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Curriculum

A Laboratory Activity Demonstrating the Antibacterial Effects of Extracts from Two Plant Species, *Moringa oleifera* and *Allium sativum* (Garlic) †

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A variety of plants synthesize natural products that either kill or inhibit the growth of various microorganisms. These plant products may serve as useful natural alternatives to synthetic antimicrobial pharmaceuticals and can be especially important in regions where commercial drugs are often not available. Despite this, the role of plants as producers of natural antimicrobial agents is often understated or even ignored in undergraduate biology curricula. In this laboratory exercise, students extract water-soluble constituents from two plants, *Moringa oleifera* (moringa) and *Allium sativum* (garlic), and determine their activity against both a gram-positive (*Bacillus cereus* strain 971) and a gram-negative (*Escherichia coli* strain K12) bacterium using a disk diffusion assay on Mueller-Hinton agar. Disks infused with commercially available antibiotics (e.g., penicillin and tetracycline) serve as controls. Following an incubation period of 24 hours, students obtain quantitative data by measuring zones of growth inhibition that develop as a result of strain sensitivity. To determine the effectiveness of the learning objectives, an unannounced quiz was administered both before and after the activity, and the students showed significant gains in their understanding of key concepts. Because this activity combines aspects of two major branches of biology—plant biology and microbiology—it is suitable for use as a laboratory exercise in courses related to either discipline, or it may be used as a laboratory component of a general biology course.

INTRODUCTION

The identification and use of medicinal plants are important components of the health and wellbeing of diverse populations in the developing world. In addition, plants that have antibacterial properties are becoming increasingly relevant in an age in which healthcare professionals are documenting growing antibiotic resistance among pathogenic bacteria. *Moringa oleifera* (moringa) is a tree that is originally from India and has been used both as a nutritional supplement and as a medicinal plant (1–5). Based on our own research (unpublished) and that of several other groups (4, 6–8), extracts of the leaves, seeds, and roots of moringa show antibacterial properties. *Allium sativum* (garlic) has also been shown to exhibit antibacterial qualities (9, 10). These inhibitory effects can be easily demonstrated in an instructive and highly visual laboratory exercise.

Several methods may be used to determine the antibacterial effects of different substances, but the disk diffusion method (11) is one of the more cost effective, straightforward, and visually striking techniques available. This method gives clear results that are easily observed and interpreted by students performing the experiment. Herein, we describe a laboratory activity in which students were instructed in how to extract antibacterial compounds from plants, aseptically plate bacteria, and test the extracts for antibacterial activity. Following a 24-hour incubation period, the students analyzed their results and collected data regarding the inhibitory effect of the tested plant extracts on the growth of gram-positive and gram-negative bacteria.

The students were given an identical unannounced quiz immediately before (pre-lab) and one week after (post-lab) the laboratory activity to assess whether their understanding of the concepts and procedures improved after performing the laboratory exercise. The students also provided an evaluation of the laboratory activity by completing a post-lab survey.

Intended audience

Due to its multidisciplinary nature, combining concepts from both microbiology and plant biology, this laboratory activity is appropriate for a range of undergraduate biology courses. These might include freshman-level "Principles

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of Biology" type courses, plant biology (botany), general microbiology, and medical microbiology. It would also be a suitable activity for any course that addresses medicinal plants, conservation biology, or tropical agriculture.

Prerequisite student knowledge

Undergraduate students who have successfully completed high school biology coursework should have sufficient background knowledge to perform this activity, assuming that a pre-laboratory lecture covering basic concepts of bacterial culture and cell structure (gram-positive versus gram-negative) is also provided (see "Faculty instructions"). In addition, students are expected to have met the laboratory safety requirements of the course, which should include coverage of the guidelines for the use of biosafety level (BSL) 1 organisms (see "Safety issues"). From their completed training, students will have a basic understanding of microbial growth and growth control and proficiency in the safe handling of live microorganisms, including the use of aseptic technique and the proper disposal of biohazard materials. Instructors should review these concepts during pre-laboratory explanation and demonstration.

