Antibiotic Potential in the Liverworts, Especially Ricciocarpus Natans (L.) and Marchantia Polymorpha (L.)

Theodore Roy Shields
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ANTIBIOTIC POTENTIAL IN THE LIVERWORTS,
ESPECIALLY RICCIOCARPUS NATANS (L.)
AND MARCHANTIA POLYMORPHA (L.)

by
Theodore R. Shields

A Thesis
Submitted to the
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Theodore R. Shields
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CHAPTER I

THE PROBLEM AND ITS BACKGROUND

Introduction to the Problem

The use of plant extracts to control diseases caused by parasites is of continuing interest to professional health services personnel. The discovery of new substances to fight new mutant strains of bacteria is definitely needed by them. An investigation of two non-vascular plants was undertaken for antibiotic substances.

The specific problems of this investigation were:

1. to detect the presence of a substance with potential antibiotic activity in the liverworts, especially Ricciocarpus and Marchantia;

2. to extract the plant tissues with solvents;

3. to test the strength and inhibition of growth of the extracts against test organisms;

4. to determine the stability and chemical structure of the compound.
CHAPTER II

LITERATURE REVIEW

Many plants and their extracts have been used for medicinal purposes even before the finding of penicillin by Fleming in 1929. Verrill (1939) states that the Indians' medicine man used various plants and herbs for his cures, among which was the liverwort. Steere (1940) also claims that it had great powers in stopping inflammation of the blood and aided in clotting. The early Chinese treated boils and controlled foot infections by using moldy soybean curd and wearing sandals which were furry with mold growth. Metchinkoff in 1899 recommended the use of *Lactobacillus* in the treatment of dysentery. In 1901, Emmerich and Low demonstrated that when liquid cultures of *Pseudomonas aeruginosa* were injected into rabbits, they protected them against anthrax. About 1924 Gratia and Dath made the first systematic search for and study of antibiotics in strains of actinomycetes. Fleming, of course, is noted for his discovery of the miracle drug, penicillin. But with Fleming's discovery, the use of plant extracts for antibiotics were given more serious thought and study (after Pelczar, 1965).

Many of the present antibiotics are obtained from microscopic molds, bacteria, and soil actinomycetes. Schmidt (1966) has recently found antibiosis in alcoholic extracts of lichens. McCleary (1966) made alcoholic extracts of fifty-four species of mosses of which eighteen showed strong antibiosis which he suggests is probably due to non-ionized organic acids and polyphenolics. Two experiments which
were quite closely related to this investigation were the examination of mosses and their relatives for antibiosis by Wolters (1964) and Pavletic (1963). Wolters obtained fungistatic action in eleven species of mosses. Pavletic indicated that gram positive bacteria were susceptible to his extract of *Marchantia*.

Nakang (1962) has made twenty per cent alcoholic extracts of *Carica papaya*, *Saraca indica*, *Nardostachys ptomansi*. These extracts were effective against gram positive *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Kushner (1962), working with more complex plants discovered bacterial inhibition with extracts from the conifers. Spohn (1965) tested extracts from both the gemmae and dead thalli of *Marchantia polymorpha* (L.), on test organisms and found some growth inhibition in his extracts.
CHAPTER III

METHODS AND PROCEDURES

Sampling Techniques

Two species of liverworts, *Ricciocarpus natans* (L.) and *Marchantia polymorpha* (L.) were used in this investigation. Extraction was accomplished using water and organic solvents (ethyl alcohol and xylol). Whole thalli, devoid of reproductive structures, e.g., gemmae or sexual sporophores, were used throughout the various experiments.

*Marchantia* thalli were grown on well decayed peat moss. The whole thallus was collected, washed to remove all foreign particles, and then air-dried for the extraction process. Thalli of *Ricciocarpus* were harvested from culture tanks in the greenhouse, washed, and air-dried.

