JOURNAL OF
EMERGING INVESTIGATORS

Article

Analysis of ultraviolet light as a bactericide of gram-negative bacteria in *Cladophora* macroalgae extracts

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SUMMARY

In recent years, antibiotic resistance has increased in pathogenic bacteria, posing a growing threat to both patients and the pharmaceutical industry. This has resulted in inflated medical expenses and higher mortality rates for individuals diagnosed with bacterial infections. A potential alternative to synthetic medications involves the use of marine algae as natural antibacterial agents. Prior research suggests that the *Cladophora* macroalgae genus may be one option, as it has high polyphenolic content that has previously exhibited antibacterial properties. However, obtaining organic samples from bacteria-ridden environments poses complications concerning microbial contamination of medicinal plants. During initial trials of antibacterial testing, the *Cladophora* algae extract was found to be greatly contaminated with gram-negative bacteria. The study was then adapted to explore ultraviolet (UV) light as a possible bactericide of this microbial growth. The experiment utilized a control with no UV light treatment along with four experimental samples exposed to UV light for durations of 15, 30, 45, and 60 minutes. We found that there was a statistically significant difference between the control mean and the means of the 30, 45, and 60-minute treatment groups. The overall data trend displays a negative correlation between the ZOF diameter and UV exposure time, supporting the hypothesis that longer durations of UV light treatment have a greater degree of antibacterial effectiveness.

INTRODUCTION

The overuse of antibiotics has led to a crisis in which dangerous infections are no longer treatable by previously reliable medications. A 2019 investigation by the Center for Disease Control (CDC) reports that more than 2.8 million antibiotic-resistant infections occur in the U.S. each year, resulting in more than 35,000 deaths nationwide (1). Creating new pharmaceutics from existing sources no longer provides a sustainable solution to this issue. Although modifying traditional medications may provide a short-term fix, this process is costly, time consuming, and does not guarantee a long-term resolution to this time-sensitive crisis (2). Unlike with pharmaceutically-produced antibiotics, drug resistance may be harder to develop with natural antibiotics, making them an ideal combatant of drug-resistant bacteria in humans (3). A growing field that offers a promising natural alternative to synthetic antibiotics are the bio-active compounds derived from marine algae (4). A prior study in this expanding area of research found that many macroalgae contain a multiplicity of biomedical defenses, such as phlorotannins, polysaccharides, and peptides that produce antibacterial, anti-inflammatory, and antioxidant properties (4). Explorations conducted in the past decade assert that certain algae have effectively developed defense systems that combat threats like bacterial pathogens, viruses, and fungal infections (5). The potential of algae to act as a medicinal agent is largely due to their high polyphenolic content. Thanks to their ability to inhibit bacterial factors such as enzymes and toxins, polyphenols act as potent compounds that work to fight diseases and foreign cells within the plant (6). Naturally, these components give way to the opportunity for marine algae to act as natural medicinal agents in the pharmaceutical industry.

Despite the potential for marine algae to act as antibiotics, researchers face challenges when it comes to obtaining a pure sample (7). Marine algae are often found in naturally contaminated environments. In most cases, medicinal organisms are obtained with high levels of microbial impurities that may disrupt testing for antibacterial effectiveness and possibly impact the commercial use of algae with medicinal potential (7). Previous research into this complication suggests that the *Cladophora* algae genus in its natural state can be associated with high levels of *Escherichia coli* bacteria growth (8). A 2003 study concluded that the *Cladophora glomerata* algae species provides a suitable environment for *E. coli* to grow under natural conditions and persist for extended periods of time (8). In order to obtain high quality plant compounds for commercial use, it is of utmost importance that reliable techniques of lavation are developed and effectively implemented into industry practices.

When we found the *Cladophora* extract to be heavily contaminated with gram-negative bacteria, the aim of this study was altered to explore ultraviolet (UV) light as a possible bactericide of the microbial contamination. This disinfection method was selected due to its known efficacy in killing bacteria. For over a century, electromagnetic radiation has been found to effectively kill harmful microorganisms by breaking DNA strands and rendering the cells inactive (9). The goal of this study was to determine the effectiveness of UV light in eliminating bacterial contagions in algae extracts. If verified, UV light could be used on a larger scale and implemented as a common practice to prevent contamination

in pharmaceutical algae plants, offering a solution to this pressing matter within the field of natural antibiotic exploration.

