Tested Studies for Laboratory Teaching
Proceedings of the Association for Biology Laboratory Education
Vol. 37, Article 4, 2016

Antimicrobial Properties of Spices

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Spices have been used for centuries to make food taste better, add nutrients, and retard spoilage. Scientists have recently proposed that spices may also kill micro-organisms, inhibit their growth, or suppress their production of toxins, implying that the development of spice use in ethnic cuisines may have been used historically to protect consumers from illness caused by pathogens (Sherman and Flaxman 2001). A variety of laboratory exercises designed to test spices for antimicrobial effects have been developed. Few of these emphasize evolutionary themes, and even fewer capitalize on the rich potential that a multitude of unique combinations of spices, microbes, solvents, and preparations can provide for an array of student-directed hypothesis-testing. Worldwide, there is tremendous variability in the use of different spices. This suggests that, if there is a relationship between spice use and antimicrobial benefits, this relationship has been realized multiple times in different cultures in the development of ethnic cuisines. This theme is inherently intriguing to many undergraduates, who may be curious about "who" uses what types of spices, and why. In this lab, we allow students to construct an investigation, including submission of a group research proposal, arising from their own personal interest in certain spices. We equip students with a standard protocol (the diffusion disk assay) to test their own unique hypotheses; the shared protocol facilitates lab management, resource use, and interpretation of outcomes, while allowing for a significant range of student-generated experiments. Students are also encouraged to compare the effectiveness of their spice extracts with other antibiotics (e.g. penicillin, erythromycin) on a target microbe (either *Escherichia coli* or *Staphylococcus epidermidis*). An extension allows students to document evidence of the microbial response through mutations - evidence of the evolution of antimicrobial resistance.

Keywords: antibiotics, antimicrobial properties, spices, student-generated hypotheses/experiments, microbiology, diffusion disk assay, evolutionary responses, microbial inhibition

Introduction

Testing the antimicrobial properties of spices used in preparing foods provides a relevant framework for undergraduate students to generate testable scientific hypotheses that are inherently intriguing to them. A multitude of independent variables can be explored: the type of spice, the method/solvent used for extraction of spice compounds, the origin and preparation of the spice (commercial, organic, fresh, dried, etc.), the microbe species the spice extracts are tested with, concentration of extract and/or microbe culture, and the part of the plant from which the spice was derived (seeds, roots, leaves, fruits), just to name a few! The basic investigation lays the foundation for abundant opportunities for students to develop interest in, and ownership of, their own ideas, and to gather open-ended results that will intuitively enable them to design and pursue subsequent experiments. The primary objective of this investigation, therefore, is to promote student engagement and to provide an opportunity for the development of student-generated hypotheses within a guided inquiry. The instructor will provide the foundational question to be explored – do spices inhibit the growth of microbes? The instructor will also coach protocols to conform in one aspect – all groups will use the standard diffusion disk assay for answering the various questions about potential inhibition of microbial growth. This renders the open-ended nature of the investigation much more manageable from the standpoint of resource use and instructor time. Prior to conducting experiments, students will complete a group

research proposal in which they select variables, treatments, sample sizes, design replications (which treatments are replicated on which plates in what arrangements), and specify procedures and supply needs appropriate to their own hypotheses.

The components of this investigation can be packaged in multiple ways to meet the needs and constraints of different programs. In general, given that microbial stock cultures are already available, one or two days are needed for growing out bacteria in broth cultures, or re-plating new cultures on agar plates. After students have prepared particular test plates with disks soaked in spice extracts applied to microbial "lawns", one or two days are needed to see results in bacterial growth. One day is sufficient if the cultures are kept in a 37^{o}C incubator. However, simply allowing more time for bacterial growth at room temperature can replace the absolute need for an incubator and it can also constitute an additional independent variable, if desired. This underlying process of prepping bacterial cultures, student preparation of test plates, and scoring results one or two days later, can be repeated as often or as little as an instructor requires for the particular questions investigated and for scheduling constraints. As presented here, the entire experiment requires four lab periods of 2 hours each for traditional biology laboratories that meet weekly. Less time is required for labs meeting more than once per week. Additional time may be required if statistical analysis is used, and will be determined by the level of student background in statistics and whether or not students have previously gained familiarity with a particular statistical software program. A few hours of instructor (or lab assistant) preparation time is required prior to each component (preparing plates, maintaining stock cultures, transferring and "growing out" microbe cultures). Allowing students to pursue additional experiments informed by their initial (three-week) tests will require additional laboratory periods. The entire exercise can be shortened to two traditional lab periods of two hours each if the last experiment (re-culturing of mutant colonies on treated plates) is omitted.

We complete this investigation in introductory biology and general education science courses that have no pre-requisites. Our instructor group includes individuals with substantial microbiological expertise, as well as those without such background. Instructors teaching this lab for the first time may require initial assistance from microbiologist colleagues, or they may initially implement the lab with more advanced students who are already familiar with some of the techniques (pipetting, inoculating agar plates).

For further examples of similar labs exploring antibiotic resistance or antimicrobial properties of spices, please refer to Bozzone (2014), Dorsett and Hammonds-Odie (2014), Hester *et al*. (2014), Joseph *et al*. (2013), Lessem (2008), Marion and Preszler (2010), and Sousa and Waldman (2013).