Preliminary runs were performed to determine if there were antibiotic substances present and also to determine which of the solvent fractions contained the substances. Tests were also made to see if different methods of extractions would produce quantitative differences in the products extracted, i.e., whether changing the order of solvents during the extraction would produce different effects. These preliminary extractions were accomplished by the use of medium sized, ground glass tissue homogenizers and a known volume of solvent. The solvents used were: ethyl alcohol (95 per cent USP), xylol (USP), and double distilled water. The temperature of the homogenized runs
was kept below 10° C. by an ice bath throughout the extractions. At first, the extractions were made using one gram of whole thallii and 20 ml. of each solvent. Later, the quantity of thallus and solvents was increased to obtain larger amounts of concentrated extracts.

For the detection of the best extraction procedure, three series of solvents were set up. Series I consisted of (a) xylol (b) water (c) alcohol. Series II consisted of (a) water (b) alcohol (c) xylol. Series III consisted of (a) alcohol (b) xylol (c) water.

In each series, one gram of thallus was homogenized in 20 ml. of the initial solvent. After the homogenization was complete, the mixture was centrifuged at 2500 r.p.m. for fifteen minutes and the supernatant liquid decanted into screw-top test tubes. Then the second solvent was added to the centrifugate and homogenized, centrifuged, and decanted as in the initial step. The third solvent was run as the second.

Ten (5.4 mm. in diameter) sterile discs cut from heavy blotter paper, were added to each tube of the final extract. Three controls consisting of 10 paper discs introduced into each of the solvents, water, xylol, and alcohol, were similarly prepared with volumes equivalent to the extracts. Both controls and the experimental tubes were then dried in a vacuum freeze-drying apparatus as described below: the tubes containing the discs were placed in the vacuum chamber (see figure 1., page 6). A vacuum pump which developed a negative fifteen p.s.i. was used. The vacuum chamber was submerged in an insulated thermos which was half filled with a mixture of crushed dry-ice and acetone. A temperature of about -100° C. was
Following the evaporation to dryness, the discs that contained the extract of each solvent series were divided into two groups. One half was immediately plated on petri dishes inoculated with *Bacillus subtilis* and *Staphylococcus aureus*. The other half was plated twenty-four hours later to test for the unknown substrate's stability. These discs were kept under refrigeration at 10°C, for the twenty-four hour period.

A second set of trials was performed to determine if the method of drying the discs would in some way influence the strength of the antibiotic substance. Extraction was done with a series (as in the
preliminary series III) of five milliliters of alcohol, followed by
five milliliters of xylol, then followed by five milliliters of water.
The supernatant of each fraction was freeze dried as described above
and the other was dried at 37°C. in an incubator. Discs from each
of these were plated on dishes inoculated with Bacillus subtilis and
Salmonella typhimurium.

Consideration was also given to the conditions for extraction.
There were two conditions under which extraction could be performed.
In the first condition, heat was required to remove and activate the
substance which was bound to the substrate. The substance reacted
with the extraction solvent, ethyl alcohol. The second condition
was one in which heat destroyed the active sites of the substance,
and thus, destroyed its antibiotic activity. Hence, both methods
of extraction were investigated. Extraction without heat was per­
formed at 0° to 10°C. by homogenizing in an ice bath, like that in
the preliminary series. Extraction with heat was accomplished by
refluxing the thalli in 95 per cent ethyl alcohol. The latter method
consisted of placing the thalli in a filter paper envelope. The
envelope was then placed in a 300 milliliter round bottom flask and
a water cooled reflux condenser was mounted in the flask with the
mouth in the open end of the envelope. The solvent was then added
and the temperature was adjusted at the boiling point of the solvent.
The material was refluxed for one hour.

Concentrations of one and three grams of thalli per five milli­
liters of 95 per cent ethyl alcohol were used in the cold homogeni­
zation extraction. Three grams of thalli in five milliliters of 95
per cent ethyl alcohol was followed by five milliliters of xylol in the reflux extraction. The supernatant fluids were centrifuged at 2500 r.p.m. for fifteen minutes and then added to tubes containing ten sterile discs. The discs were then freeze dried. These discs were then plated on nutrient agar plated containing the different test organisms. *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium*, and *Staphylococcus aureus* were used as the test organisms to give a broad spectrum of antibiosis and to see how gram positive and negative bacteria react to the extract.