Learning time

To complete this activity, one two-hour laboratory period is necessary to collect the plant extracts, swab sterile plates of Mueller-Hinton agar with the provided bacterial cultures, and spot the plant extract and antibiotic-infused disks onto the plates for incubation at 37°C. Following a 24-hour incubation, either experimental results should be collected immediately by the students, or the plate cultures should be refrigerated at 4°C for data collection in the next laboratory period. Students should be able to measure and interpret their results in 15 to 30 minutes.

Learning objectives

After successful completion of this laboratory exercise, students will be able to:

1. Extract water-soluble products from plants of potential medicinal value and determine their antibacterial activity.
2. Establish bacterial cultures on petri plates using aseptic technique.
3. Quantify antibacterial effects of plant extracts and known antibiotics by identifying, measuring, and comparing zones of growth inhibition on a bacterial plate culture.
4. Compare growth inhibitory effects of plant extracts and known antibiotics on a gram-positive versus a gram-negative bacterium.
5. Formulate a hypothesis to explain the function of antimicrobial products in plants.
6. Articulate potential uses of plants to combat bacterial infections.

PROCEDURE

Materials

Proper personal protective equipment (PPE), including gloves, lab coats, and eye protection, is necessary for students performing this activity. In addition, each pair of students will require the following materials: two Mueller-Hinton agar plates, one nutrient (or tryptic soy) broth culture each of *Bacillus cereus* str. 971 and *Escherichia coli* str. K12, a bent-rod bacteria spreader (or two sterile swabs), a 1,000-μL micropipette and four sterile tips, two screw-cap centrifuge tubes (15 mL), two small beakers, two disposable sterile petri dishes, two Luer-Lok syringes (10 mL), two syringe membrane filters (sterile, 0.2–0.45 μm), four blank paper disks (sterile), two tetracycline disks, two penicillin disks, one mortar and pestle, one pair of forceps, one 10-mL graduated cylinder (or a 10-mL pipette with pipettor), one weighing spatula, two moringa seeds, one clove of fresh garlic, and 5 mL of 60°C water (Fig. 1A). If necessary, the students can share a tabletop microcentrifuge, ethanol, forceps, and a Bunsen burner. To save time, one half of the students can prepare the moringa extract, and the other half can prepare the garlic extract.

Student instructions

Detailed student instructions to perform this activity are provided in Appendix 3. Briefly, the students worked together as a class to prepare filter-sterilized moringa and garlic extracts in water (Fig. 1B-E). They then plated a "lawn" of each of the provided bacteria (one plate of *E. coli* and one plate of *B. cereus*) using either an ethanol-flamed bacteria spreader or sterile swabs. Ethanol-flamed forceps were then used to apply both extract-soaked and antibiotic-infused disks to each inoculated plate (Fig. 1F-H), and the plates were incubated for 24 hours at 37°C. After incubation, the students measured zones of growth inhibition around each disk (Fig. 1I) and completed a post-lab analysis, which included recording their data and answering the post-lab summary questions.

Faculty instructions

The instructor may obtain the moringa seeds from several possible sources. We obtained our seeds from www.echonet.org. Other vendors may be available via the Internet. Fresh garlic may be purchased from a local grocery store. Fresh (approximately 24-hour) bacterial cultures should be provided for the students to conduct this activity (see Appendix 4 for more details). All other materials are standard laboratory supplies.

Present a pre-lab lecture to ensure the students are familiar with basic microbiological concepts (Appendix 4). If it has not already been covered in the course, the pre-lab

