An Analysis of Antiseptic and Antibiotic Properties of Variously Treated Mosses and Lichen

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Abstract

Lichen, a symbiosis between algae and fungus, and sphagnum moss are both able to fight against bacteria. In this experiment, we tested whether moss and lichen have antibiotic or antiseptic properties on human-affecting bacteria, and to find out which types of mosses and lichen have the strongest of these properties. We collected samples of fruticose lichen, foliose lichen, crustose lichen, red sphagnum moss (Sp. 1) and green sphagnum moss (Sp. 2) from Bryant Bog and placed them into 160 Petri dishes with bacteria. We also recorded the pH of each type, to see if it had a relationship with bacterial growth. Although pH was not found to be related to antiseptic and antibiotic properties, treatments of each taxon were found to make a difference in the inhibition of bacterial growth. The abilities lichen and sphagnum moss to fight off bacteria were determined after they were plated for five days with gram-positive bacteria in an incubator at 35℃. From our test, we were able to draw the conclusion that fruticose, when crushed, was a strong antibiotic compound, and ground green sphagnum moss (Sp. 2) was a strong antiseptic compound.

Introduction

Sphagnum moss has for decades been used to dress wounds due to its exceptional absorbent power (Porter 1917). It was used in both World War I and World War II to conserve cotton for other uses. However, its absorbency may not be its only practical use. Sphagnum moss is also thought to have an ability to inhibit gram-positive bacteria growth (Painter 1991). Nine species of sphagnum from southwestern British Columbia showed effectiveness against gram-positive bacteria, whereas none tested were active against gram-negative bacteria (S.J. Kang *et al*. 2007). The bacteria-fighting mechanism of sphagnum mosses is not entirely clear, but some have suggested that it may be related to the moss' uronic acids, which are believed to be what gives sphagnum its bacteria-inhibiting properties (Clymo 1963). Others suggest that it may be due to its acidic properties (Stalheim *et al.* 2008). Sphagnum moss was found to have strong abilities to exchange cations with its surrounding environment, in addition to uronic acids, causing sphagnum moss to have high acidity levels (Clymo 1963).

Lichen may also play a role in fighting specific types of bacteria, as past studies have found that some acidic lichen compounds have similar molecular appearances to antibiotic substances, which created a gateway to the discovery of an active antibiotic compound in lichen (Burkholder and Evans 1944, 160). It was also discovered that many of the antibiotic substances present within lichen are acids, such as usnic acid (Burkholder and Evans 1944, 160). Yet, when Burkholder and Evans tested lichen against gram- negative bacteria, most of the lichens appeared to have little to no antibiotic activity, however, small zones of inhibition were observed with gram-positive bacteria (Burkholder and Evans 1944).

Both gram-negative bacteria and gram positive bacteria are found, in abundance, within the human body (Kenneth 2008); however, gram positive is more susceptible to growth

inhibition by antibiotics and antiseptics. This is mainly due to the fact that gram-positive bacteria has a single cell membrane that lends less protection from antibiotic substances then the double membrane found in gram-negative bacteria (Ghuysen 1994).

In past studies, many researchers did not distinguish between whether the moss or lichen killed bacteria or just suppressed its growth (Burkholder and Evans 1944, Clymo 1963). In this paper, substances that are capable of killing bacteria are referred to as antibiotic, whereas substances that only inhibit bacteria growth are called antiseptic (Callahan 2001, Weber *et.al*., 2007). For example, tetracycline is a common antibiotic—it kills bacteria, whereas hand sanitizer is commonly used today as an antiseptic and only prevents the spread of bacteria.

We tested the antibiotic and antiseptic abilities of moss and lichen collected from University of Michigan Biological Station against gram-positive bacteria. We selected two different species of moss and distinguished them by their different colors- red and green. For the purposes of our study, species identification was not necessarily important because we only wanted to know if there were differences between two species of moss. It is also easy to distinguish between them in the field and they could be identified later if need be. We selected lichens of three different forms—fruticose, foliose, and crustose—to compare potential differences in inhibitory abilities among visibly different lichens. The purpose of this experiment was to determine whether moss and lichen have antibiotic or antiseptic properties on human-inhabiting bacteria, and to find out if there are differences in effectiveness across species and treatments of sphagnum mosses and lichen. We predict that moss and lichen will have antiseptic properties and that those properties will be correlated with pH. Our results, if conclusive, could be useful for wilderness survival techniques and also serve as a potential source of antibiotic or antiseptic therapies.