Additional experimental series were made using the reflux method with 95 per cent alcohol as the only solvent. To increase the concentration of the extract, the reflux time was extended and larger quantities of thalli were used keeping the volume of alcohol the same. Drying the extract on discs was improved by the use of a flash evaporation unit.

**Evaluation Procedure**

The plating of all the discs was done on 10 ml. of double strength nutrient agar per petri dish. The double strength nutrient agar consisted of eight grams of dehydrated nutrient broth, fifteen grams of nutrient agar, and 1000 milliliters of distilled water. This was autoclaved for twenty minutes at fifteen p.s.i. The agar plates were inoculated with the selected test organisms. The plates were incubated for twenty-four hours at 34°C. The petri dishes were then examined for zones of inhibition (no growth) or reduction of growth around the discs. These areas were measured with a millimeter ruler.
The radius of growth reduction or inhibition from the edge of the discs to the nearest region of bacterial growth, called the outer radius, was then recorded. Zones of reduction (3 mm. or larger) and zones of inhibition (2 mm. or larger) were considered signs of antibiosis.

In order to determine a comparative efficiency of the extracted antibiotic discs, Bacto-sensitivity discs of various concentrations were used. These were penicillin (10 units and 2 units), streptomycin (10 mcg. and 2 mcg.), tetracycline (30 mcg. and 5 mcg.), and neomycin (5 mcg.).

Identification Procedure

For the determination of the potential antibiotic substance two methods were used - analysis by IR and separation and identification by chromatography. Analysis by IR was performed on the powdered extract after flash evaporation.

Separation and identification of the extracts by chromatography were performed by the ascending method with the use of various concentrations of the extract and various solvent fronts. Strips were made of Whatman No. 1 paper cut to the dimensions of 1 inch by 21 inches. The chromatographs were run for seventeen to twenty-four hours in the solvents. The solvent solutions were: (a) N-propyl alcohol - acetic acid - water in the ratio of 9:1:10, (b) phenol - water in the ratio of 100 milliliters of 88 per cent liquid phenol to 2 ml. of water, and (c) N-butanol - acetic acid - water in the ratio of 40:10:50.
The strips were dried. Several strips were developed with ninhydrin (0.1 to 0.25 per cent ninhydrin in butanol). The remaining strips were used for a microbiological assay to determine the Rf of the unknown antibiotic. These strips, after chromatographic separation, were dried, then discs 5.4 mm. in diameter, were punched at regular intervals from the initial spot to the solvent front. These discs were plated on petri dishes which were inoculated with the previously mentioned bacteria.
Demonstrated Antibiosis of Cold Homogenization Extracts with Three Different Solvents

The order in which the solvents were used for extraction did not seem to interfere with or enhance the removal of the substance. The data in Table I (see page 30) show that only the discs which were extracted in 95 per cent alcohol produced any substantial evidence of antibiosis. Both *S. aureus* and *B. subtilis* showed significant reduction and inhibition of growth by the alcoholic extract, especially Series II and Series III, respectively. The extracts of alcohol showed more reduction and inhibition than those of the alcohol control, which indicated that the alcohol alone was not causing the antibiosis. The water extracts showed no antibiosis and in several trials, the growth of the bacteria was more pronounced. The xylol extracts showed very little reduction of bacterial growth, usually less than 1 to 2 mm. which was considered as no effect.

