Screening for the Antibacterial Activity of Medicinal Plants

Resource Type: Curriculum: Laboratory

Publication Date: 4/7/2004

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Abstract

Plants with suspected medicinal value will be identified, collected, and dried. Organic plant compounds will be extracted with ethanol. The plant extract will be dispensed to blank disks and dried to evaporate the solvent. The antibacterial activity of the plant extracts will be determined using the Kirby-Bauer method. A standardized bacterial suspension will be swabbed uniformly onto the surface of the Mueller-Hinton agar. The plant disks will be placed at equidistant points on the agar surface, and plates will be incubated at 35^{o}C for 24 hours. Zones of inhibition, indicating antibacterial activity, will be measured in millimeters.

Activity

Invitation for User Feedback. If you have used the activity and would like to provide feedback, please send an e-mail to MicrobeLibrary@asmusa.org. Feedback can include ideas which complement the activity and new approaches for implementing the activity. Your comments will be added to the activity under a separate section labeled "Feedback." Comments may be edited.

INTRODUCTION

Learning Objectives.
At the completion of this activity, students will be able to extract suspected antimicrobial compounds and screen them for activity using a modified Kirby-Bauer method.

Lab Skills Addressed.
After the activity the student will demonstrate ability to:

1. Use aseptic techniques in handling microorganisms and other materials
    - Sterilize glassware, media, and other materials
    - Maintain sterility of materials
    - Perform aseptic transfer
    - Subculture microbial samples
2. Use appropriate microbiological media and test systems
    - Isolate colonies
    - Record experimental observations
3. Estimate the appropriate bacterial density using a MacFarland standard
4. Use standard microbiology laboratory equipment correctly
    - Use the standard metric system for weights, lengths, diameters, and volumes
    - Light and adjust a laboratory burner
    - Use the autoclave, oven, laminar flow hood, and incubator

Laboratory Thinking Skills.
A student successfully completing this activity will demonstrate an increased skill level in

1. Cognitive processes
    - Search available literature on the topic
    - Formulate objectives
    - Develop the research plan
    - Follow an experimental procedure
2. Analysis skills

- Record and organize data systematically
- Collate and present data descriptively as tables or paragraphs
- Make appropriate conclusions based on the results

3. Communication skills
   - Discuss and present experimental results
4. Interpersonal and citizenry skills, including
   - Work effectively in teams so that the tasks, results, and analysis are shared effectively
   - Manage time so that overlapping tasks are done simultaneously by members of the group
   - Integrate experimental results and folkloric claims on the bioactivity of medicinal plants

Background.
Students should have basic knowledge and skills in

- Literature search on what plants to use. Books, journals, or internet sites that offer information on ethnobotanicals (plants used by native folks for traditional medicine) are useful references.
- Aseptic techniques

PROCEDURE

Materials.

- filter paper
- forceps*
- ruler
- 25-ml pipette*
- gloves (optional)
- Pasteur pipettes*
- cotton swabs*
- pipette bulbs
- Schleicher & Schuell 740E blank disks, 6.35 mm*
- 20-ul pipette
- 100-ul micropipette tips*
- petri dishes*
- alcohol lamp
- test tubes*
- inoculating loop
- Erlenmeyer flasks
- plant material
- funnel
- 10% lysol solution in beaker
- applicator sticks*
- antibiotic sensitivity disk (ofloxacin)
- Mueller-Hinton broth*
- Mueller-Hinton agar
- distilled water*
- absolute ethanol (95%)
- MacFarland standard 0.5
- autoclave
- oven
- stove
- water bath
- coffee blender or scissors
- spot plate

Note: those items with an asterisk should be sterilized.

Organisms.
Staphylococcus aureus ATCC 25923
Escherichia coli ATCC 25922
Pseudomonas aeruginosa ATCC 27853

Student Version.
Day 1
Extraction of plant leaves (3, 6)

- Collect the plant material when the weather is dry.
- Remove diseased parts or parts with insect bites.
- Spread the plant material on newspaper and keep in a shady and well-ventilated area, turning them often until crispy dry. Drying times will vary depending on the type of tissues and water content in them, amount of material, size, etc. Ordinarily, nonsucculent leaves about 1 by 2 inches in size will be ready to use in 1 to 2 days.
- Remove and discard the stems; comminute the leaves in a coffee blender.

- Weigh 10 grams of the comminuted material; place in an Erlenmeyer flask.
- Add just enough 95% ethyl alcohol to cover the comminuted material.
- Cover the Erlenmeyer flask with aluminum foil and allow the mixture to stand for 24 hours with occasional shaking.
- Filter the material and place the filtrate in a petri dish.
- Expose the filtrate under a laminar flow hood until almost dry. This dry material is called the crude plant extract.

Sterilization of materials

- Sterilize the following materials at 121^{o}C, 15 psi for 20 minutes: petri dishes, test tubes, applicator sticks, cotton swabs, distilled water, and media.
- Sterilize the blank disks in the oven at >100^{o}C for 2 hours.