Student Outline

The Spice of Life: Does Spicy Food Help Keep You Healthy?

Spices have been used for centuries to make food taste better, add nutritional value, and even prevent meat from spoiling (Lai and Roy 2004, Rahman *et al.* 2011). Spices are plant products and are derived from various parts of the plant, including roots, flowers, seeds, and fruits. The distinctive aroma and flavor of each spice is due to a variety of secondary compounds produced by the plant (Sherman and Flaxman 2001). Secondary compounds are often complex chemicals that are not essential for the plant's metabolism, but may be used for defense from natural enemies such as herbivores and microbial pathogens. Is the ultimate reason that people also use secondary compounds from plants for protection from micro-organisms? If spices kill micro-organisms, inhibit their growth, or suppress their production of toxins, then spices may have been used historically (and currently) to protect us from illness caused by pathogens (Sherman and Flaxman, 2001). Several recent scientific studies have demonstrated that some spices do have antimicrobial properties in that certain spices can kill harmful bacteria in food, and that they can aid in food preservation (Takikawa *et al*. 2002, Tajkarimi *et al.* 2010, Dorman and Deans 2000, Ceylan 2003, Lai and Roy 2004, Ozcan *et al*., 2006). It is intriguing that, around the globe, there is tremendous variability in the use of different spices, suggesting that, if there is a relationship between spice use and antimicrobial benefits, that this relationship has been realized multiple times in different cultures in the development of ethnic cuisines. In this lab, you will be testing extracts that you make from some common spices for evidence of antimicrobial action. Finally, as you compare effectiveness of different spices in "suppressing" a target microbe (either *Escherichia coli* or *Staphylococcus epidermidis*), you will collect evidence documenting the response of the microbe itself - evidence of the evolution of antimicrobial resistance. For an excellent summary of the problems associated with the evolution of antibiotic resistance, please refer to the EvolED project supported by the National Science Foundation http://evoled.dbs.umt.edu/lessons/background.htm#appliedref, and/or read about antibiotic resistance at the Centers for Diseases Control (http://www.cdc.gov/drugresistance/index.html), or the National Institutes of Health (https://www.nlm.nih.gov/medlineplus/antibioticresistance.html).

Microbes reproduce and mutate quickly, enabling them to efficiently adapt to new environments (Greulich *et al*. 2012, Hermsen *et al.* 2012). Antimicrobial resistance is the ability of microbes to grow in the presence of a chemical (drug) that would normally kill them or limit their growth. We know now that antibiotics are not as effective at killing bacteria as when they were first introduced. Many infectious diseases are increasingly difficult to treat because of antimicrobial-resistant organisms, including HIV infection, staphylococcal infection, tuberculosis, influenza, gonorrhea, candida infection, and malaria. According to the Centers for Disease Control and Prevention (U.S. Dept. of Health and Human Services 2013), antibiotic resistance in the United States is killing more than 90,000 hospital patients a year and costs an estimated $20 billion a year in excess health care costs.

Although it is a popular misconception that antibiotics don't work as well as they used to because humans have developed a tolerance for antibiotics, the primary reason is because the microbes are no longer inhibited or killed by the drug because they have evolved resistance to the effects of the drug. Mutations that allow a microbe to resist the effects of an antibiotic provide a selective advantage to those microbes. Sometimes the resistance characters are simple mutations, involving a change in just a single gene! The result, whether an alteration in a single gene, or a more complex mutation, is that the resistant microbes differ genetically from their parents. This difference in genetic makeup is what leads to evolution.

We say that the population has evolved resistance due to natural selection by antibiotics because we know that the gene frequency at the mutation site has changed in this population. What we "see" in the bacteria is that there has been a shift from a susceptible population to a resistant population; we know that what underlies this shift is a fundamental genetic difference, hence evolutionary change has occurred (Croucher *et al.* 2011, Davies and Davies 2010, Lupo *et al.* 2012). The use of antimicrobials, even when used appropriately, creates a selective pressure for resistant organisms. Selection of resistant micro-organisms is exacerbated by inappropriate use of antibiotics, for example, patients who do not complete antibiotic doses as prescribed or healthcare providers with inadequate diagnostic information, or wishing to placate an insistent patient. It can also be exacerbated by hospital conditions where sick patients, often receiving heavy doses of antimicrobials, may be in close contact. Unfortunately, this is a fertile environment for the spread of antimicrobial-resistant "germs", and the result has been that more than 24 types of bacteria are now resistant to one or more types of antibiotics that had previously been effective against them. Some, such as *Enterococcus, Pseudomonas,* and *Mycobacterium tuberculosis* are resistant to nearly every antibiotic we have invented (Davies and Davies 2010).