The independent variable for this study was the duration of UV light exposure. Antibiotic sensitivity discs were used to compare the effectiveness of UV light in killing gram-negative bacteria in extracts from *Cladophora* algae. The dependent variable in this investigation was the size of the zone of facilitation (ZOF). This is the circular area of bacteria growth surrounding each sensitivity disc. We hypothesized that longer durations of ultraviolet light exposure would have a greater degree of antibacterial effectiveness on the *Cladophora* algae extract. Ultimately, the data displayed a statistically significant variation between the control mean and the means of the 30, 45, and 60-minute treatment groups, supporting the hypothesis.

RESULTS

After conducting the original experiment, we found that instead of inhibiting the growth of *S. epidermidis*, the extract-infused discs facilitated a dense growth of an unknown bacteria **(Figure 1)**. We then performed a gram stain and determined that the unknown growth was a strain of gram-negative bacteria, presented microscopically as short red rods (data not shown). Although not confirmed, we presumed that this gram-negative bacteria could be a strain of *E. coli*. Due to nearly identical molecular structure to images found online, we concluded that the unknown bacteria were likely from the *E. coli* species (13). Prior studies suggest that *Cladophora* provides a stimulating environment for *E. coli* growth both in natural and laboratory environments (14).

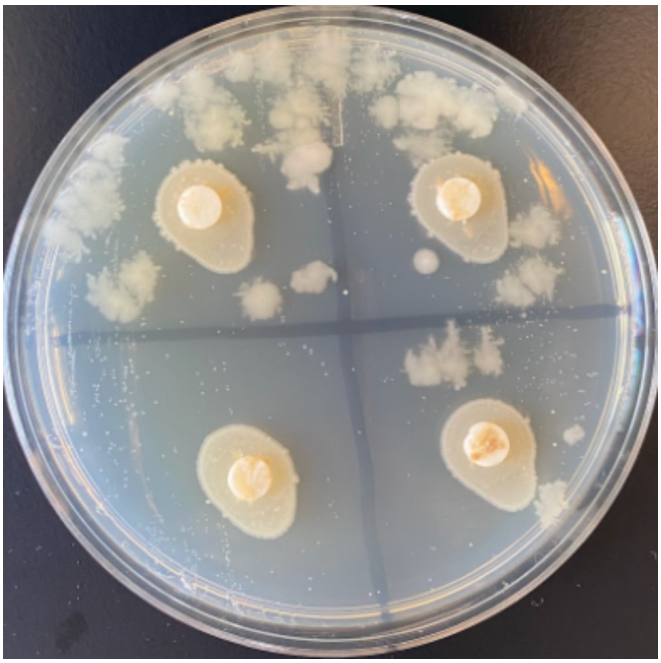

Figure 1: Discs with algae extract on *S. epidermidis*. This is an example of the unknown growth that surrounded the extract-infused discs after using the procedure in the first experiment. The dense Zone of Facilitation (ZOF) is expected to be a strain of *E. coli* bacteria. The surrounding colonies are the *S. epidermidis* bacterium plated before incubation. The procedure and hypothesis were then altered after receiving this unexpected result.