Preparation of culture media

- Prepare Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) according to the manufacturer's directions.
- Prepare six plates. Dispense 20 ml of the sterilized MHA per plate.
- Allow the agar to harden. Refrigerate. Prior to use the next day, equilibrate to room temperature.

Preparation of MacFarland standard (2)

McFarland standard 0.5 (equivalent to approximately 1.5 x 10^{8} CFU/ml)

- Add 0.05 ml of 1% BaCl, aqueous (chemically pure) to 9.95 ml of 1% H_2SO_4 (chemically pure)
- Mix the two solutions and store in a clear screw-capped glass tube. Seal with parafilm and label.

Day 2

Preparation of plant disk

- Weigh 10 mg of the crude extract and dissolve with 2 ml of water or ethyl alcohol. Mix with a vortex.
- Dip a sterile blank disk into the mixture and touch the disk to the side of the tube to remove the excess. Place the plant disk in a petri dish.
- Do the same for 14 disks. Do not allow the disks to touch each other.
- Expose the plate with the disks under the laminar flow hood to evaporate the alcohol.
- For controls, dip 10 disks in either water or alcohol, whichever was used to dissolve the extract. Allow to evaporate under the laminar flow hood.

Preparation of inoculum

- Pick three similar colonies from the *Staphylococcus* plate and suspend in 5 ml of MHB.
- Incubate the suspension for 2 hours or until the turbidity matches that of a MacFarland standard 0.5. Add broth if the suspension is more turbid than the standard. Incubate further if the suspension is lighter than that of the standard.
- Do the same for *Escherichia coli* and *Pseudomonas aeruginosa*.

The Method (1, 4)

- Label two plates Sa (*S. aureus*), two plates Ec (*E. coli*), and two plates Pa (*P. aeruginosa*).
- Dip a sterile swab into the adjusted *S. aureus* suspension. Touch the swab to the sides of the tube to remove excess fluid.
- Swab the entire surface of an Sa MHA plate. Swirl the swab along the sides of the plate.
- Draw a template of a petri dish on a piece of white paper. Mark four equidistant points on the template, corresponding to two plant disks, one water or alcohol control, and one antibiotic control. Each set of Sa plates will have four plant disks, two water or alcohol controls, and two antibiotic controls.
- Mark a pointer on one side of each bottom plate. This will be the starting point when applying the disks.
- Apply the disks with sterile forceps following this orientation: plant disk 1, plant disk 2, water or alcohol control, and antibiotic control. Press the disks lightly.
- Allow the extract to diffuse for 30 minutes.
- Invert the plates and incubate at 35^{o}C for 18 to 24 hours. Repeat the above steps for the second Sa plate, Ec, and Pa plates.

Day 3

Reading of results

- Using a ruler, measure the diameter of the zone of inhibition around each disk (in millimeters). Get the average of the readings.
- Complete the laboratory report.

Procedure – Instructor Version.
The class may be divided into groups, and since the tasks are overlapping, each individual may be assigned a task.

Day 1
Prior to this activity, literature may be surveyed for folkloric claims on the antimicrobial activity of plants or claims that certain plants can treat infection. Take note of plant parts used. Such plants must be properly identified and collected. They may be used dried or fresh. (When using latex, this plant part does not need to be extracted. It is used as is.) If no blender is available, stainless steel scissors may be used to cut the plant material into pieces. If leaves and stems are used, they are processed separately.

All tasks such as extraction, preparation of culture media, isolation of test organisms, and sterilization of glassware and other materials should be done on day 1. Prior to sterilization, the swabs, sticks, and forceps may be inserted into test tubes and covered with aluminum foil.

Approximate times for each step are listed below:
Preparation and sterilization of materials: 2.5 hours
Extraction: 24 hours
Plant disk preparation: 1 hour
Inoculum preparation: 4 hours
Kirby-Bauer method: 1 hour
Incubation: 24 hours
Reading of results: 20 minutes
Interpretation: 20 minutes
Reporting: 20 minutes per group

Day 2

To test for the solubility of the plant extract, use an applicator stick to get a portion of the crude extract and place it in the well of a spot plate. Add 2 to 3 drops of distilled water and observe if the extract is soluble. If not, get another portion and add 2 to 3 drops of ethyl alcohol. It is better to use water; however, ethanol is acceptable as long as the alcohol is evaporated prior to placement on the agar.

The plant disks may be prepared by immersing them in the extract solution, or a micropipette may be used to dispense 20 ul (or 0.02 ml) per disk. When water is used, it is not necessary to dry the disks completely. Starting with 10 mg of crude extract in 2 ml of water or ethanol will yield 5 mg/ml solution.

$$\frac{10\text{ mg}}{2\text{ ml}} = 5\text{ mg/ml}$$

Therefore, a disk with 20 ul of the extract solution will yield a concentration of 100 ug.

