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Effects of Shade on Antibacterial Production in *Aloe Vera* Plants: A Model Course Based Undergraduate Research Experience for First- and Second-Year Chemistry and Biochemistry Students

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Abstract As a means to promote student engagement in chemistry, this proposed course-based undergraduate research experience (CURE) provides an opportunity for students to research a topic that is particularly prevalent in national public health headlines. Antibiotic resistance is a public health issue of increasing concern as result of the overuse and often misuse of antibiotics. For this reason, it is important to explore a variety of avenues to discover novel antibiotics, including isolating bioactive natural products from plants and other organisms. Organisms such as plants, bacteria, and fungi have long been utilized for their antibacterial properties, but little is known for how the majority of these natural remedies are affected by varying growth conditions. Herein, we propose a method through which students in first- and second-year chemistry or biochemistry laboratory courses can manipulate growth factors of *Aloe vera*, an antibiotic-producing plant, to probe how those manipulations affect antibiotic production and therefore *Aloe vera*'s effectiveness as an herbal medicine. *Aloe vera* plants were grown under varying shade conditions, and their gel was extracted and evaluated for antibiotic activity using both a broth microdilution assay and an agar diffusion assay against *Staphylococcus aureus* and *Escherichia coli*. Through this model, students are able to develop critical thinking skills, derive a hypothesis, and use analytical and biochemical techniques to evaluate their hypothesis.

Keywords: *natural product, CURE, antibacterial activity*

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1. Introduction

It has been shown that students are more likely to select careers in STEM in the first years of their undergraduate education and that inquiry-based problem solving early in education also leads to an increase in motivation for students to persist in STEM and pursue undergraduate research experiences. [1] Therefore, it is essential to give students an opportunity to engage in STEM research early in their undergraduate educations so as to improve retention especially for underrepresented students. Course-based Undergraduate Research Experiences have been repeatably demonstrated as a way to engage students in the actual process of science, improve retention, and improve learning within a course. [2,3] CUREs use projects rooted in problem solving, which allows students to experience a collaborative learning environment and the ability to be in control of hypothesis development and project design. Both of these closely mimic what a student would experience in a research laboratory both in academia and in industry. [4] Therefore, despite students having not completed all of their foundational disciplinary courses, it is important to implement course-based undergraduate research opportunities in lower-level laboratory courses. This allows students to grasp the excitement that comes with the opportunity to understand something that is completely unknown and feel the responsibility of contributing to a project where the applications have the potential to benefit the public. [5] For these reasons, a medicinally relevant project provides an opportunity to research and contribute to a field that students will undoubtedly have been previously exposed to in their personal lives or in the media.

Antibiotic resistance is currently classified by the World Health Organization as one of the top public health threats of the twenty-first century, accounting for 35,000

deaths in the United States annually. [6,7] This rapidly escalating issue can be attributed mainly to the misuse and overuse of prescribed antibiotics and the use of antibiotics in agriculture, which both lead to current antibiotics becoming ineffective over time and further highlights the necessity for the continued discovery of novel antibiotics. [8] A majority of the antibiotics currently on the market are directly or indirectly derived from natural products, which are produced by a variety of organisms, including microorganisms such as bacteria, fungi, and plants, each remaining a robust source of potential antibiotics today. [9] From 1981 through 2014, 65% of approved small molecule drugs and 73% of approved antibiotics were either discovered or derived from these natural sources. [10]

In particular, plant sources of antibiotics are of great interest, as these are widely available, generally tend to have fewer side effects, and are relatively inexpensive to source. [11] While organic syntheses have become increasingly advanced, natural products are molecularly complex and their total syntheses tend to be long and low yielding. For this reason, scouring nature for compounds with medicinal qualities can be advantageous. Many herbs and botanical plants have been tested for their antibacterial activity, with results showing they include some active compounds. [12] One plant in particular that has been shown to have antibacterial properties is *Aloe vera*.