Demonstrated Stability of the Extract

The discs which were alcohol extracted and plated immediately after drying showed a slightly greater potency (fewer resistant colonies) than those which were plated twenty-four hours later. The data in Table I, (Series I, II, and III) show this difference quite well. The alcohol extract of Series I, of *S. aureus* changed very little after twenty-four hours. Only a few resistant colonies appeared
near the discs. The alcohol extract of Series II, on *S. aureus*
decreased in stability from an average of three millimeters reduction
to no reduction. The alcohol extract of Series III, on *B. subtilis*
showed a strength of 4.0 millimeters inhibition by plating immediately,
as compared to an average of three millimeters inhibition of those
plated twenty-four hours later.

Demonstrated Antibiosis of Freeze Drying Versus Heat Drying Methods

With reference to Table II, page 32, especially the response
by *B. subtilis*, the discs which were freeze dried showed better anti-
biotic potential than those which were heat dried. The heat drying
method was less effective as noted by a few resistant colonies.
Again, the alcohol extracted discs showed better antibiosis. Water
extracted discs again showed a pronounced growth of bacteria. Xyol
extracted discs showed no antibiosis by either freeze drying or heat
drying. All the discs plated with *S. typhimurium* in this series tended
to show little or no antibiosis. However, in some instances growth
was more pronounced.

Demonstrated Comparison of Refluxing Versus Cold Homogenization
Extraction Method

A comparison of heat extraction, namely refluxing, and that of
cold homogenization extractions, were obtained, (see figure 2 for
results). The extract of three grams of thalli by cold homogenization
(3 GH) showed less potential than the extract of three grams of
thalli refluxed in alcohol (3 GR ETOH) against *S. aureus* (figure 2A).
FIGURE 2. RELATION OF THE STRENGTH OF THE PREPARED DISCS TO COMMERCIAL SENSITIVITY DISCS ON STAPHYLOCOCCUS AUREUS

FIGURE 2 B. RELATION OF THE STRENGTH OF THE PREPARED DISCS TO COMMERCIAL SENSITIVITY DISCS ON SALMONELLA TYPHIMURIUM
FIGURE 2 C. RELATION OF THE STRENGTH OF THE PREPARED DISCS TO COMMERCIAL SENSITIVITY DISCS ON BACILLUS SUBTILIS

FIGURE 2 D. RELATION OF THE STRENGTH OF THE PREPARED DISCS TO COMMERCIAL SENSITIVITY DISCS ON ESCHERICHIA COLI

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The extract of 3 GH of *S. typhimurium* (figure 2B) showed no antibiosis while that of 3 GR ETOH showed a strong inhibition (an average of four millimeters). The extract of 3 GH on *B. subtilis* (figure 2C) showed a slightly stronger antibiosis than the extract of 3 GR ETOH. On *E. coli* (figure 2D), the reflux extraction with alcohol showed stronger potential (an average of four millimeters reduction) than the cold homogenized one (an average of 1.5 millimeters reduction).

**Demonstrated Spectra of Bacteria Susceptibility to the Extract**

The alcohol extracts used against *S. aureus* (figure 2A) produced about 2 to 3 mm. inhibition while those plated against *S. typhimurium* produced 5 millimeters of inhibition (figure 2B). *S. aureus* is gram positive while *S. typhimurium* is gram negative. *B. subtilis* (figure 2C), which is gram positive, showed inhibition of about 2 mm. while *E. coli* which is gram negative, showed a reduction in growth of about 4 mm. Both of these studies gave an indication that gram negative bacteria are slightly more susceptible to the antibiotic than the gram positive bacteria.

**The Results of the Strength of Extracted Discs to Commercial Sensitivity Discs**

A comparison of extracted discs (the refluxed alcohol preparations) with commercially prepared discs was made (see figure 2). The discs which were plated against *S. aureus* (figure 2A) did not match the strength of inhibition of the commercial discs including streptomycin (10 mcg.), tetracycline (30 mcg.), and penicillin (10 units), but
showed a weak zone of inhibition. Platings of *S. typhimurium* (figure 2B) showed stronger zones of inhibition against the extracted discs when compared to all the commercial discs used. Platings of *B. subtilis* (figure 2C) showed that the extracted discs were weaker in inhibition than the commercial discs. Platings of *E. coli* (figure 2D) showed that the extracted discs were better inhibitors than the streptomycin (10 mcg) or penicillin (10 units), but less so than tetracycline (30 mcg).