Methods

Bacterial Cultures

We created agar from a mixture of distilled water, dehydrated bacto-agar and chicken boullion at a ratio of 48:1:1. We heated the mixture to a boil on a hot plate and then let it cool to 50°C before pouring it into Petri dishes, where the agar cooled until solid at room temperature. We plated a check swab and incubated the plate for three days at 35°C to mimic the temperature of a human body.

After three days of incubation, bacteria were visible on the Petri dish. In order to determine whether the bacteria were gram-positive, meaning it had a single cell membrane, Gram's staining method was used. We heat fixed a smear onto a microscope slide, and a carbol gentian violet solution was applied for 60 seconds. We then applied Lugol's iodine solution for 60 seconds. The slide was dipped into an alcohol solution for 30 seconds, and then rinsed clean with tap water. A safranine solution was applied for 120 seconds, and the slide was again rinsed with water. We allowed the slide to dry, and the bacteria appeared violet, a sign that it lacked the outer cell membrane that gram-negative bacteria would have. Bacteria from the first colony was transferred onto another Petri dish and allowed to incubate for two days. Next, the gram-positive bacteria were transferred to each of 160 Petri dishes, using a wire loop sterilized with ethanol.

Moss and Lichen Collection and Treatments

We obtained sphagnum moss at Bryant Bog in Pellston, Michigan. We collected both red and green sphagnum moss by the handful and placed them into gallon plastic bags. In addition, we also collected water samples by squeezing moss samples into 250 mL plastic jar. The squeezed

moss was also collected into plastic bags and was later laid out to dry. A portion of both types of dried moss were ground using a mortar and pestle.

We collected lichen from Bryant Bog; fruticose, foliose, and crustose lichen were collected from the bark of white pine trees and placed into plastic bags. Some of each type of lichen was ground using a mortar and pestle. Both the moss and lichen sat in bags or out to dry for five days. To test the pH of lichen, we ground it and allowed it to sit in distilled water for thirty minutes before using a pH meter to obtain the pH. This method was based on a similar method that is used for soil.

Moss treatments included four states: wet with bog water; dry; wet with distilled water (which was dried moss re-wet); and ground. Controls included Petri dishes with cotton placed in the dishes, wet and dry, to simulate the weight and texture of wet and dry moss, and Petri dishes with pencil shavings to simulate ground moss (Table 1). We also had four agar plates with nothing but bacteria.

Lichen treatments included the lichen's original state and a ground state. For controls, we used pencil shavings and ground pencil shavings to simulate weight and texture of normal and ground lichen (Table 2).

Experiment 1: Inhibition Test

To test and quantify the amount of bacteria growth mosses and lichen could inhibit, we spread gram-positive bacteria onto 80 agar plates. At the same time we placed a 2 cm diameter circle of moss, lichen, or a control on four plates for each treatment. We let the plates incubate at 35°C for 5 days. To quantify areas of agar where bacterial growth was inhibited, we used a grid and counted the area of each plate with no growth in square centimeters.

Experiment 2: Kill Test

On another 80 plates, we plated gram-positive bacteria and allowed the culture to grow for 2 days. Then we placed the moss and lichen treatment in a 2 cm diameter circle on top of the bacteria in the center of each plate, using four plates for each treatment. After 5 days of growth, we used toothpicks to transfer bacteria from within a zone just outside the moss and lichen treatments to gridded agar plates. We allowed these new colonies to incubate for 2 days and the noted whether or not each colony of bacteria grew or not. We assumed that if bacteria did not grow when transferred to a new agar plate, then the moss or lichen had killed it.

Statistical Tests

For Experiment 1, we used unequal variance t-tests to test for differences in the mean area inhibited between each type of moss and lichen and the cotton or pencil shavings, as well as between each treatment for each type of moss and lichen and their controls. Next we used a Brown-Forsythe (as an alternative to ANOVA because our sets had unequal variance (Brown and Forsythe 1974)) to test for differences in effectiveness between treatments, and then used Games-Howell to locate the inequalities.