As a source for novel and medicinally-active small molecules, *Aloe vera* has a number of convenient attributes, including its lack of excessive maintenance needs and its ability to thrive in a plethora of environments. As a plant with a wide variety of purposes, *Aloe vera* can provide a solution for an array of health concerns, ranging from topical burns to conditions including irritable bowel syndrome. Previous research has conveyed the antibacterial benefits of *Aloe vera*, reporting 76.9% inhibition of *Escherichia coli* (*E. coli*), 75% inhibition of *Staphylococcus aureus* (*S. aureus)*, and 40% inhibition of *Pseudomonas aeruginosa* when compared with the controls of the study. [13] The gel extract of *Aloe vera* is comprised of approximately 98%-99% water and 1%-2% active compounds. [14] Considering that the active compounds in *Aloe vera* are naturally very dilute, simply the detection and quantification of antibacterial activity from raw *Aloe vera* gel indicates this plant produces compounds worth exploring.

Previous literature has outlined how the properties of *Aloe vera* can be altered as a result of manipulating the plant's growing environment. [15,16] For this reason, it was of heightened interest to determine if the already established antibacterial effects of *Aloe vera* are augmented as a result of varying light exposure during the growth period. The benefit of confirming a correlation between growth conditions and antibiotic potency means that this information can then be considered for other plants that produce antibiotics.

Herein, we describe the development of a method for analyzing the effect of light exposure restriction during growth on *Aloe vera*'s antibiotic activity. We have designed this method to be readily adaptable to a CURE program for first- and second-year students for the analysis of the effect of different growth conditions on biologically active, plant based natural product production. This CURE has been designed to be easily adaptable to a 3-4 week long project (with two months of growth time) that can be imbedded in a quantitative chemistry or introductory biomedical laboratory course that focuses on the interdisciplinarity of the analysis of natural products and the variables that effect their production (Figure 1). This method gives students a relatable application for research and relies upon student's engagement with scientific databases and literature to establish a foundation for their proposed project. Similar student-led projects have been implemented at the University of North Carolina Asheville, and elsewhere, with responses of significant student engagement and results. [4]

Through the implementation of this project, students will gain experience in generating hypotheses, variable manipulation, statistical analysis, dilution, and sterile technique when working with bacteria. Furthermore, the prerequisite skills required to carry out this project are minimal, creating a hypothesis-driven curriculum applicable to students early in their undergraduate educations. In the project described herein, shade was implemented as the variable of choice, however other variables such as variations to soil conditions are easily applicable to this project, allowing students to develop unique hypotheses based on literature research of potential variables.

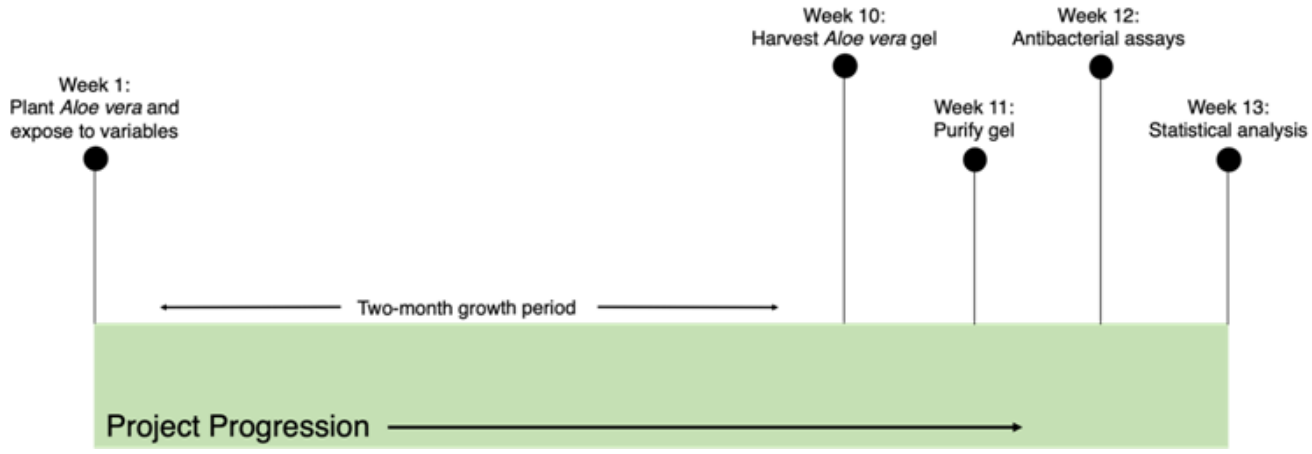

Figure 1. Ideal progression of *Aloe vera* project throughout the course of a semester