Since *B. subtilis* was quite susceptible to the alcohol extract, a majority of the tests were performed with this organism. A comparison of responses to commercial sensitivity discs with those of the experimentally extracted discs is seen in Figure 3.

**Figure 3 A. Photographic Comparison of Extract with Commercial Sensitivity Discs. Extract versus Streptomycin.**

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FIGURE 3 B. PHOTOGRAPHIC COMPARISON OF EXTRACT WITH COMMERCIAL SENSITIVITY DISCS. EXTRACT VERSUS TETRACYCLINE.

FIGURE 3 C. PHOTOGRAPHIC COMPARISON OF EXTRACT WITH COMMERCIAL SENSITIVITY DISCS. EXTRACT VERSUS PENICILLIN.
FIGURE 3 D. PHOTOGRAPHIC COMPARISON OF EXTRACT WITH COMMERCIAL SENSITIVITY DISCS. EXTRACT VERSUS NEOMYCIN.

Two mcg. of streptomycin produced zones of inhibition from 8 to 9 mm. against *B. subtilis* as compared to the alcohol extract which produced zones of 3 mm. inhibition (figure 3, part A). Tetracycline with levels of 5 mcg. produced inhibition zones of 15 mm. in size compared to 5 mm. zones of inhibition using the alcoholic extract (see figure 3, part B). The sensitivity discs containing 10 units of penicillin showed inhibition zones of 7 to 8 mm. as compared to 4 to 5 mm. zones of inhibition with the alcohol extracted discs (figure 3, part C). The alcohol extracted discs showed slightly less antibiotic potential (3 to 4 mm. inhibition) than the 5 mcg. neomycin discs against *B. subtilis* (figure 3, part D).
Identification of the Antibiotic Extract by IR Analysis and Paper Chromatography

The extract was flash evaporated leaving a grease-like substance with a dark green color. The IR zones were not sharp enough at the highest peaks for a direct interpretation of the molecular structure. There were 3 zones at about 3 microns, another at 3.5 microns, and a third at about 6.3 microns. The zone at 3 microns of medium strength was estimated to be either an amine or an alcohol (OH) group. The medium strength zone at about 3.5 microns indicated either an alkane or an aldehyde group. The weak zone around 6.3 microns was interpreted as a possible amine or an aromatic group, but was not considered to be the antibiotic substance (see figure 4).

FIGURE 4. IR ANALYSIS OF THE ALCOHOLIC EXTRACT

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There were no apparent separations during the chromatography process of the alcohol extracts with both N-propyl alcohol – acetic acid – water (9:1:10) and phenol – water (100 ml. of 88 per cent liquid phenol : 2 ml. of water) as solvents. In the N-propyl alcohol – acetic acid – water series, and the phenol – water series, the extracted substance moved the same distance as that of the alcohol control and the paper control. Either 20 or 30 lambda of the extract was spotted on the paper strips. The extract was obtained by refluxing 9 grams of thalli in 15 ml. of alcohol for two hours. In an experimental series in which phenol – water was the solvent, the 20 lambda unknown from the alcohol extracted discs showed a slightly stronger antibiosis by microbiological assay than the alcohol and paper strip controls. Ninety lambda of the alcoholic extract spotted on paper strips was chromatographed in butanol – acetic acid – water (40:10:50). Discs were punched at regular intervals along the chromatographed strips and plated against B. subtilis. Only on those discs taken from the end of the sequence near the solvent front showed inhibition. Another ninety lambda of the alcohol extract was chromatographed in N-propyl alcohol – acetic acid – water (9:1:10), and discs were punched along the axis of the solvent flow and plated against B. subtilis. Only those discs in the proximity of the solvent front showed inhibition of bacterial growth. The alcohol control discs showed no significant inhibition of growth.
CHAPTER V