For Experiment 2, we ran a Chi-square test on results of the gridded agar plates to test for significant differences between “bacterial growth” and “no growth” on the plates. In running this test, we assumed that 100% of the plates would show “bacterial growth”. To determine whether pH was a factor in area inhibited, we ran a Spearman’s correlation comparing pH of the moss or lichen and area of bacteria inhibited.

Results

Our findings indicated that the types of moss and lichen we tested do, in fact, exhibit antibiotic and antiseptic properties. Experiment 1 showed that most treatments for the taxa were significantly effective at inhibiting bacterial growth (Table 3). In comparing the effectiveness of taxa to inhibit bacterial growth regardless of treatment, the area of inhibition for each taxa was significantly larger than that of the controls (Table 4).

There seemed to be a trend in the effectiveness of treatment by treatment type, so we combined data by treatment type (across taxa). Because we had unequal variance among our samples, we used a Brown-Forsythe test instead of an ANOVA test to compare the effectiveness of each treatment in inhibiting bacterial growth (i.e., we tested dry, whole moss and lichen; dry, crushed moss and lichen; wet moss in bog water; and wet moss in distilled water). There was a significant difference ($p = 0.03$). Using a Games-Howell test to make individual comparisons among treatments, we found "dry, whole" and "dry, crushed" were both statistically more effective at bacterial inhibition (and were equivalent) ($p = 0.04$) than "wet bog" and "wet distilled", which were also equivalent (Figure 1).

We noticed that in general, fruticose had the highest area inhibited (Figure 2). To see if this was a significant trend, we used t-tests to make comparisons of the area inhibited by each taxa (disregarding treatments). There were significant differences in two cases; fruticose inhibited more growth than red moss ($t=2.48$, d.f. = 22, $p=.021$), and fruticose also inhibited more growth than foliose ($t=2.561$, d.f. = 14, $p=0.023$ –Table 5 and figure 3). Perhaps if we had more samples we could see a more definite pattern.

We wanted to see if pH and affected area were related, so we measured the pH of moss and lichen (Table 6). The Spearman's coefficient for the correlation between average area

inhibited and pH was close to 0, indicating that pH and size of inhibited area of bacteria are not correlated (Figure 4).

In Experiment 2, we ran Chi-square tests to test our null hypothesis: that all colonies would grow. Again, we ran tests for taxa, disregarging treatments, so we could see broad trends. Results indicate that fructicose ($p=0.046$), and crustose lichen ($p=0.046$) grew significantly less than expected, suggesting they exhibited antibiotic properties.

In summary, results of our experiments suggest that green moss and red moss exhibit antiseptic properties, while fruticose and crustose exhibit antibiotic properties. Foliose lichen displayed predominantly antibacterial properties, but the results were not significant enough to say one way or another. Red moss had one treatment that was completely ineffective and was not used in the Chi-square. We did not test for significance across treatments, but noticed that three out of four species were antibiotic in the ground treatment, and we thought this was an interesting result that could be looked further into.

Discussion

We were interested in testing antiseptic and antibacterial properties in moss and in lichen because both are abundant in the forests and bogs near the University of Michigan Biological Station, and could possibly offer an alternative to modern medicines involving bacteria inhibition. Identifying the exact species of sphagnum we tested proved difficult, but did not matter with our experiment as we were only interested in finding out about the bacterial inhibition abilities of common mosses in the area.

We thought acidity would be positively correlated with area of bacteria inhibited, but our results show that pH is probably not a significant factor in effectiveness. The question remains as to which mechanisms are inhibiting bacterial growth or killing bacteria. These mechanisms are not likely to be something excreted or on the surface of moss or lichen because the ground treatments were most effective, meaning that the mechanism is likely a substance existing within the cell structure. There are some metabolites within lichen that have been proposed to protect lichen from herbivores (Lawry 1989). This is a possible antibiotic element that could be residing within the cells of lichen that were released upon the crushing of its structure.