2. Materials and Methods

2.1. General

Below we will describe the specific methods used evaluate the effects of shade on antibiotic production in *Aloe vera* and describe how these methods can be adapted for a CURE. Due to the nature of plant growth, an ideal timeline would consist of planting and variable application early in the course, allowing approximately two months for the plants to grow before harvesting (Figure 1). During this time, students can then participate in lessons to hone the skills that they will need for the remainder of the project. All reagents were purchased from commercial vendors at ≥ 99% purity, and all growth media and pipet tips were sterilized via autoclave at 121°C for one hour. All work was carried out under aseptic (sterile) conditions under flame, and surfaces were sterilized using 70% ethanol in water and 10% bleach. Full-strength tryptic soy broth (FSTSB) was prepared with 30.0 g tryptic soy powder (BD Bacto) and 1 L water, and then autoclaved. Bacteria were quadrant-streaked and grown initially on 10% tryptic soy agar (TSA, 3 g BD Bacto TSB powder, and 20 g Bacto agar per 1 L deionized water) for 36-48 h at 25 °C.

2.2. Plant Selection and Equipment

A total of 6 mature *Aloe vera* plants were purchased from a local garden center, and a manufactured shade growth cloth (Amazon B00LBUO22K) was used as a method to uniformly shade plants which were grown under an established grow light (GE Ecolux 32W Daylight 6500K) consisting of artificial light for ten hours a day (9am-7pm). Mature plants were selected based on size, consisting of leaves at least six inches in length. Plants were grown for two months under the established conditions, with variables including full light, no light, and ~20% increments of shade, measured throughout the growing period by a light probe (Li-Cor Li-250A light meter). A baseline shade cloth was purchased, which provided 20% shade upon analysis by light meter, and then the 20% shade increments were established by uniformly layering cloth to increase shade percentage. The exact percentage of light exposure was monitored by reading light exposure via a light probe, and proportionally determining true shade value. Plants were watered according to the regiment of the species.

2.3. Plant Harvesting and Extraction

After nine weeks, the plants were harvested. A surgical knife was used to remove a leaf from each plant by making an incision at the base of the leaf. By slicing lengthwise, the gel of the leaf was exposed, and removed with a spatula. After separating the gel, 1 mL aliquots of raw gel were prepared and centrifuged (14,000 xg for 4 minutes). The supernatant was collected, and the pellet was discarded to remove contaminants.

2.4. Bacterial Inhibition Assay

All work was carried out under standard practices for sterile conditions. To prepare a cell death assay for assessment of antibacterial activity, overnight cultures of each bacteria were grown, consisting of a single bacterial colony, picked from tryptic soy agar plates (TSA, BD Bacto), inoculated into 10 mL of FSTSB and incubated overnight at 37°C. For the purposes of this experiment, the chosen bacteria strains used were *E. coli* and *S. aureus.* Assays were carried out in clear, round-bottom 96-well microtiter plates with samples ran in quadruplicate (n = 4) so as to be able to calculate the standard deviation and apply a Q-test to remove outlier data points if necessary (Figure 2). To each well 10 µL bacteria inoculum in FSTSB, 10 µL gel extract and 80 µL FSTSB were added. 1 µL of chloramphenicol stock, consisting of chloramphenicol solubilized in DMSO (5,000 µg/mL), was used as the positive control. 1uL of 100% DMSO was used as the negative control. Plates were incubated at 37 °C, and absorbances at 590 nm were read every hour for 24 hours on a Biotek Synergy HTX Multi-mode plate reader.