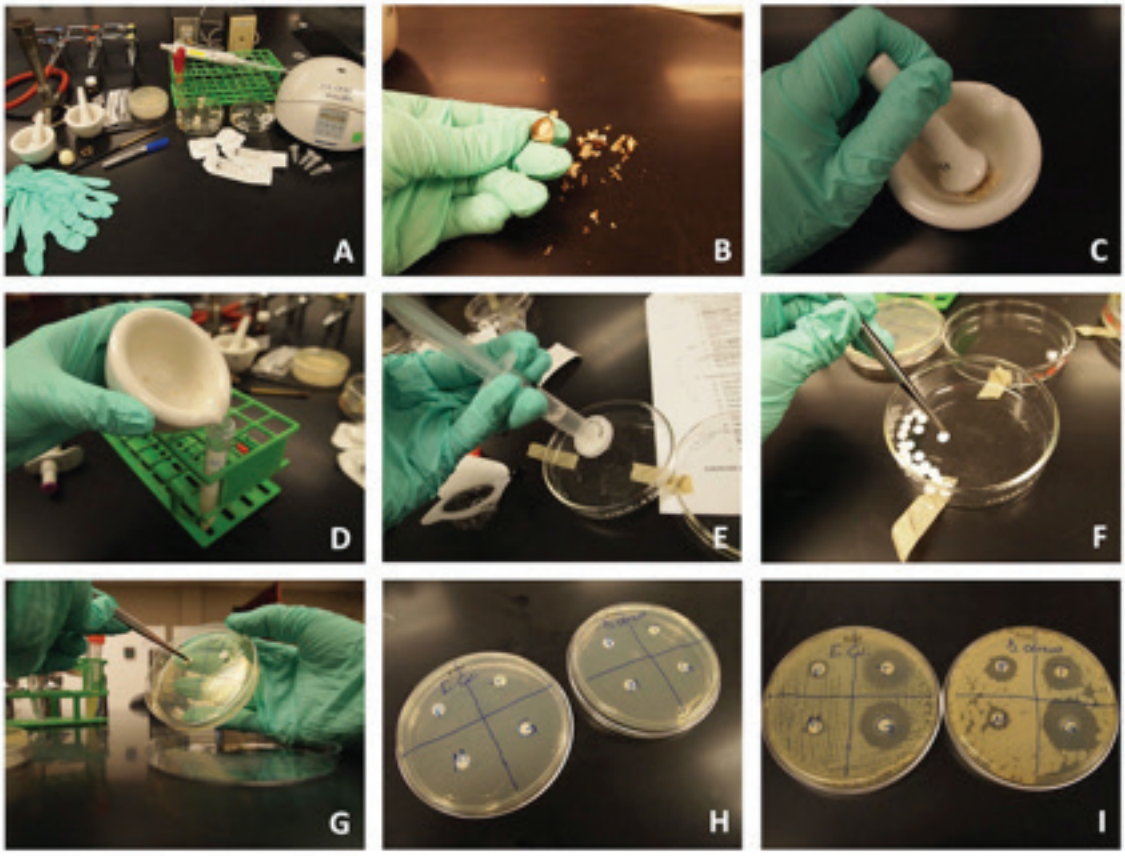

FIGURE 1. Images of laboratory procedures. (A) Materials needed for the activity; (B) Moringa seed with hull removed; (C) Mortar and pestle grinding of moringa seed; (D) Ground moringa seed slurry being poured into a 15-mL centrifuge tube; (E) Slurry supernatant sterilized through a membrane filter (0.2–0.45 μm); (F) Sterile disks soaked in sterile extracts; (G) Plating extract-soaked disks onto inoculated plates; (H) Disk placement on plates of either *E. coli* (left) or *B. cereus* (right); (I) Zones of inhibition apparent on plates following 24-hour incubation (37°C).

lecture should include an introduction to artificial culture media and the use of proper growth conditions to cultivate bacteria. Students should also be made aware of the structural differences between gram-positive and gram-negative bacteria, as these often determine the efficacy of antibiotics that affect cell wall biosynthesis, such as penicillin, versus those that target other processes in the bacterial cell, such as tetracycline, an inhibitor of protein synthesis. In addition, students should be given basic background information regarding plants that produce antimicrobial compounds and the use of moringa and garlic as medicinal plants.

The students should also be reminded of their training regarding the safety precautions for handling bacterial cultures, including their proper disposal, and an instructor demonstration of aseptic technique is recommended. As it is a potential source of injury, students should also be shown the correct technique for ethanol and flame sterilization of forceps and inoculating tools.