Studies done by Burkholder and Evans (1945) concluded that acidity was a possible factor in antibacterial activities of lichen, yet this conclusion conflicts with our findings. Our research shows that the mechanism causing antibacterial and antiseptic properties is probably not acidity or cell surface structures. Despite the conflict between past research and our research, there is still a possibility that acidity is responsible for antibacterial and antiseptic qualities in moss and lichen. It's possible the difference is due to faulty assumptions on our part—we assumed that pH would remain constant across treatments within species and the controls would all have a neutral pH. Perhaps more extensive research could prove whether acidity truly is a

factor in bacteria inhibition for sphagnum and lichen. A larger sample size and more types of moss comparisons may help show significance between acidity and bacteria-inhibiting qualities.

Our findings regarding the antibiotic and antiseptic properties of moss and lichen have practical applications. Sphagnum, being more antiseptically effective, should be placed on a wound immediately to inhibit bacteria growth. Perhaps the use of moss for wounds was mainly for absorbent properties and was convenient because of high abundance and short drying time, as well as a possible benefit of preventing infection. Lichen on the other hand, can be used to kill bacteria rather than simply inhibit its growth and works best when ground into a powder and applied at the time of injury. If we had monitored the incubating plates daily, we may have had a better idea about the vital time period revolving around use of moss and lichen on a wound.

Birds use moss and lichen for practical applications as well. Researchers found that some birds, such as the western bluebird, the pygmy nuthatch and the tree swallow, choose certain plant material for its antimicrobial properties in order to protect against biodegrading microbes (American Society for Microbiology 2004). Protective substances in such plant material, such as usnic acid and ascorbic acid match acidic substances in moss and lichen, suggested that the use of moss and lichen in nests is a practical application. Studies done by Burtt and Ichida (1999) found that *Bacillus licheniformis* is a type of bacteria that can cause feather degradation in birds. *B. licheniformis* is also known to be gram positive, meaning that moss and lichen used in a nest could have possible bacteria-inhibiting benefits for birds.

The absence of significance in some types was interesting. For example, dry whole red moss had no effect on bacteria, whereas other treatments of red moss did, and all treatments of green mosses did. Mold grew on some agar plates that contained red moss, possibly causing competition between bacteria, and skewing results for Experiment 2. However, we did find that

all four Petri dishes containing one type of organism and treatment usually had the same effect on bacteria, suggesting consistency among taxa and their respective treatments. We believe that if we had used more samples, we could have shown that most trends we observed were significant.

Future research on this topic is not limited to the methods we used. Past studies of sphagnum's antiseptic properties used moss in a suspension of saline solution, inoculated with bacterial cultures, with samples withdrawn at intervals for plate counts (Painter 2003). Another idea is to keep our agar plate method, but plate common antiseptic and antibiotic substances along with all taxa and their controls, in order to determine which have greater bacterial inhibition abilities. Our experiment was relatively simple, but our results were complicated. We were fortunate to find patterns between taxa and their ability to inhibit or kill bacteria. Our results were promising, and offer a reason for further study.

Tables

Table 1*

	Moss type 1	Moss type 2	Moss control
Wet, bog water	8	8	8
Wet, distilled water	8	8	8
Dry	8	8	8
Dry, ground	8	8	8

Total= 96

Table 2**

	Crustose	Fruticose	Foliose	Lichen control
Original	8	8	8	8
Ground	8	8	8	8

Total= 64
Grand total= 160

***Table 1 summarizes the number of Petri dishes used for moss. There were eight Petri dishes for each treatment; four for Experiment 1 and four for Experiment 2.
**Table 2 summarizes the number of Petri dishes used for lichen. There were eight Petri dishes for each treatment; four for Experiment 1 and four for Experiment 2.