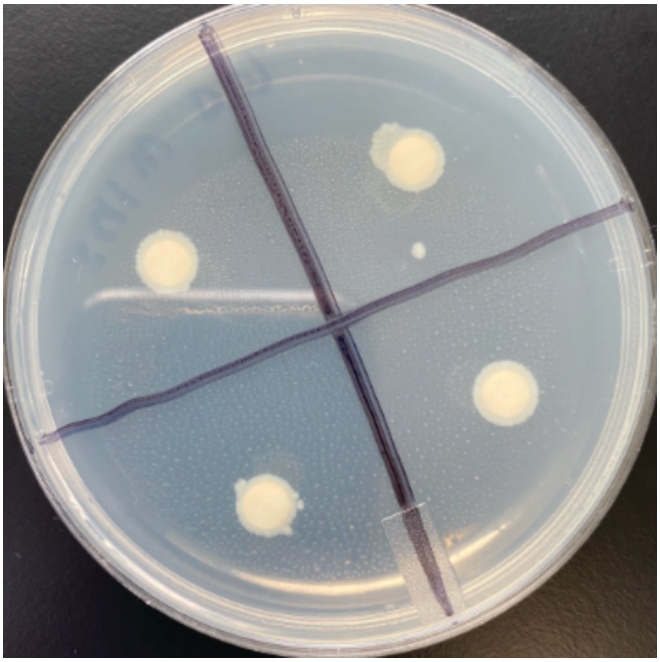

Figure 2: Extract-infused discs on sterile agar plate after incubation. This is an example of the bacteria growth (ZOF) that occurred around the sensitivity discs infused with extract treated with UV light for 60 minutes. This growth is likely the remaining *E. coli* bacteria not killed by the UV treatment.

The study was then altered to analyze the efficacy of UV light as a bactericide. The ZOF was defined as the total circular area around the disk where the sample produced visible bacterial growth **(Figure 2)**. A large ZOF indicated a less potent effect of the UV treatment, while a ZOF of zero indicated complete antibacterial capabilities. We compared the mean ZOF measurements across the five treatment groups **(Figure 3)**.

The data suggested that longer durations of ultraviolet light treatment result in a smaller ZOF diameter. The control group had a significantly larger ZOF than all the UV treatments except for 15 minutes ($p = 0.07684$). Overall, there is sufficient evidence to reject the null hypothesis for UV exposure times of 30, 45, and 60 minutes, however, we failed to reject the null hypothesis for a treatment time of 15 minutes.

DISCUSSION

After finding the *Cladophora* extract to be contaminated with gram-negative bacteria during antibacterial testing, the study was shifted to analyze ultraviolet light as a bactericide of the microbial growth in the algae extract. We hypothesized that longer durations of ultraviolet light exposure would have a greater degree of antibacterial effectiveness, resulting in smaller ZOF measurements. The data supported the hypothesis, suggesting that UV light acts as an effective antibiotic treatment in *Cladophora* algae extract for durations of 15, 30, 45, and 60 minutes.

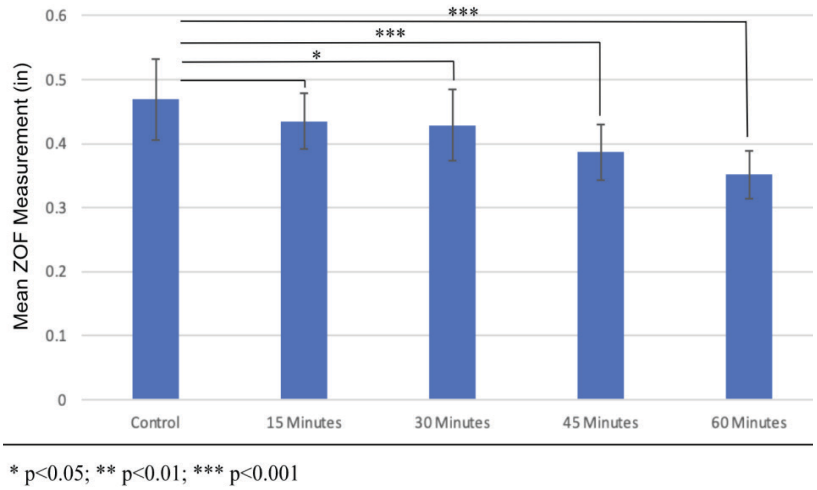

Figure 3: Zone of facilitation measurements. Discs with algae extract were placed on sterile plates and treated with UV light for 0, 15, 30, 45, or 60 minutes. Zone of facilitation was measured, and the mean is represented by bars. Statistical analysis was performed using the One-Way ANOVA test and the *p* values are indicated. Error bars represent the standard deviation in the data.

The complications encountered in this study demonstrate many of the challenges that arise during preparation of medicinal organisms in the field of natural antibiotic research. Pharmaceutical plants and marine algae are at high risk for containing biological contaminants due to environmental factors that impact the quality of organic samples (7). Moreover, *Cladophora*'s role in promoting *E. coli* growth enhances the risk for dangerous contagions in sources of natural antibiotics. Risk evaluation and elimination of microbial abundance in medicinal organisms has hence become an important objective in the establishment of current Hazard Analysis and Critical Control Point initiatives (7). The goal of this research was to contribute to the knowledge and ongoing investigation into decontaminants and possible lavation methods used in pharmaceutical preparation techniques.