Suggestions for determining student learning

Before beginning the activity, the students were given an unannounced Pre-Lab Quiz (Table 1; also see Appendices 1 and 2). The quiz was designed to assess the students' existing knowledge of plants as a source of antibacterial products and how the activity of these products can be measured. After completing the activity and obtaining their results, the students were required to complete a post-lab analysis (Appendices 3 and 4), which included several summary questions to encourage critical thinking and assess student comprehension. The post-lab analysis, with its accompanying collection of data and answers to the summary questions, was due at the beginning of the next lab period. To gauge their retention of key concepts introduced the previous week, the students were then given the same Pre-Lab Quiz (unannounced and completed without the aid of notes) as a Post-Lab assessment.

Sample data

Students measured zones of inhibition 24 hours after plating and incubation. They were able to see the effect of moringa and garlic extract and compare it with penicillin and tetracycline. Classroom results were generally uniform since all students shared the same extracts and tested the same strains of bacteria. Several observations were made,

including the effects of penicillin and tetracycline on gram-positive versus gram-negative bacteria and the effects of garlic and moringa extracts on the two bacteria. The students could clearly see that tetracycline was more effective against both gram-positive and gram-negative bacteria than penicillin (Fig. 2). Garlic was also effective against both types of bacteria, whereas moringa was more effective against gram-positive than gram-negative bacteria (Fig. 2). Table 2 shows an example of quantitative data typical of that collected by the students.

Safety issues

The bacterial strains used in this activity (*E. coli* strain K12 and *B. cereus* strain 971) are classified as BSL 1 organisms. (Note that several alternative bacterial strains or species may be suitable for use in this activity, but some of these are classified as BSL 2 organisms (see "Possible modifications" below) and, as such, must be handled accordingly in a properly equipped lab.) The students were trained in the safe handling of these microorganisms according to the ASM Biosafety Guidelines (https://www.asm.org/images/asm_biosafety_guidelines-FINAL.pdf) described by Emmert et al. (12). The students acknowledged their training by signing a safety agreement and adhered to standard laboratory safety procedures, which included disinfecting lab surfaces before and after completing the activity and using proper personal protective equipment (because of the potential splash hazard associated with the manipulation of plant extracts and liquid bacterial cultures, PPE should include eye protection). Contaminated pipet tips and swabs, and all bacterial cultures were disposed of by autoclave sterilization after use.

Finally, for this activity, it is necessary for the students to sterilize laboratory utensils (e.g., forceps and bacteria spreader) by dipping in ethanol and flaming with a Bunsen burner. Ensure that student training includes how to properly carry out this procedure.

TABLE 1.
Pre- and post-lab activity quiz, including the learning objective to which each question corresponds.

Question Number	Quiz Question	Corresponding Learning Objective
1	Some plants have been shown to have medicinal value. How might you test a plant for production of antibacterial compounds?	1
2	What are the requirements for growing bacteria in the laboratory, and what is the significance of aseptic technique in this process?	2
3	What do clear zones around antimicrobial disks on a bacterial petri plate culture indicate?	3
4	Bacteria are often categorized as either *gram-positive* or *gram-negative*. Why might an antibacterial compound inhibit the growth of gram-positive but not gram-negative bacteria?	4
5	Why do some plants produce chemicals that have antimicrobial properties?	5
6	How might humans make use of antimicrobial plant products to combat infectious disease?	6