5 mg:1 ml = x:0.02 ml
x (1 ml) = 5 mg (0.02 ml)

$$x = \frac{5\text{ mg } (0.02\text{ ml})}{1\text{ ml}} = 0.1\text{ mg}$$

or 100 ug (1 mg = 1,000 ug; therefore, 0.1 x 1,000 = 100 ug)

When using glass petri dishes, 20 ml of agar is enough to give an agar depth of 4 mm. If using disposable plates, pour 20 to 25 ml of agar onto the plate, insert a toothpick vertically through the agar, and measure the moist portion. A 4-mm depth is recommended. The parameters in this activity have been standardized to ensure reproducibility of results.

The American Type Culture Collection microorganisms recommended here have been used because they are sensitive and will predict the response of common gram-positive and gram-negative clinical isolates.

The Kirby-Bauer method has been found to be cheap, rapid, and simple to perform. It has a long and consistent track record of assessing the antibacterial activity of drugs.

Day 3
A zone of inhibition indicates antibacterial activity. However, this activity is only a qualitative procedure.

Safety Issues.

- The recommended microorganisms pose potential health risks as opportunists and may not be suitable for (or permitted in) all laboratory settings. Alternative microorganisms that may pose significantly fewer risks are *Bacillus subtilis* and *Sarcina lutea.*
- Put on gloves and a laboratory coat before beginning experiment.
- Cap the bacterial slant and refrigerate after subculturing.
- Keep hands away from the face or any part of the body while doing the activity.
- Wash hands thoroughly after handling the materials and rub hands with 70% alcohol.
- Wipe laboratory tables with 70% alcohol or a disinfecting solution after use.
- Report to the professor any spilled agar, inoculum, broken glassware, etc.

ASSESSMENT AND OUTCOMES

Suggestions for Assessment.
The activity is assessed by the oral and written reports presented by the students. Reports that indicate negative activity of the plant extract do not mean failure in the activity. A commercial antibiotic disk is included in the experiment as a positive

control. In this case I have suggested Ofloxacin because it would exhibit activity against *S. aureus* and *E. coli*, and therefore, will signify skills learned.

Field Testing.
This activity is always a part of my course in general and medical microbiology. The class is usually composed of 50 to 55 pharmacy or microbiology undergraduate students. My graduate students number 10 to 15; however, for this group we proceed to quantitative analysis (MIC determination) using clinical isolates and equipotency testing. The activity never fails to excite both the students and me as an instructor.

Student Data.
After all the reports have been submitted some students opt to collate the data and present the results in science fairs and conventions.

Example of data

Susceptibility of *Jatropha multifida* latex to tester strains (5)

Tester strain	Mean zone of inhibition (mm)[a]	Interpretation
S. aureus ATCC 25923	13.6	Sensitive
E. coli ATCC 25922	0	Resistant
P. aeruginosa ATCC 27853	9.3	Sensitive

[a]Via disk diffusion method with a disk diameter of 6.35 mm.

SUPPLEMENTARY MATERIALS

Possible Modifications.
Those who want to proceed to a quantitative assay may opt to do the agar dilution method which is more cumbersome, but is the best high throughput assay to test a large number of clinical isolates against a single plant extract.

References.

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Appendices.
Example of a Laboratory Report

Curriculum Resources

Example of a Laboratory Report

Plant material:_____;_____
(Scientific name) (common name)

Plant part used:_____

RESULTS

Drugs	Zone of Inhibition (mm)		
	S. aureus	*E. coli*	*P. aeruginosa*
Alcohol extract 1 2 3 4 Average			
Control Antibiotic 1 2 Average			
Distilled water 1 2 Average			

Questions:

1. Which of the two extracts is the more effective against any of the test organisms?
2. Which of the 3 organisms is the most sensitive?
3. Is the activity of your extract broad or narrow in spectrum? Explain your answer.
4. Is there a folkloric practice associated with the plant you studied?
5. How is it applied as a therapeutic substance?
6. What do you mean by inappropriate antibiotic use? Why is it hazardous?
7. Explain why in vitro results do not sometimes correlate with in vivo or clinical results.

8. How does this activity apply to biotechnology?

Answer Guide for the instructor.

1. A plant extract that exhibits a clear zone of inhibition against any of the 3 tester strains is considered bioactive.

2. The biggest zone of inhibition is exhibited by the most sensitive organism.

3. The activity is broad if the plant extract is active against both gram positive and gram negative tester strains, and narrow if it is active against either strain.

4-5. The answer will depend upon the folkloric claim.

6. Inappropriate antibiotic use is the use of antibiotics without the benefit of in vitro sensitivity testing. The hazard involved is the emergence of resistant antibiotic- resistant microbes.

7. Many factors play a role in the clinical aspect: the immune status of the host, the density of infecting microbe, etc.

9. Biotechnology is just a tool used to produce new products. In this activity the sensitivity of microorganisms was used to test the bioactivity of plants. Plants are usually used for food, clothing, and shelter. This activity focuses on the use of plants for medicinal purposes.