Please review one or more of these helpful animations on the evolution of antibiotic resistance:

Mutations-selection: the bacteria resist

http://www.youtube.com/watch?v=zjR6L38yReE sponsored by www.evolution-of-life.com

Antibiotic Resistance, at ABPI, the Association of the British Pharmaceutical Industry:

http://www.abpischools.org.uk/page/modules/infectiousdiseases_medicines/medicines3.cfm

Science Bulletins – MRSA – Evolution of a Drug-Resistant Superbug: American Museum of Natural History: http://www.youtube.com/watch?v=iLhSk_0tWJ4

Background on Bacteria Used in This Lab

Escherichia coli (*E. coli*) is one of the most important research organisms on the planet, and probably the most well-known microbe to the general public. It is a rod-shaped, gram-negative, facultative anaerobic bacterium that lives in the intestinal tracts of mammals and birds. Most *E.coli* strains (genetic varieties) are harmless, and in fact, have important roles in normal human digestive function. However, some strains can cause serious food poisoning and related illnesses. Because it is a gram-negative bacterium, *E. coli* has a thinner cell wall than *Staphylococcus* and lacks the amount of carbohydrates found in gram-positive bacteria. The differences in the composition of bacterial cell walls have implications in the types of antibiotics prescribed to combat infections by these different bacteria. *E. coli* is an important model organism in the fields of biotechnology and microbiology and displays enormous genetic diversity and a very rapid rate of reproduction.

One species of bacteria that has been of great concern in studies of antibiotic resistance is the spherical-shaped *Staphylococcus aureus*, which can result in a wide variety of human infections (Laabei *et al.* 2015). It causes superficial skin lesions such a boils, more serious infections such as pneumonia and meningitis, and urinary tract infections. *S. aureus* is a major cause of hospital-acquired infection of surgical wounds and infections associated with indwelling medical devices. It also causes food poisoning by releasing poisonous substances, called toxins, into foods and it can cause toxic shock syndrome by releasing toxins into the blood stream. Many strains, or types, exist and are frequently carried on the skin, in the nose, and in the gastrointestinal tract of healthy persons. *S. aureus* cells are about 1 µm in diameter, and usually occur in grapelike clusters. During growth, *S. aureus* produces round, raised, opaque colonies with a golden color. It is classified as a gram-positive bacterium, which means that it has a thick cell wall that contains a carbohydrate called peptidoglycan.

Over 40 species of *Staphylococcus* exist within the genus. As a safety precaution, you will be working with a closely related species of bacteria called *S. epidermidis*. We will use *S. epidermidis* as a model for the related gram-positive *S. aureus,* which also infects humans. Unlike *S. aureus*, this bacterium has not evolved to cause disease, but maintains a benign relationship with its host, usually colonizing the skin, armpits and nares (nose). It is similar to *S. aureus* in that it is gram-positive and colonies occur as grape-like clusters. Although *S. epidermidis* is not usually pathogenic, patients with compromised immune systems are at risk of developing infection, especially in hospital environments. *S. epidermidis* can form biofilms on catheters and surgical implants and is a frequent contaminant of specimens analyzed by diagnostic laboratories.

The Kirby-Bauer Method of Measuring Zones of Inhibition (Diffusion Disk Assay)

One way that clinicians examine strains of microorganisms to determine their sensitivity to various antimicrobial agents is to perform a sensitivity-disk method, also known as the Kirby-Bauer method. The Kirby-Bauer method utilizes paper discs that have been saturated with an antimicrobial agent. These discs are placed on a 'lawn' of a particular organism (from a previously standardized dilution). The antimicrobial agent diffuses from the disc and generates a gradient of the antimicrobial agent in the medium surrounding the disk. If the microorganism is sensitive to the antimicrobial agent, there will be a visible zone of inhibition, where microbial growth will not occur due to the presence of the antimicrobial agent.

If the microorganism is resistant to the antimicrobial agent, the microbial growth will not be affected by the presence of the antimicrobial agent, and microbial growth will be visible at the edges of the disks. Following the measurement of all zones of inhibition, the clinician reports the results to the physician as "sensitive" or "resistant" to each antimicrobial agent tested. The physician can then make the decision about which antibiotic is the best for treating the patient's illness. Similarly, paper discs used in the Kirby-Bauer method can be infused with other substances that are hypothesized to have antimicrobial properties – such as spices! In this exercise, you will be able to make your own spice-infused paper discs, and test the effectiveness of spices in inhibiting the growth of *E. coli, S. epidermidis*, or both.

Spices, Antibiotics, and Evolution

The evidence for the evolution of antibiotic resistance in certain microbes is overwhelming, and is already having dramatic impacts on the delivery of health care (Davies and Davies 2010). If spices also have antibiotic properties, would we expect microbes to have evolved in response to the selective pressure caused by generations of spice use in human consumption? Can we find evidence of mutations that confer resistance to spices in common bacteria (e.g. Pryce *et al.* 2000)? If so, how might we expect the selective pressures for the evolution of resistance to spices to differ from those that lead to the evolution of resistance to drugs, and why? Refer to these questions as you complete your group research proposal.

The Spice of Life Experiments

Bring your group research proposal, which now includes comments from your instructor regarding how you will test your hypothesis on the effects of spices on the growth of bacteria. Some groups will have selected one species of bacteria that

will be used to test different spice preparations, and other groups may have opted to test the effectiveness of a given spice on two different species of bacteria. In order to streamline use of materials, and in order to allow groups to be able to compare results with one another, most protocols will be modified somewhat to take advantage of a framework that allows you to complete your experiment in the allotted time, with materials that are available. The details of your protocol, however, are unique to your group, so plan carefully!