Table 3:
All types compared to controls

	Red moss		Green moss		Fruticose		Foliose		Crustose	
	Mean area	Sig	Mean area	Sig	Mean area	Sig	Mean area	Sig	Mean area	Sig
Controls	2.77	**0.000**	1.9	**0.002**	4.13	**0.001**	1.7	**0.022**	2.97	**0.004**
T value	4.478		3.657		5.655		2.922		4.199	

(Bold) **p<=.05**

Table 4: All treatments compared to controls

	Red moss		Green moss		Fruticose		Foliose		Crustose	
	Mean area	p-value	Mean area	p-value	Mean area	p-value	Mean area	p-value	Mean area	p-value
Wet_bog	2.64	**0.10**	1.05	**.032**	-	-	-	-	-	-
Wet_distilled	0.807	0.32	3.4	***.003***	-	-	-	-	-	-
Dry_ground	4.233	**0.061**	3.15	0.12	4.69	***0.014***	2.65	***.000***	3.7	**0.065**
Dry_whole	3.38	**0.079**	0.000	1.00	3.58	**0.059**	0.806	0.32	2.24	***0.006***

(Bold) **p<=.1**, (Bold and italicized) ***p<=.05***

Table 5: All types compared to all other types

	Green Moss		Red Moss		Fruticose		Foliose		Crustose	
	T	Sig	T	Sig	T	Sig	T	Sig	T	Sig
Green Moss	.	.	1.07	0.29	-1.34	0.193	1.068	0.297	-0.21	0.839
Red Moss			.	.	-2.48	0.021	0.208	0.873	-1.2	0.242
Fruticose					.	.	2.561	0.023	1.139	0.274
Foliose							.	.	0.175	0.198
Crustose									.	.

p<=.05

Table 6

Organism	pH
Bog water, away from moss growth	5.91
Red moss	4.33
Green moss	5.20
Crustose	5.53
Foliose	4.62
Fruticose	4.73

Figures

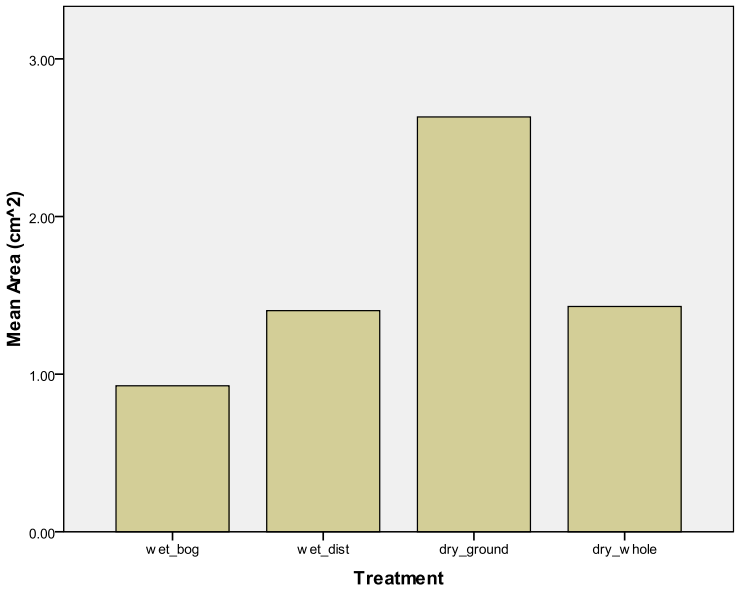

Figure 1: A graph of mean inhibited area for each treatment. The dry, ground treatment has the highest average inhibited area, followed by dry, whole. Both of these were shown to be statistically equivalent.