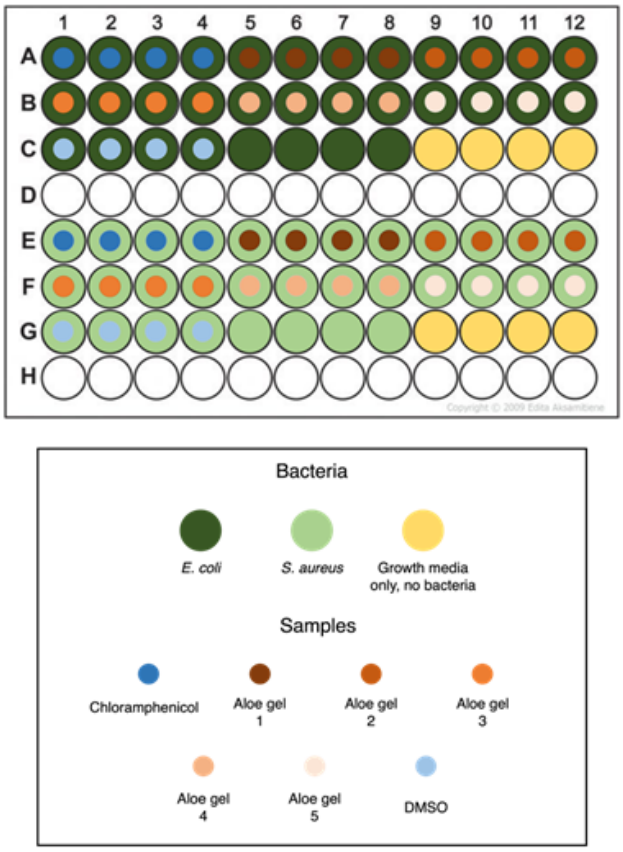

Figure 2. Plate layout for bacterial inhibition assay

2.5. Statistical Analysis of Bacterial Inhibition

Through using the Gen5 Software accompanying the Biotek HTX Multi-mode plate reader, absorbance output values for each well of the 96-well plate were exported. In interpreting this data, a mean of all replicate points (n=4) and a standard deviation were calculated using basic functions available through Excel (Microsoft). Using the calculated mean and standard deviation, confidence intervals were also determined at 95%. Considering that FSTSB is characterized by a light brown color, the absorbance value of the FSTSB only wells must be subtracted from all sample means. This was accomplished

by subtracting the mean absorbance for the FSTSB only wells from the mean of the wells of interest. In the interest of making a clear comparison between the positive control and the samples of interest, the means of all samples were normalized to chloramphenicol. This was accomplished by subtracting the mean value of chloramphenicol from the mean value of each sample, essentially making the value of chloramphenicol at each time point equal to zero. In interpreting the data, a lower mean absorbance value indicated fewer cells, which indicated a more potent antibiotic. Data was plotted for graphical interpretation, as shown in Figure 3 and Figure 4.