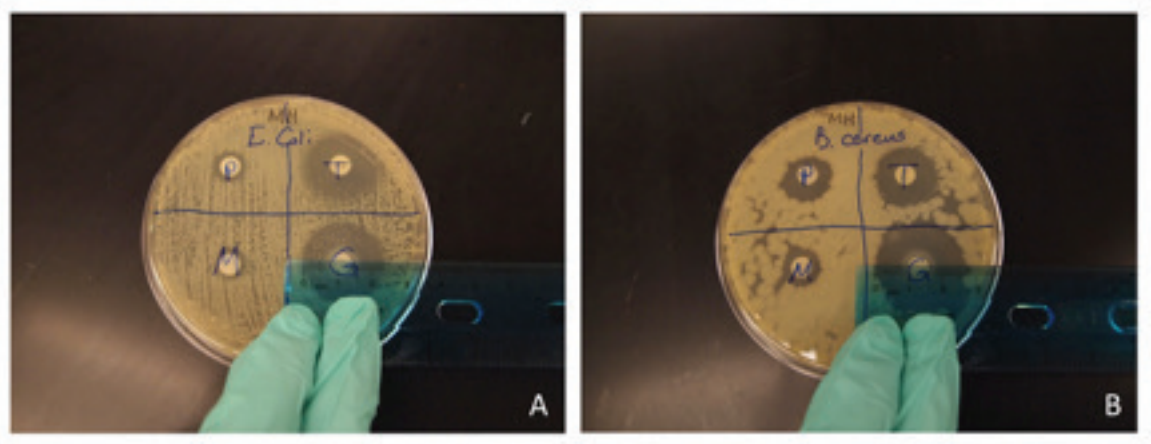

FIGURE 2. Measuring zones of inhibition after 24-hour incubation. Inhibition of bacterial growth was evaluated by measuring the diameter (mm) of each zone of inhibition. Comparisons of individual antibacterial agents can be made between the gram-negative *E. coli* (A) and the gram-positive *B. cereus* (B). Disks clockwise from top left: P, penicillin; T, tetracycline; G, garlic; M, moringa.

DISCUSSION

Field testing

This laboratory exercise is routinely conducted in our microbiology and plant biology courses. The students are able to see how these two major disciplines in the biological sciences integrate and complement one another. The students benefit from a rich learning experience as they form their own hypotheses and are able to observe clear results within 24 hours. They learn to work in teams and analyze data. The survey shows that they were excited to see the results and gained an appreciation for the antibacterial qualities of two plant species. Students were exposed to some of the benefits of medicinal plants in our lives and in research. In addition, as a result of exposure to this activity, several students have continued in undergraduate research laboratories that are currently exploring the use of other plant extracts to inhibit bacteria. Students were encouraged that people around the world who may not have access to commercial antibiotics could potentially use medicinal plant extracts as an alternative.

Evidence of student learning

The pre- and post-lab activity quiz score improved from a 75% average before completing the lab activity to an 87% average after completing the activity, for a total increase in student scores of 12%. Although the sample size was modest (29 students), the students showed significant improvement in correctly answering quiz questions 3, 4, and 5 (Fig. 3). Although improvement in correctly answering questions 1, 2, and 6 was also noted, the difference did not reach statistical significance (Fig. 3). We attribute this to the high level of prerequisite knowledge of the participating students. All of the students who completed this lab activity were upper-level undergraduate students who had already completed several college courses in the biological sciences. In addition, by the time this lab activity was conducted, the students had been attending the lecture portion of their respective plant biology or microbiology course for several weeks and had already begun to establish a solid fundamental knowledge of these disciplines.

The observed increase in the quiz score after completing the activity indicates that by conducting the experiment and answering the summary questions during the post-lab analysis, the students gained a better understanding of the concept of bacterial inhibition using the disk diffusion method, as well as the effects of penicillin and tetracycline on representative gram-positive and gram-negative bacteria. The students also observed that some plant extracts are capable of inhibiting bacterial growth. The students were able to collect their data in 24 hours and, by measuring zones of inhibition, interpret their results and make conclusions.

Nearly all students reported that they really enjoyed this laboratory activity and found it to be highly instructional. Using a Likert-type scale, each student was asked to respond to a five-question survey (included as Appendix 5). A summary of the responses is shown in Figure 4.

Possible modifications

Instructors may choose to use sterile water instead of (or in addition to) a second antibiotic as a negative control. Such an addition will demonstrate that any observed antimicrobial effects are a product of the extract itself and not a component within the disks. If seeds of *Moringa oleifera* are unavailable, *Moringa stenopetala* may be used as a substitute since extracts from both plants have similar effects. Although the activity focuses on the control of microbial