I. Lab 1: Preparing Spice Extracts

A. Identify The Following Materials at Your Work Station for Preparing Spice Extracts

- spices identified in your Group Research Proposal
- mortar and pestle
- 1 teaspoon measuring spoon
- electronic scale
- distilled water
- 100% methanol
- weigh boats
- 10 mL graduated cylinder
- 2-10 mL Büchner flask or filtration flask
- clear tube to connect vacuum and Büchner flask or filtration flask
- funnel that fits Büchner flask (or filtration flask)
- filter paper that fits funnel
- sample vial rack holders
- additional materials as specified in your Group Research Proposal and approved by your instructor
- protective gloves and protective laboratory safety glasses or goggles

B. Spice Extract Preparation

In your Group Research Proposal, you will have specified whether you are preparing spice extracts in water, in methanol (a polar solvent), or both. Use only the procedure(s) that you specified in your proposal. Wear protective gloves and safety glasses.

1. Water Extraction

a) Prepare the part of the plant or spice that you will use. Using a teaspoon, weigh boat, and an electronic scale, obtain the mass of approximately one teaspoon of spice. Record the mass of each preparation to the nearest 0.01 g, below, adding lines as needed.

Name of spices and their mass:

1). _____ _____g

2). _____ _____g

b) Transfer the spice from the weigh boat into a mortar and grind well to a very fine powder.

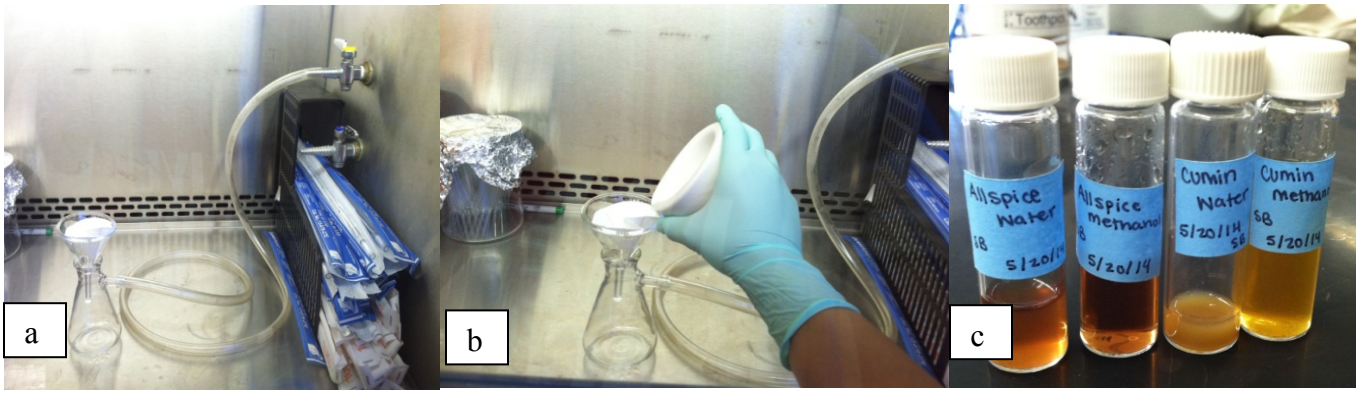
c) Next, add 10 mL of distilled water and then continue grinding and mixing the solution for approximately 15 minutes. This may seem tedious but it is absolutely essential to break tissues to release chemical compounds from the spice.

d) Filter each preparation separately in the vacuum filtration system in the hood similar to that shown in Fig. 1a. Be sure to clean the apparatus and change filter paper between filtering different samples.

e) Carefully, transfer the solution from the mortar into the funnel and slowly turn on the vacuum to speed up the filtration process (Fig. 1b).

f) Record the mass of an empty sample vial in the space below. Then, measure the volume of the collected spice extract using a graduated cylinder. Finally, transfer the filtered spice extract into the sample vial and screw on the top. Repeat for each of your samples after each one is filtered. Each vial should be carefully labeled and should appear similar to those in Fig. 1c.

g) Store your sample vials labeled with your name, date, and extract type in a refrigerator until the next laboratory period.



Figures 1a-c. 1a. filtration flask connected to vacuum pump in a hood, 1b. transferring the sample to the filtration flask, and 1c. sample extract vials with labels. Photographs by S. Balcazar 2014.

2. Methanol Extraction

Note: Methanol is a toxic alcohol. When pouring methanol and when mixing/grinding your spice with methanol in a mortar, do so ONLY in a ventilated hood while wearing protective gloves, eye protection, and an lab coat. Avoid splashing by grinding gently and firmly.

a) Follow the same procedures as given under B.1. Water Extraction, items a) – g). with extra precaution to not spill or splash methanol.

C. Calculating the Density of your Spice Extract Prepared in Methanol

First you will need to determine the density of water (if you made spice extract in water) or the density of methanol (if you made spice extract in methanol). Circle water or methanol on the entry for each sample. Then, use the following guidelines for determining the density of each of your spice samples. If methanol was used, divide the spice density by 0.7918 (last column in Table 1) to determine the density of the sample in g/mL.

1. Spice #1 in water/methanol: _____
   Measured volume of collected spice extract:_____mL
   Mass of empty vial: _____ g
   Mass of vial with extract: _____ g
   Calculated Density (g/mL) _____
2. Spice #2 in water/methanol: _____
   Measured volume of collected spice extract:_____mL
   Mass of empty vial: _____ g
   Mass of vial with extract: _____ g
   Calculated Density (g/ml) _____

Table 1. Spice extract preparations completed.