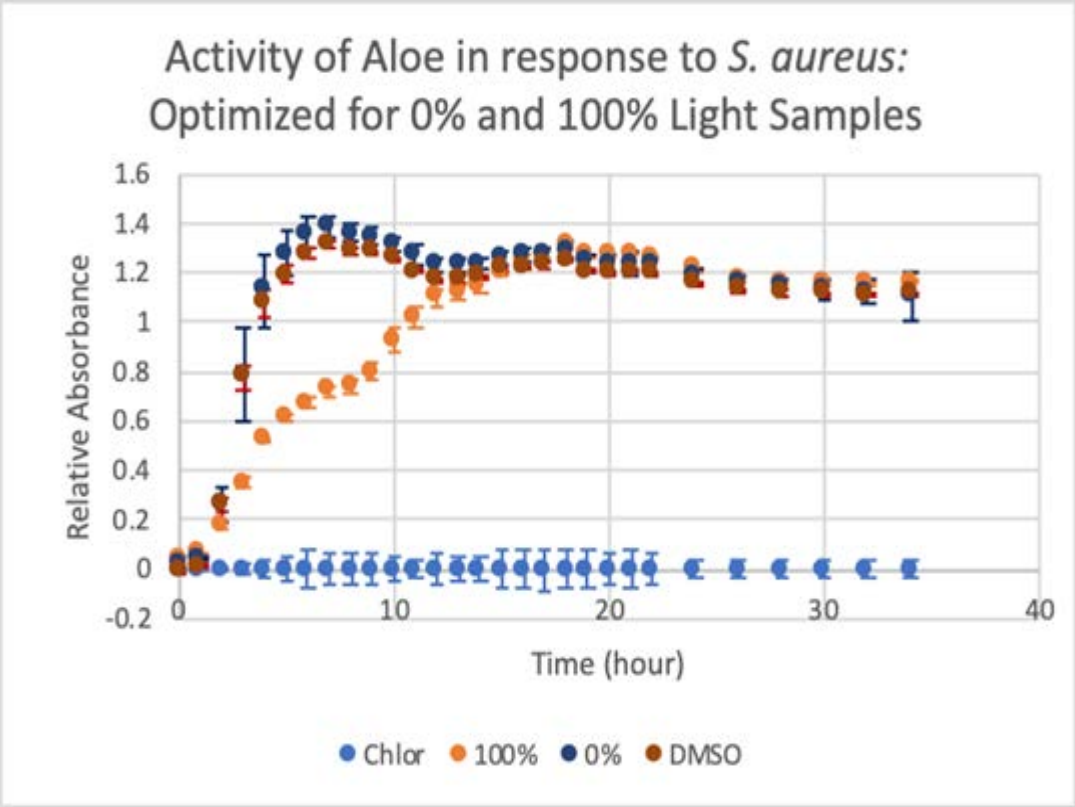

Figure 3. Plot of relative absorbance, normalized to chloramphenicol, versus time in hours, in response to *S. aureus*