To overcome contamination from microorganisms, several technologies such as ethylene oxide gas, gamma irradiation, and steam heating have been used (10). However, these methods have disadvantages such as toxic by-products and lowered efficacy of the medicinal properties in the plant. The results of this investigation suggest that ultraviolet light may be used as a decontamination technology that accomplishes the same purposes as traditional techniques without the possible drawbacks. This is because UV light is a non-thermal approach that uses the electromagnetic spectrum to deactivate microorganisms and has been confirmed to be effective against bacteria within the range of 200-280 nm (11). This occurs due to the ability of ultraviolet light to penetrate the cell membrane and to damage the DNA or RNA of microorganisms, thus preventing their reproduction.

A future direction of the study includes investigating various wavelengths of UV light. Although the wavelength we used falls within the suggested range for UV effectiveness (254 nm), the study was not able to test shorter wavelengths of UV irradiation. Naturally, this subject demands extensive additional research. Due to a time constraint of 17 weeks, we were not able to test the antibacterial effectiveness of *Cladophora* after UV decontamination. This subject requires additional exploration, both in the investigation of UV light as a bactericide, as well as marine algae as a natural antibiotic compound. If the findings of this study continue to be expanded upon, UV light could be used on a larger scale and implemented as a common practice to prevent contamination in pharmaceutical algae plants.

MATERIALS AND METHODS

Green macroalgae determined to be a species of the *Cladophora* genus were collected from a Michigan freshwater pond. We collected a sample from the harvested plant material and compared the cell structures using a microscope to *Cladophora* purchased from Carolina Biological Supply Company. The sample was then air dried and ground into a powder consistency using a mortar and pestle. In order to obtain bioactive compounds from the organic material, we performed a water extraction modeled after a similar study (12). To begin, 100 grams of air-dried plant powder was extracted using 400 mL of distilled water as a solvent. The sample was allowed to soak undisturbed for a period of 6.5 hours. After the maceration period was complete, the water was filtered through a grade 1 Whatman No.1 Filter Paper under vacuum conditions using a sink aspirator connected to a side arm Erlenmeyer flask. After filtration, 150 mL of water-algae extract remained for experimental use. Algae extraction was performed twice, one for each experiment.

Determining Antibacterial Effectiveness

The first procedure aimed to determine the antibacterial effectiveness of the *Cladophora* algae extract. To conduct experimental trials, agar plates were first inoculated with *Staphylococcus epidermidis* bacteria. Blank sensitivity discs were then dipped into the extract sample and placed in each quadrant of the plates using forceps. The forceps were

sterilized using a flame to ensure no outside contaminants affected the results. The agar plate was then incubated at 37 °C for 24 hours.

Analyzing Ultraviolet Light as a Bactericide

In order to test ultraviolet light as a bactericide of this contaminant, the same extraction technique was performed, and the sample was divided into five 30 ml treatment groups. The control group was not treated with any UV light. The four experimental samples were treated at different durations of UV light exposure, including 15, 30, 45, and 60 minutes. This was done using a Cetrix ChargeMax UV disinfection cabinet (254 nm). The same disc diffusion and plating methods were used as described in the first procedure; however, the agar plates remained sterile in this experiment, excluding the *S. epidermidis*. After an incubation period of 24 hours, the diameter of each ZOF was measured using a digital caliper.

Statistical Methods

To determine statistical significance, a One-Way ANOVA hypothesis test was performed using the Social Science Statistics calculator. With a F-ratio of 23.913 and p-value < 0.001, the variation between sample means was significant ($p < 0.05$). To produce pairwise comparisons, the Post Hoc Tukey HSD test was conducted. All but three pairs (T_1:T_2 (control vs. 15 minutes), T_2:T_3 (15 minutes vs. 30 minutes), and T_4:T_5 (45 minutes vs. 60 minutes)) demonstrated significant results. In the context of the study, it is particularly important to consider that the UV treatments of 30, 45, and 60 minutes resulted in a significantly reduced ZOF as compared to the control ($p < 0.05$), while the control vs 15 minute comparison fell short at $p = 0.07684$.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Ed Robinson for consultation on developing the procedure.

Received: June 7, 2022
Accepted: August 3, 2022
Published: November 7, 2022

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