Spice Type	Solvent	Mass of total sample (to 0.01g)	Solvent volume used (mL)	Density of spice in sample (g/mL)	Methanol conversion -divide density by 0.7918 (g/mL)

II. Lab 1: Preparation of Bacterial Broth Cultures

A. Identify the Materials Needed for Preparation of Bacterial Broth Cultures

- nutrient agar slant or petri dish cultures of *S. epidermidis* and *E. coli*
- nutrient broth
- sterile toothpicks
- 70% ethanol
- forceps
- incubator (37°C)
- sterile bent plastic or glass rod
- sterile culture test tubes and tube racks
- sterile 5 mL pipette
- petri dishes
- petri dishes prepared with LB media OR Mueller-Hinton agar media
- sterile LB mix without Agar in broth solution OR Sterile Mueller-Hinton in broth solution
- protective gloves and safety goggles

B. Determine the Number of Bacterial Cultures in Broth That You Will Need

1. If your research protocol specifies use of one species of bacterium (e.g. *E. coli*), then you will prepare only one broth culture. If your research protocol specifies two species (*E. coli and S. epidermidis*), then you will prepare a separate broth culture, one for each species. Because the density of each broth culture will be different, you must use the same broth culture to inoculate all of your test plates for a given species of microbe to be able to directly compare results among different treatments that this species is subjected to.
2. Label a culture tube for each type of bacteria you will use with your initials, the date, and the bacterial species.
3. Transfer 2 mL of liquid LB media (no agar) or liquid Mueller-Hinton media (no agar) into each tube using a sterile 5 mL pipette**. Use the same media type for all of your cultures throughout the entire experiment** (either LB or Mueller-Hinton).

C. Aseptically Transfer Growth from One Bacterial Stock Plate (e.g. *E. coli*) to the Broth Culture Tube

Follow the procedures very carefully so that you do not contaminate either the stock culture or the new broth culture with bacteria from other sources (your hands, the table, the air, etc.). Read steps 1. - 6. BEFORE proceeding.

1. Put on sterile gloves and safety goggles.
2. Sterilize a pair of forceps by dipping the ends in 70% ethanol. Continue holding the sterilized forceps so that they do not touch any other surfaces until you are finished with the transfer.
3. Use the sterile forceps to grasp one sterile toothpick from the toothpick container, replacing the lid of the toothpick container without letting it touch the table.
4. Now grasp one end of the toothpick with your gloved hand. Pick up the stock culture of *E. coli* (or *S. epidermidis*) and remove the lid momentarily while you touch the other end of the toothpick to the agar surface of the stock plate where you can see bacterial growth. Without gouging the surface of the agar, slide the toothpick smoothly across the surface to remove a small amount of bacterial growth from the surface of the agar. Replace the lid of the stock culture.
5. Remove the cap of the broth culture you prepared without setting the cap down on the table. Drop the toothpick into the culture tube. Re-cap the tube.
6. Gently swirl the tube to break up the bacterial cells.
7. Repeat steps 1-6. for the other bacterial species only if your research protocol specifies comparison of two bacterial species for your experiment.
8. Incubate the cultures overnight in an incubator shaker at 37^{o} C.
9. When bacterial growth is apparent and the broth media appears cloudy, your instructor will instruct you to store the broth cultures in a refrigerator until the next lab period. Cultures should be allowed to come to room temperature when used for setting up your experiments.

III. Lab 2: Preparation of Test Plates for First Experiment on Inhibition of Growth by Spices

A. Identify the Materials Needed for Preparation of Test Plates for Your First Inhibition Experiment

- your vials of spice extract that you prepared previously
- 70% ethanol

- forceps
- sterilized blank paper disks
- LB medium OR Mueller-Hinton medium in agar prepared in petri dishes
- *E. coli* and/or *S. epidermidis* broth culture tube that you prepared previously (remove from the refrigerator when you come to lab to bring to room temperature)
- sterile 5 mL pipette
- 100 µL pipette and pipette tips
- microcentrifuge tubes
- biohazard waste
- glass/plastic bent rod
- protective gloves and safety glasses
- parafilm

B. Determine the Number of Test Plates You Need to Prepare

1. Each plate can be divided into thirds or quarters for replicates of more than one treatment. When you mark the plates use a permanent marker and **mark the bottom of the plate,** not the lids. Each plate you prepare should have one of the sections devoted to a "control" treatment (e.g. a plain sterile paper disk with no spice on it). Why do we run a control in each plate instead of simply assigning one plate (out of several that may be used for our experiment) to contain a control disk?
2. In your research proposal, you specified how many replicates for each treatment you would include in your experiment. It is important to have several replicates for each type of treatment in order to make an experiment more representative of what the range of outcomes may be. Also, without replication, we cannot use statistics to analyze our data, since replication allows us to estimate sampling error. For example, if you are comparing the effectiveness of allspice (extracted in water) and cumin (extracted in water) as inhibitors of bacterial growth, you can prepare one replicate disk for *each* spice, and the control disk, and place one of these disk types in each of the 3 sections of a plate divided into thirds. Then, if you prepare 6 plates in exactly the same manner, you now have a replicated experiment that includes 6 replicates *each* of: control, allspice in water, and cumin in water. Your instructor will advise you on maximum number of replications per treatment relative to the supplies available.
3. If your experiment specified comparing two types of bacteria, you will need to separate bacteria such that they are always on different plates, and replicate tests with each bacterial species several times.
4. Sketch a figure in your lab notebook showing your own plan for application of replicate disks and control discs to the test plates you will prepare. This may be slightly modified from your Group Research Proposal, as recommended by your instructor.

