least amount of rainfall - which indicates relatively high antibacterial activity (*Figure 1*). Following that month, there was a drop in bacterial inhibition as the rainfall increased for the plants white sage and yerba santa. Black sage and jimson weed, on the other hand, had an increase in activity. However, it is possible that other factors contributed to this contrasting result.

Figure 2b provides evidence that black sage was the least impacted by change in rainfall compared to the other plants. As the rainfall fluctuated significantly, the change in antibacterial activity of black sage across these months was minimal while for all other plants, there were dramatic changes. This suggests that black sage is more resistant to changes in rainfall, in other words, more drought tolerant. However, further study with multiple replicates is required to affirm this hypothesis.

Jimson weed was rather irregular in that it did not exhibit any inhibition against *B. subtilis,* and it had minimum inhibitory action against *S. epidermidis* in October despite the low rainfall. This result may be due to the age of the leaves during that time as there may not have been sufficient time for secondary metabolites to accumulate. Jimson weed leaves collected for the October experiment had significantly smaller, lighter leaves to the point of unrecognizability. Later during the November data collection, the jimson weed leaves were matured and had the recognizable leaf color and morphology. Furthermore, jimson weed had high inhibitory action against both *B. subtilis* and *S. epidermidis* in the month of November (*Figure 2d*). This suggests that higher concentrations of antibiotics are produced in more mature leaves rather than young leaves. Further study comparing young leaves to mature leaves is needed to confirm this observation. As rainfall stayed steady between the months of November and December, antibacterial activity decreased. Then in January where the rainfall was the highest, there was zero inhibitory action against either bacteria. This supports the hypothesis that as rainfall cumulatively increases, inhibitory action against bacteria decreases. It may also suggest that jimson weed has an optimal rainfall level or season where it produces the most antibiotics - given that significant antibacterial activity was observed only in the months of November and December. This could be confirmed with an additional study with jimson weed and varying water levels.

White sage had fluctuating trends in relationship to rainfall. As mentioned in the results, the inhibitory action of white sage against *S. epidermidis* had a distinct inverse trend to that of cumulative rainfall over the months - demonstrating how rainfall negatively affects the production of antibiotics (*Figure 2a*). In regards to white sage against *B. subtilis*, the highest inhibitory action was in October with the lowest rainfall, then successively decreasing antibacterial activity as rainfall increased, with the exception of January. In January, there was a slight increase in activity. Since

there was not a decrease in activity as expected with higher rainfall, this does not support the hypothesis that drought stress increases antibiotic production.

Yerba santa's antibacterial activity also fluctuated with the change in rainfall. Looking at the activity against *S. epidermidis*, yerba santa exhibited the highest inhibitory action in October with the lowest rainfall compared to the following months (*Figure 2c*). When there was steady rainfall from November to December, inhibition against *S. epidermidis* remained consistent as well. With the increase of rainfall in January, however, there was a 0.2 cm increase in the diameter of inhibition which does not support the hypothesis. Activity against *B. subtilis* correlated with rainfall differently. While there was a high level of activity in October that decreased in November with the increase of rainfall, the activity had a great increase (greater than that of October) going into December despite the consistent rainfall. This activity remained consistent into January following a great increase in rainfall. The overall observation is that the antibacterial activity fluctuated greatly with no change in rainfall while remaining consistent with dramatic changes in rainfall. Reasons for this pattern are unknown, but could suggest that yerba santa responds to low levels of rainfall while not being as affected by higher levels.

In regards to oregano grown in a controlled environment, the data supports that increasing drought stress results in an increase in antibacterial activity (*Figure 3*). Both trials run at different time periods (December 2021 and January 2022) show this trend, although with some differences in range of inhibition and difference between groups. For the trial run in December 2021 (*Figure 3a*), higher contrast is seen between the three groups of drought treatment compared to the trial in January 2022 (*Figure 3b*). Additionally, the inhibitory action of oregano grown with 25% watering capacity in December 2021 was higher than the same oregano in January 2022 despite having the same treatment. There are several possible reasons for this difference. In January 2022, many leaves of the oregano turned purple, which may be due to poor soil drainage, nutrient content, or watering, in addition to temperature change (Ballanti, n.y.). It is possible that prolonged drought treatment caused this, however, purple leaves were also prominent on oregano grown with 100% watering capacity (no drought stress). Therefore, other abiotic factors may have altered the amount of secondary metabolites, including antibiotics, produced. Another possibility for this difference includes the movement of plants that occurred in between the two trials (changing locations due to travel), and the change in climate that was a result of traveling. The temperature difference was about 10 degrees between the two locations (60°F average vs 70°F average indoors), so it may have had an impact on the range of antibacterial activity and tolerance to drought stress. From these observations, it can be noted that while this was intended to be a study in a controlled environment, several abiotic factors were not controlled.

Overall, the results for the indoor plants study agree with the Azhar et al. study of 2011 in that the plant groups put under the most drought stress exhibit the highest antibacterial activity compared to the lesser stressed groups. Additionally, the varying trends between rainfall and antibacterial activity displayed by the outdoor plants agree with the study of Kachoei et al. (2012) as they also observed inconsistent results among their treatment groups.

In addition to the suggested further studies mentioned above, a finer tuned focus on one or two plant models with several replicates for each drought condition would be needed so that statistical analysis could be used to possibly confirm the patterns observed in this exploratory study. This would be a more accurate assessment of the plant species' response to drought stress. When conditioning the plant model to drought stress in a controlled environment, better regulating the other factors such as temperature and nutrient content would be a better representation of the effect of drought stress on oregano. This could be achieved in a well-controlled greenhouse with monitored levels of nutrients and temperature to ensure no stress in those aspects.

As a further study on secondary metabolites, purifying the crude extracts to isolate compounds would be useful in identifying the compounds responsible for the antibacterial effects exhibited in this type of study. This could be done by preparing first a crude extract by similar methods or by using other organic solvents such as methanol that could be more potent in extracting compounds from the plant material not extracted by ethanol. From there, separation techniques such as high-performance liquid chromatography (HPLC) and flash chromatography could be used to obtain pure compound solutions (Sasidharan et al., 2010). For identification, infrared spectroscopy could be utilized to determine active compounds in the solution which would include antibacterial molecules. As was observed with the oregano plants, those experiencing the most drought stress exhibited the highest antibacterial activity, which signifies a high concentration of antibacterial molecules. With that, purifying extracts of plants under drought stress would increase the chances of isolating important compounds that could be used in medicine and other plant-based products.

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