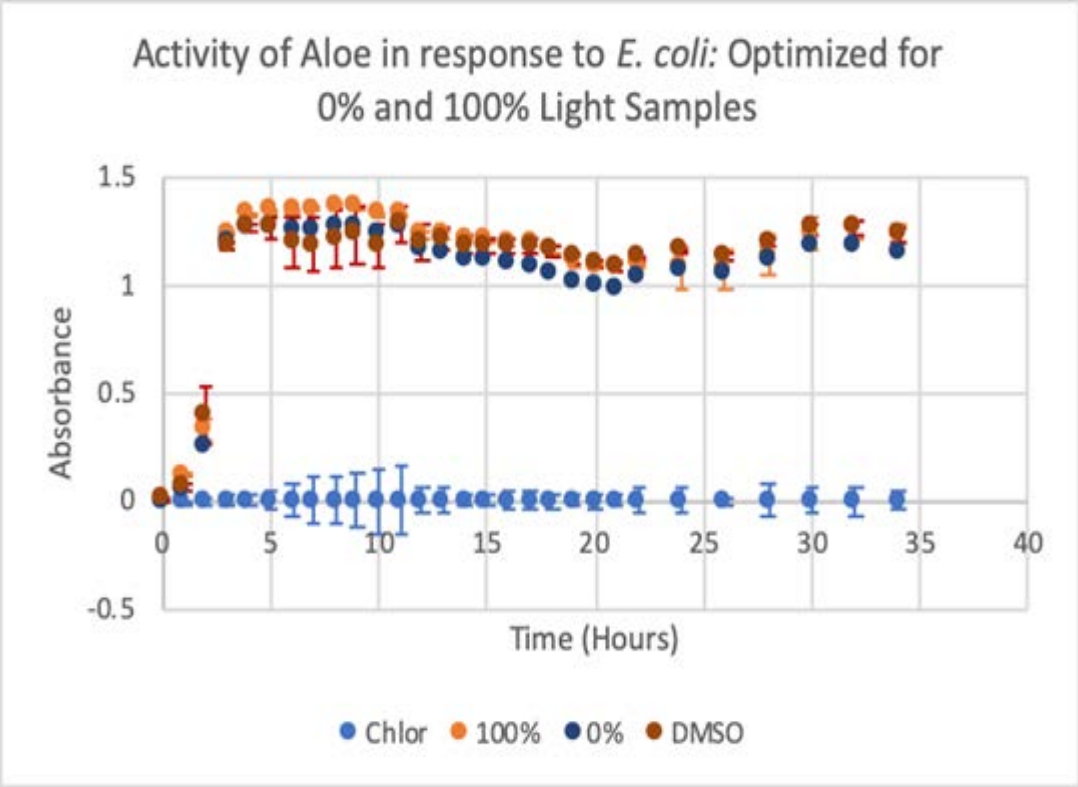

Figure 4. Plot of relative absorbance, normalized to chloramphenicol, versus time in hours, in response to *E. coli*

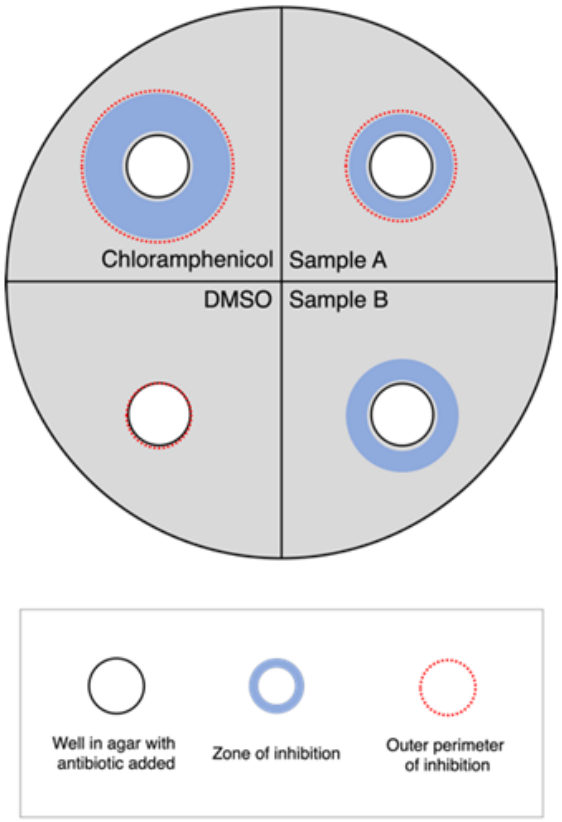

Figure 5. Example plate layout for disk diffusion method

2.6. Agar Diffusing Bacterial Inhibition Assay

As an alternative to the liquid based antibacterial assay, an agar well diffusion method was implemented, which does not require the use of a microtiter plate reader and makes this CURE more accessible to a wider variety of courses. For this method, agar plates were prepared with 10% TSA. Overnight cultures were also prepared, as previously described. The plates were then dried briefly by running through a flame. Approximately 200 µL of overnight culture of either *S. aureus* or *E. coli* in FSTSB was added to the plate, manually rotating the plate until the culture was evenly spread throughout the plate. Excess liquid was then removed. A grid was drawn on the back of the plate to indicate where each sample would be added. A hole was punched in the gel at the center of each quadrant using the wide end of a sterile polystyrene 20-200 µL pipet tip. Following this, 50 µL of sample were added to each well, using chloramphenicol as a positive control and DMSO as a negative control. For aloe wells, 50 µL of purified gel was added with a syringe. For the controls, stocks were made consisting of 1uL antibiotic (1000 µg/mL in DMSO) and 99 µL FSTSB. (Figure 5).

2.7. Hazards

The advantage to a biologically-based research project like the one proposed here is that the chemical hazards are less severe, and the presence of fumes is unlikely. However, it is important that students understand the challenges associated with handling bacteria. Strains of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 29213) were used for this project. *E. coli* was BS Level 1 and *S. aureus* was BS Level 2. All bacterial work was performed under sterile conditions, and killed by treating with 10% bleach for 20 minutes.