DISCUSSION

Isolation and Stability of the Extract Showing Antibiosis

The results in Series I (extract from Ricciocarpus) indicated that the alcohol extract was the only one to show substantial evidence of antibiosis. Waksman (1947) stated that antibiotic agents differ greatly in their solubility. Some are water soluble; others are alcohol soluble and only slightly soluble in water; and some are acids that react with alkali solutions to form soluble salts. The order in which the solvents were used for extraction did not seem to interfere with or enhance the removal of the substance. The alcohol extracts showed growth reduction and inhibition over and above that of the control, which indicated inhibition of bacterial growth. Waksman also indicated that alcohol extracts from plants form esters which are bactericidal. This, he asserted, occurred by the breakdown of larger metabolic products during extraction or by the release of a compound which is already present in the plant cells.

The heavier growth of bacteria around the water extracted discs was due to the removal of compounds such as sugars and amino acids which when plated on the inoculated petri dishes stimulated growth.

The discs that contained alcohol extracts showed stability after refrigeration for twenty-four hours. The zones of inhibition were equal to the zones of those discs that were plated immediately. It was also evident that the outer radius was attained within twenty-
four hours after plating and that this outer radius did not increase after the initial inhibition area was observed. This indicates that the process of diffusion of the substance into the medium had occurred rapidly at first and apparently reached an equilibrium within the first twenty-four hours. The extract was stable and strong enough to inhibit growth for several days.

The extract prepared by the freeze drying technique produced larger zones of inhibition than those prepared by heat drying at 37°C in an incubator. The other method of drying, flash evaporation, showed no interference with antibiosis. The use of a vacuum eliminates the need of high temperatures for drying the discs and furthermore, it saves time.

With the use of heat extraction (refluxing) greater inhibition was observed. This might again be due to the formation of an ester between alcohol and some substance in the cellular material. It was also possible that the reflux method produced extracts with a greater concentration of the antibiotic substance than was obtained by the cold homogenization technique. This interpretation implies that the molecular weight would be the same for the constituents of both types of extracts and that the difference in antibiotic effect was a function of concentration only. However, an alternative interpretation was that, if the zones of inhibition of the cold homogenization extracts were different in size than the refluxed ones, and if these zones were consistent in size, then these substances would have different rates of diffusion. Since the rate of diffusion is somewhat proportional to molecular weight, the results would indicate that
the intercellular constituents derived by the cold homogenization process would have a greater molecular weight (slower diffusion) than those derived from the reflux method. If this reasoning is sound, then these substances are not the same and may not even be related. However, the size of the antibiotic compounds had received some study. According to Woodbine (1962) there are low molecular weight substances in plants which are antibiotic.

It is likely that the substance obtained from the reflux method may be the breakdown products of other molecules which originally had no antibiotic effect, and the substances derived from the cold homogenization process were broken into harmless constituents as a result of heat drying. This was indicated in the second series of experiments when it was observed that heat drying of the discs at 37°C, which were cold extracted reduced their antibiotic potency.

Interpretation of Bacterial Sensitivity to the Extract

Waksman (1947) claims that most gram positive bacteria are more sensitive to the majority of antibiotics than are gram negative bacteria. In this investigation, however, the results indicate that gram negative bacteria were more susceptible than gram positive bacteria to the alcoholic extract from liverworts. Still, both gram positive and gram negative bacteria were found to be susceptible to the alcohol extract of the liverworts. Contrary to the findings of this investigation, in which all of the test organisms were susceptible to the extract, Pavletic (1963) found that gram positive bacteria were susceptible to their extract of Marchantia, but that gram negative
bacteria were not susceptible to the extract.