C. Preparing Spice Extract Discs and Transferring Bacteria to Test Plates

This step requires aseptic technique, as you did previously. Read steps 1 – 10 before beginning the aseptic procedure.

1. Wearing gloves, sterilize forceps by rinsing with 70% ethanol over a waste beaker.
2. Use the sterile forceps to grasp a disk from the container of sterilized blank disks. Drop 6-10 disks into *each* vial of spice extract. Let the disks soak for at least 20 minutes prior to application on bacteria plates.
3. Make sure your test petri dishes are divided and marked correctly, as described in part B. Label each one- third (or each quarter) of each plate. Each plate should be labelled with the bacterial species, your name and the date. Each section of a plate should contain the treatment name (e.g. cumin in water, or pepper leaves, etc.) or control.
4. Using a sterile 5 mL pipette, transfer 1 mL of *E. coli* (or *S. epidermidis*) from your culture tube with liquid broth into a small microcentrifuge tube. Then replace the broth culture tube in the rack.
5. Using a 100µL pipette, transfer 25µL of bacteria in liquid to ***each*** section of the agar plate.
6. Then using a sterile bent glass/plastic rod spread the bacteria in each plate to create a bacteria lawn. Re-sterilize the bent glass/plastic rod with 70% ethanol solution after completing each individual plate. This will help prevent cross-contamination from one plate to another.
7. Replace the lid and turn the plate upside down and let it sit for at least 5 minutes to dry.
8. If (and only if) you are using two bacterial species for your experiment, repeat steps 3-7 using the *S. epidermidis* (or *E. coli*) culture to create a bacteria lawn in each of the plates to be tested on this species.
9. Refer to the figure you made in your lab notebook that shows the "map" for placement of paper disks on each plate. The bottom of each plate should be labelled accordingly. Now, use forceps sterilized in ethanol to

carefully and **aseptically** place the test disks (controls and those containing your specified spice extracts) onto the surface of the plates, one at a time. Be sure to let the forceps dry for a few seconds each time you re-sterilize them between disk applications so that excess ethanol does not drip on your plates. Gently, touch the surface of each disk with the forceps, immediately as you apply it to the plate, to ensure good contact between the disk and the medium.

10. On each plate there should be a section reserved for a blank, sterile control disk. Place the cover on each plate after all disks are applied to each. Discard any unused or empty microcentrifuge tubes in a biohazard waste bag.
11. Once all of your plates are prepared, carefully invert the plates and then place in an incubator at 37° C for 24-48 hours, or at room temperature, as your instructor recommends. Your instructor will provide information on when they are to be taken out, wrapped and sealed with parafilm, and placed in the refrigerator.

D. Predictions Regarding this First Test of Inhibition of Bacterial Growth.

Refer to your Group Research Proposal and write some predictions for what you expect to occur if your working hypothesis is supported by the data. Around what type of disks do you expect the greatest zone of inhibition, and why? Around what disks do you expect the smallest zone of inhibition, and why? Do you think any disks will result in no apparent zone of inhibition? If so, which ones, and what would explain such a result?

Do you expect the zones of inhibition to be the same for the **same type of disks** on all of the petri dishes that contain that type of disk? Why or why not? What would cause such a result?

IV. Lab 3: Measuring Zones of Inhibition on the First Test of Inhibition of Bacterial Growth

A. Identify the Materials Needed for Measuring Zones of Inhibition

- a calculator
- small cm ruler to calibrate optical micrometer
- your previously prepared test plates (petri dishes)
- protective gloves and safety glasses or goggles

B. Measuring Zones of Inhibition

1. Check that the edges of each of your petri dishes are wrapped securely in parafilm.
2. Place one of your petri dishes upside down on a dark surface such as your lab table.
3. Line up the cm ruler with the edge of one paper disk and measure the distance from the edge of the disk to the outer edge of the zone of inhibition to the nearest mm (Fig. 2).

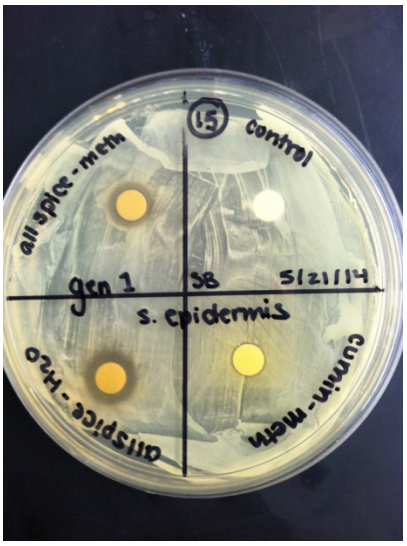


Figure 2. Sample plate showing zones of inhibition around (from widest zone to most narrow zone) allspice extracted in water, allspice extracted in methanol, cumin extracted in methanol, and control disk. Photograph by S. Balcazar, 2014.