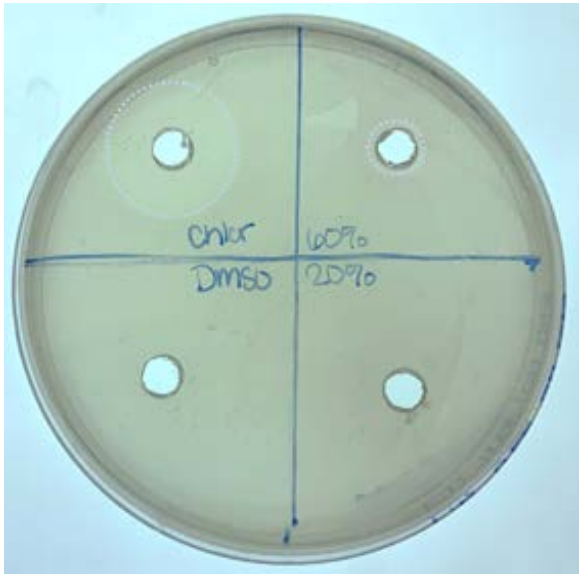

Figure 6. Results of disk diffusion assay in response to S. aureus, with inhibition patterns outlined. Value labels correspond to the theoretical shade values

3. Results

Using the two antibacterial assay methods described, we found that shaded growth does affect *Aloe vera* antibiotic production against *S. aureus*. In the instance of *S. aureus*, it became evident that the aloe grown in 100% light conditions was more potent than the aloe grown in 0% light (Figure 3). Alternatively, it appeared that inhibition of *E. coli* was stronger with aloe grown in 0% light, though the difference between the two was less than that for *S. aureus* (Figure 4).

The findings of the disk diffusion assay carried out with *S. aureus* complimented the results of the multi-well plate assay, indicating that the *Aloe* grown in 100% light has stronger antibacterial properties than that of 0% light (Figure 6). In addition, the intermediate light values showed the varied inhibitions that were experienced with the multi-well plate assay.

4. Discussion

Based on the outlined project, it was determined that in the context of *Aloe vera*, antibacterial properties can potentially be manipulated by altering shade growth conditions. This was shown to be true in *S. aureus*, and to a lesser extent in *E. coli*. This is a compelling argument that antibacterial potency of plant based natural products can be augmented by varying the growth environments.

This discovery provides the opportunity to apply this in the classroom to a variety of variables of the student's choosing. Students have the opportunity to do preliminary research to form a hypothesis to justify their variable of choice, choosing the growing conditions to manipulate. Potential variables include:

- pH
- water exposure and quality
- humidity
- fertilizer exposure and composition
- temperature

Finally, this project opens avenues to explore compound isolation and other more advanced undergraduate techniques. *Aloe vera* is a particularly convenient plant for this purpose, as it is available year-round at most hardware stores, and the process of extraction is relatively straight-forward, alleviating the need for liquid-liquid extraction or other methods to isolate the internal components that other plants require. Aside from its potential as an antibiotic, *Aloe vera* has a demonstrated of other medicinal properties, which can easily be implemented in further assays. [17,18]

5. Conclusion

Antibiotic resistance is a public health concern that is only projected to heighten in the coming years. Given that the responsibility for antibiotic discovery is primarily in the hands of academia, it is important to develop novel methods to approach this daunting task. By giving students a project where the foundation is laid in a globally relevant issue, this drives the interdisciplinary collaboration and perspectives that these issues require. It has been shown that antibiotic potency can be augmented through the manipulation of growth conditions. In expanding this project to a wide variety of growing conditions in the classroom setting, this can help gain insight into manual control of antibiotic expression.

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Statement of Competing Interests

The authors have no competing interests.

List of Abbreviations

Escherichia coli (*E. coli* or EC); *Staphylococcus aureus* (*S. aureus* or SA); tryptic soy broth (TSB); full strength tryptic soy broth (FSTSB); dimethyl sulfoxide (DMSO); gram (g); liter (L).

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