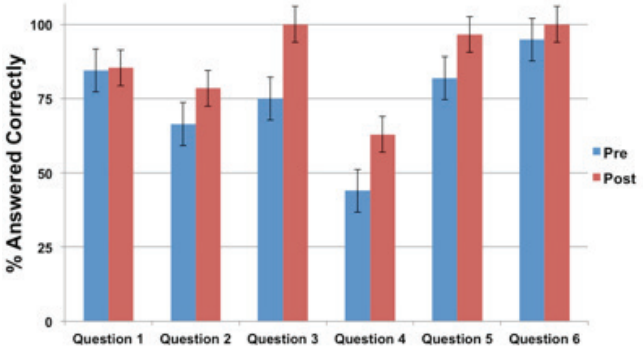

FIGURE 3. Average improvement of student performance on the pre- and post-lab activity quiz. Significant gains in student understanding were evident for questions 3, 4, and 5. The questions presented to the students are given in Table 1 and in Appendices 1 and 2. Data were compiled from the responses of 29 students. Statistical differences were determined by converting the pre- and post-lab quiz scores to percentages, which were then normalized for the statistical analysis. In this form, the chi-squared test could be applied using the CHITEST function of Excel. The resulting *p* value calculated for these 29 sets of pre- and post-lab scores was 4×10^{-38} (<0.05).

TABLE 2.
Example of typical data collected and reported by the students.

Bacterial Species	Zone of Inhibition (diameter, mm)			
	Moringa Seed Extract	Garlic Extract	Penicillin	Tetracycline
Bacillus cereus	11	15	6.5	12
Escherichia coli	7	20	7.5	18

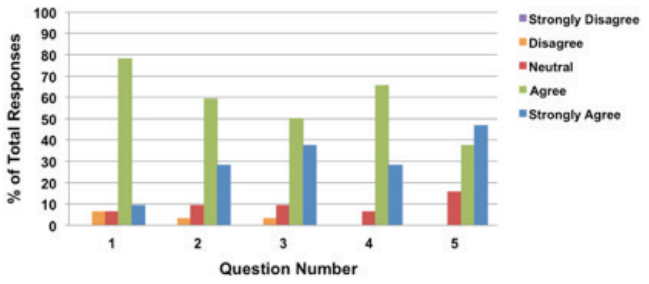

FIGURE 4. Student evaluation of the laboratory activity. The survey results showed that almost 90% of the students strongly agreed or agreed that they enjoyed the activity (question 1), the instructions were easy to follow (question 2), and they were interested in the results (question 3). Nearly 94% of the students strongly agreed or agreed that the techniques were relevant and useful (question 4). Perhaps most importantly, about 85% of the students agreed (nearly one-half strongly agreeing) that the exercise highlights the value of medicinal plants as potential producers of antibacterial agents (question 5), which was identified as a specific goal of the activity (Objective 6). The questions as presented to the students are given in Appendix 5.

growth using biosynthetic products from moringa and garlic, other plants having known antibacterial properties could conceivably be used, including cinnamon, carob, and green tea. Alternatively, students may also find it interesting to compare the antibacterial properties of extracts from fresh versus processed plants. For example, whereas extract from fresh garlic shows impressive antibacterial activity (Fig. 2), extracts from canned garlic or garlic pill supplements have little or no effect on bacterial growth (data not shown).

Another possible modification would be to substitute the bacteria used in the activity. It is generally best to use one gram-negative and one gram-positive bacterium for the activity, but the actual species used can vary. For example, BSL 1 strains of *Serratia marcescens* could be used instead of *Escherichia coli* strain K12 since they are both gram-negative. Similarly, provided the students have completed training in the safe handling of BSL 2 organisms and the laboratory space has been approved for their use, *Staphylococcus aureus* could be used in place of *Bacillus cereus* since they are both gram-positive. We have also observed plant extract-induced growth inhibition of *Corynebacterium xerosis* (BSL 1), *Bacillus subtilis* (BSL 1), *Staphylococcus epidermidis* (BSL 1), and various species of *Listeria* (BSL 1 or 2, depending on the strain), *Micrococcus* (BSL 1), *Streptococcus* (BSL 2), and *Enterococcus* (BSL 1 or 2, depending on the strain).

SUPPLEMENTAL MATERIALS

Appendix 1: Pre- and post-lab activity quiz
Appendix 2: Pre- and post-lab activity quiz; instructor key
Appendix 3: Student laboratory report with post-lab assessment questions: antibacterial effects of plant extracts
Appendix 4: Student laboratory report with post-lab assessment questions: antibacterial effects of plant extracts; instructor key
Appendix 5: Laboratory activity student evaluation survey

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