4. Continue to record the width of each zone of inhibition for each disk, including the control disk, and record your results in a Table such as the sample in Table 2. Adjust the rows as needed.
5. Sometimes, the zone of inhibition is not circular when bacterial growth is uneven on different sides of the disk - if this occurs, ask your instructor or a TA for advice on measurement. It may be advisable to measure the distance at several points around the disk, and then determine the mean width of the zone of inhibition. Some zones of inhibition may be very narrow - do not neglect to measure carefully! If there is absolutely no zone of inhibition at all, record the value as zero.
6. In the event that not all of the paper disks have the same diameter, your instructor may advise you to measure the diameter of the (disk + the zone of inhibition) around it, and then to subtract the diameter of the paper disk from this quantity, in order to allow accurate comparisons for zones of inhibition around different disks.

Table 2. Results of first experiment on inhibition of bacterial growth.

Treatment	Replicate Number	Total Diameter of Zone + Disk (mm)	Disk Diameter (mm)	Zone of Inhibition (Total Diameter - Disc Diameter), mm

V. Lab 3: Analysis of Results - How Is Bacterial Growth Affected by Spice Extracts?

A. Statistical Analysis for Comparing Two Samples at a Time.

1. For your experiment, you are comparing the outcome of two kinds of treatments (for example growth of *E. coli* in allspice extracted in methanol as compared with growth of *E. coli* in cumin extracted in methanol). The treatment is the independent variable. For some groups, the independent variable may be the bacterial species tested or the type of preparation, or the part of the plant that was used to extract the spice. Identify your (two) treatments for the independent variable you are testing.
2. The dependent variable is the outcome we have measured, which is the width of the zone of inhibition.
3. If one (or more) of our treatments has inhibited bacterial growth, then we would expect that the average zone of inhibition is greater for the treated disks than it is around the control disks. We might also expect that the average zone of inhibition around one treatment group is greater than the average zone of inhibition for the other treatment group. For example, if we hypothesized that cumin is a more powerful inhibitor of *E. coli* than allspice, we would expect that zones of inhibition measured around *E. coli* grown in the presence of cumin are greater, on average, than those measured around *E. coli* grown in the presence of allspice. We will use the independent samples t-test to determine if the means of two samples are significantly different from one another. In addition to comparing the two treatments with one another, we will test each treatment with the control.
4. State your own group's working hypothesis here (refer to your group research proposal): _____ _____
5. Write your null hypothesis, for purposes of conducting an independent samples t-test comparing your two treatments, here: _____ _____

6. Write your null hypothesis, for purposes of comparing a treatment group with a control group, using the independent samples t-test:

   _____

   _____

7. Using a graphics and statistical software program recommended by your instructor, construct a bar graph with standard error bars showing the mean zones of inhibition for each of your two treatments and the controls. Include a copy of the bar graph in your lab notebook.
8. Conduct three separate independent samples t-tests using the recommended software, as assigned by your instructor. Include copies of all output data, and a copy of your data file, in your lab notebook.
   a) Treatment 1 (e.g. cumin in methanol) compared to control disks
   b) Treatment 2 (e.g. allspice in methanol) compared to control disks
   c) Treatment 1 (e.g. cumin in methanol) compared to Treatment 2 (allspice in methanol)

VI. Lab 3: Culturing Resistant Mutants from the First Experiment on Bacterial Growth in The Presence of Spice Extracts and/or Antibiotics

A. Identify Materials Needed for Re-Culturing Mutant Bacterial Colonies

- your petri dish cultures of bacteria from the first experiment (the same cultures for which you have previously measured zones of inhibition)
- sterile toothpicks
- 70% ethanol
- Forceps
- Incubator at 37^{o} C
- Sterile test tubes and test tube rack
- Sterile broth tubes with LB media (no agar) or Hueller-Minton media (no agar) in solution, in racks
- 5 mL sterile pipette and bulb

B. Identification of Resistant Colonies

1. Determine which plates from the first experiment show evidence of resistant bacterial growth. Resistant colonies will appear as small spots of isolated colony growth **within** the zone of inhibition In other words, they are bacteria that have managed to survive within the zone of inhibition where most bacterial growth was inhibited. Such colonies arise through a mutation (or perhaps several) that occur(s) in a single bacterium. As this mutant reproduces repeatedly over a day or two, the mutation is transferred to all of its offspring, and to their offspring, and so on, resulting in a visible "colony" of bacterial clones, all descended from the mutant ancestor (Fig. 3).

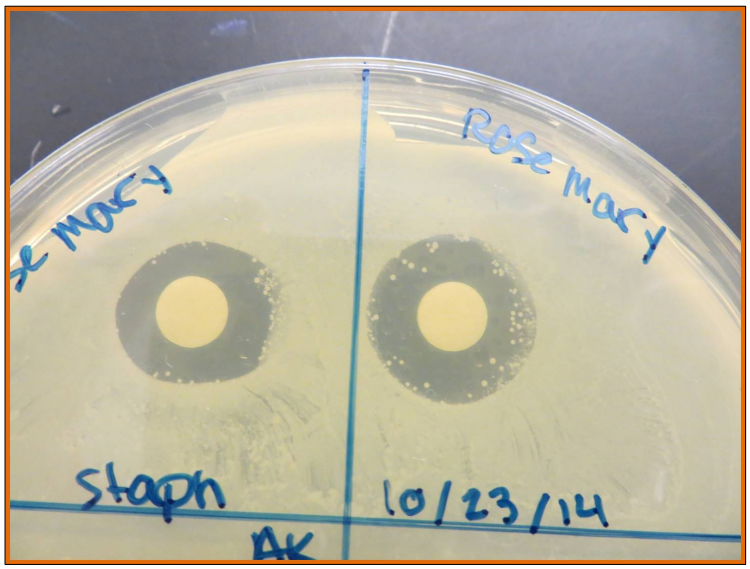


Figure 3. Several resistant colonies are evident within the zone of inhibition around both disks infused with rosemary extracted in water. Photo by K. Winnett-Murray 2014.