On *B. subtilis* and *S. aureus* (both gram positive) the extracts were weaker than the commercial discs. *E. coli* and *S. typhimurium* (both gram negative) were inhibited by the alcohol extract at a similar level of response to the commercial discs. Streptomycin, penicillin, tetracycline and the alcohol extract against *S. typhimurium* bear this out. Streptomycin, penicillin, and the alcohol extract against *E. coli* gave the same response. The extract did not show complete toxicity on the bacteria. By comparing the potency of the alcohol extract with that of commercially prepared antibiotics against common test organisms, the extract of liverworts is of sufficient strength to warrant its possible use as an antibiotic.

The extract is not of protein nature since the results of IR analysis and chromatography did not indicate the presence of a molecule that size. Waksman (1947) found that very few antibiotic substances were proteins. Foreign proteins in a cell are usually quickly rejected and destroyed. No spots were brought out or developed by ninhydrin that would indicate the presence of proteins or amino acids. Therefore, it was not considered to be protein.

To see if the antibiotic substance remained with the solvent after chromatography, microbiological assays were then run on the areas of the solvent fronts. Controls were used to include both blank paper and the blank paper plus the same quantity of alcohol as was used in the extract. Inhibition of growth by the chromatographed extract was slightly higher than the controls, showing that some of the antibiotic agent was present in this region of the chromato-
graphed strip. Only those discs obtained from this region showed antibiosis by microbiological assay. The results of this experiment indicate a further need for the identification of the active compound. Some suggestions for methods and other approaches follow.

Other methods of extraction would improve on the isolation of the product as well as aid in obtaining quantitative data. The extract's potency might be extremely helpful in determining a specific use for it. This potency in micrograms or other quantitative units could be obtained by a dilution technique and the drying of specific aliquots of the extract on paper discs. Larger concentrations of the substance should also be obtained. This would require a means of condensing the substance without its deterioration. Expressing or grinding the leaves to remove the intracellular fluids during continuous flash evaporation on discs would enhance the concentration of the substance. As an aid during extraction, it would be beneficial to examine the pH. Kushner (1962) found that the pH during extraction controls the release of the antibiotic substance. pH differences might be accomplished by incorporating the extract in a buffered nutrient agar for plating or by buffering the extracted media.

Further areas which could be pursued would be to investigate the response of other gram negative and gram positive bacteria to the extract. This would give additional information in the antibacterial spectrum of the potency of the substance.

In the realm of testing the effects of antibiotics on plants, a new method has recently been developed using coleoptiles of rye, oats, barley, wheat, and other grasses. This method has made it
possible to determine the substances which stimulate and those which inhibit growth in plants. Perhaps such a method could be adapted to examining the antibiotic substances of liverworts. Examination of other liverworts and mosses might reveal other sources of antibiotics, and their uses.
CHAPTER VI

SUMMARY AND CONCLUSION

Two liverworts, *Ricciocarpus natans* (L.) and *Marchantia polymorpha* (L.) were investigated for intracellular constituents with antibiotic potential. This was accomplished through several different experiments: the best method of testing; a method of extraction; the potency of the substance against bacteria; and the identity of the substance.

Cold homogenization extractions were made using alcohol, xylol, and water. The results of bacterial assays showed that the alcohol soluble fraction possessed inhibition properties against *Bacillus subtilis*, *Salmonella typhimurium*, *Escherichia coli*, and *Staphylococcus aureus*.

A second series of investigations was performed to determine if variations in the method of drying the discs would make significant differences in their potency. The vacuum freeze drying technique produced a greater radius of inhibition than heat drying at 37°C. in an incubator. Flash evaporation later proved to be an extremely useful means of drying the discs at a low temperature without affecting the potency of the extract. The alcohol extract was found to be stable during a twenty-four hour period under refrigeration prior to testing it against bacteria.

A third series of investigations was performed to compare products which were extracted by the cold homogenization technique to that
obtained by the reflux method. Reflux extraction produced better
inhibition than that of cold homogenization.

The refluxed alcohol extract was effective against both gram
positive and gram negative bacteria. The gram negative bacteria
were slightly more susceptible.