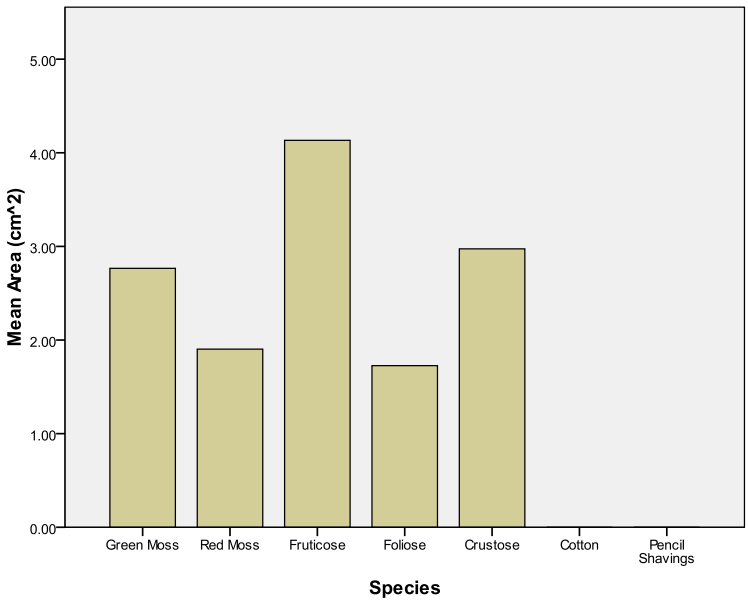

Figure 2: A graph of mean inhibited area for each taxa. Fruticose lichen had the highest mean inhibited area.

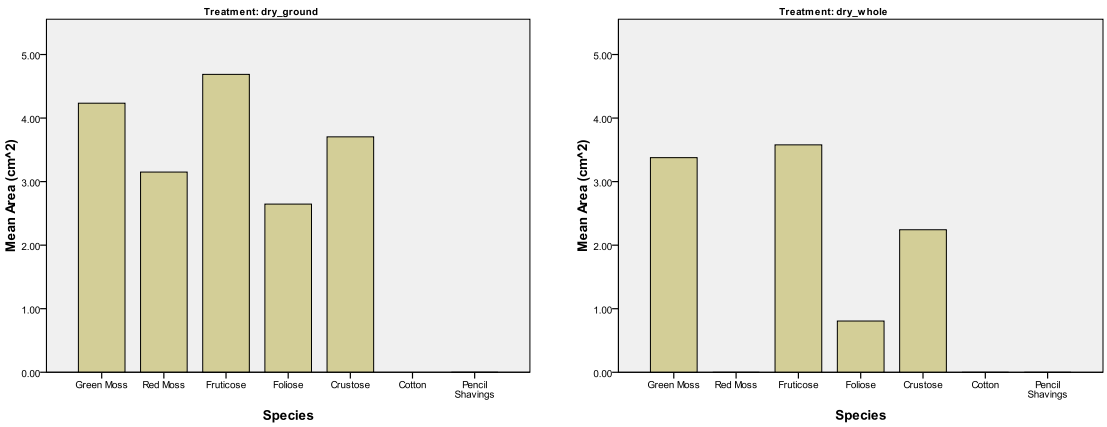

Figure 3: A graph of mean inhibited area for each species when only looking at the dry ground and dry whole treatments. In both treatments, fruticose had the highest average inhibited area.

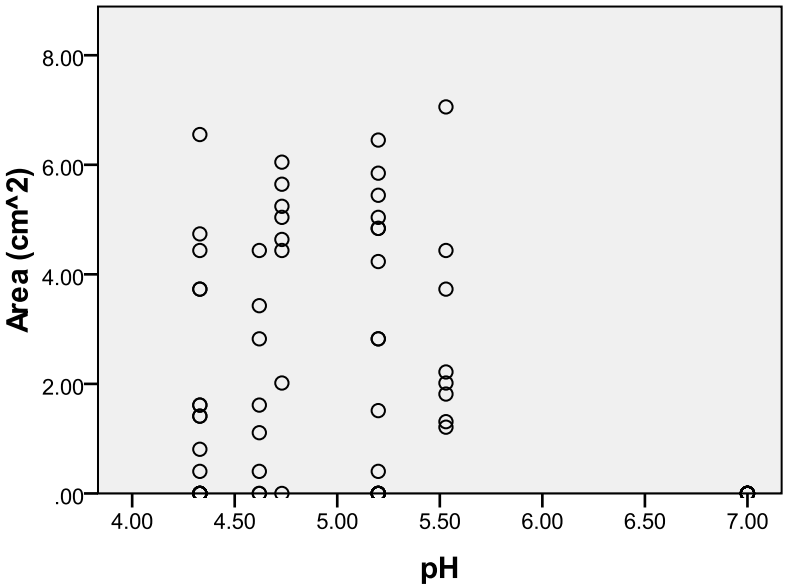

Figure 4: A graph of pH plotted against control to see if the two variables were correlated. In a Spearman's correlation test, we found they are not likely to be related. R^2: -0.2, p=0.009

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