A comparison of the alcohol extract to commercial antibiotics
was made. Tests against Salmonella typhimurium showed similar results
with the alcohol and the commercial forms - streptomycin (10 mcg),
penicillin (10 units), and tetracycline (30 mcg). The alcohol extract
when tested against Escherichia coli showed inhibition which was
slightly better than 10 mcg. of streptomycin and 10 units of penicillin,
but was considerably less effective than 30 mcg. of tetracycline.
This study showed that the extract from liverworts was of sufficient
potency to warrant its possible use as an antibiotic.

Identification of the substance was attempted with IR analysis
and microbiological assay of the chromatographs. The substance was
tentatively identified as an alkane or an aldehyde group.
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   J. Insect Path. 4(2) : 155-161.


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   D. Appleton-Century Company, 56.

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    Bei Moosen, (The Distribution of Antifungal Properties in Mosses),

    Washington, D.C.: Proceedings of the University of Nottingham
    Ninth Easter School in Agricultural Science, 195-230.
TABLE I: Data derived from cold homogenization extracts of *Ricciocarpus natans*, and used to indicate the best extraction procedure. The extracts from three different solvent orders were assayed against *Staphylococcus aureus* and *Bacillus subtilis* at zero and twenty-four hours after extraction.*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Solvent</th>
<th>Order</th>
<th>0 hrs.</th>
<th>24 hrs.</th>
<th>Solvent</th>
<th>Order</th>
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<th>24 hrs.</th>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>S. aureus</td>
<td>xylool</td>
<td>0.3</td>
<td>0</td>
<td>1.1</td>
<td>water</td>
<td>0</td>
<td>1.2</td>
<td>0.3</td>
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<tr>
<td></td>
<td>water</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>ETOH</td>
<td>3.2</td>
<td>0.9</td>
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<td></td>
<td>ETOH</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>xylool</td>
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SERIES III

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<td>xylool</td>
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<td>water</td>
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<td>0</td>
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SERIES III

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<tr>
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<td>0</td>
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SERIES III

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<td>B. subtilis</td>
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<td>3.0</td>
<td>3.0</td>
<td>water</td>
<td>0</td>
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<tr>
<td></td>
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<td>0.9</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td></td>
<td>water</td>
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<td>0</td>
<td>xylool</td>
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TABLE I (con't.)

CONTROLS

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<th>24 hrs.</th>
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<td>ETOH</td>
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<td>1.0</td>
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<tr>
<td></td>
<td>water</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>xylol</td>
<td>1.5</td>
<td>1.5</td>
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</tbody>
</table>

* Area of inhibited growth measured in millimeters as the outer radius from the edge of the paper disc.
TABLE II: The difference found on the effects of freeze drying versus heat drying with Ricciocarpus extracts.*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Solvent</th>
<th>Freeze Dried</th>
<th>Heat Dried</th>
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<tbody>
<tr>
<td><em>B. subtilis</em></td>
<td>alcohol</td>
<td>2.7</td>
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<td></td>
<td>xylol</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>water</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>alcohol</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>xylol</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>water</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Area of inhibited growth measured in millimeters as the outer radius from the edge of the paper discs.
**TABLE III:** 6 grams of *Marchantia* thalli per 10 ml. of alcohol, refluxed for 2 hours and flash evaporated on 20 paper discs, plated and compared to commercial antibiotics. *Bacillus subtilis* was the test organism.*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration</th>
<th>Extract</th>
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<tbody>
<tr>
<td><strong>Streptomycin</strong></td>
<td>8.5</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>Tetracycline</strong></td>
<td>15</td>
<td>5</td>
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<tr>
<td><strong>Penicillin</strong></td>
<td>7.5</td>
<td>4.5</td>
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<tr>
<td><strong>Neomycin</strong></td>
<td>6</td>
<td>3.5</td>
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<tr>
<td><strong>Control</strong></td>
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</table>

* Area of inhibited growth measured in millimeters as the outer radius from the edge of the paper discs.*