2. If you have many resistant colonies, select one or two examples for further experimentation. Since resistant colonies within a zone of inhibition would indicate that bacteria otherwise inhibited by the test treatment can include mutants, you will now be investigating whether those mutants can begin resistant lineages that will not be affected by the treatment. Select the mutant colonies you are most interested in, from this perspective, and write your rationale for your selection here:

   _____
   _____
   _____

3. Once you have selected the plates where there is evidence of mutant colonies, collect the same number of sterile glass test tubes as the number of mutant colonies you wish to further test. Label **each** test tube according to the resistant type and species. For example if you have an *S. epidermidis* colony that is resistant to rosemary (as shown in Fig. 3) label the test tube 'Rosemary-resist *S. epidermidis*' along with your initials and the date. The number of bacteria tubes may vary from group to group.

C. Transferring Cells from Resistant Colonies Resulting from the First Experiment to Broth Cultures.

1. Put on gloves and safety glasses.
2. Transfer 2 mL of LB or Hueller-Minton liquid media into **each** of the labeled sterile test tube you will use with a 5 mL sterile pipette. Place the test tubes in a rack at your work station.

   Aseptically transfer a portion of one mutant colony to a single broth culture tube, following the procedures very carefully so that you do not contaminate either the original culture or the new broth culture with bacteria from other sources (your hands, the table, the air, etc.). Read steps 2 - 6 **before** proceeding.
3. Sterilize a pair of forceps by dipping the ends in 70% ethanol. Continue holding the sterilized forceps so that they do not touch any other surfaces until you are finished with the transfer.
4. Use the sterile forceps to grasp one sterile toothpick from the toothpick container, replacing the lid of the toothpick container without letting it touch the table.
5. Now grasp one end of the toothpick with your gloved hand. Pick up the petri dish containing your first mutant colony and remove the lid momentarily while you touch the other end of the toothpick to the agar surface of the test plate where you can see the mutant colony of interest that you previously identified. Without gouging the surface of the agar, slide the toothpick smoothly across the mutant colony to remove a small amount of it from the surface of the agar. Replace the lid of the petri dish culture.
6. Remove the cap of the broth culture you prepared without setting the cap down on the table. Drop the toothpick into the culture tube. Re-cap the tube.
7. Gently swirl the tube to break up the bacterial cells.
8. Repeat steps 2-6.for each of the other mutant colonies you will test.
9. Incubate the cultures overnight in an incubator shaker at 37^{o} C.
10. When bacterial growth is apparent and the broth media appears cloudy, your instructor will ask you to store the broth cultures in a refrigerator until the next lab period. Cultures should be allowed to come to room temperature when used for setting up your experiments.

VII. Lab 4: Testing Growth of the Mutant Bacterial Strain(s) in the Presence of Spice Extracts and/or Antibiotics

A. Identify Materials Needed for Preparation of Second "Generation" Test Plates

- your saved vials of spice extract that were prepared previously
- 70% ethanol
- forceps
- sterilized blank discs
- LB or Mueller-Hinton media prepared in agar in petri dishes
- broth tubes of resistant mutant bacteria that you prepared previously
- sterile 5 mL pipettes - several for each group
- 100 μL pipette and pipette tips
- several microcentrifuge tubes
- biohazard waste basket
- glass/plastic bent rod
- protective gloves and safety glasses or goggles
- parafilm

B. Preparing Plates for Growth of Resistant Strains of Bacteria

1. Decide how many plates you will need to perform a replicated experiment testing inhibition of bacterial growth in the presence of your selected spice extract for *each* of the mutant colonies you have been culturing in broth. Recall that replication is necessary in order to gauge variability in the results as well as to be able to conduct inferential statistical tests on your data. A reasonable number of replicates for each mutant strain might be 5-12; your instructor will advise your group relative to availability of materials.
2. You will be able to have up to 3 replicates of each mutant strain on a single petri dish, plus a control disk, by dividing each plate into quarters. Remember to include one control disk on each and every plate. You should test your mutant strain "against" the same inhibitor from which it was originally produced. For example, if you have cultured a mutant strain that was originally *E. coli* growing in the presence of rosemary extract, you will now re-test that strain with the same rosemary extract again - why? If the strain is indeed comprised of *E. coli* resistant to the inhibitory effects of rosemary, what would you expect to observe in this second experimental test?

   _____
   _____

   At the same time, a mutant strain of *E. coli* that is resistant to rosemary might also be resistant to other spices. Decide in consultation with your instructor, and based on availability of materials with consideration to replication, what you will test in this second experiment, *in addition to* a re-test of your mutant strain on the *same* type of spice on which it was originally cultured. Write your rationale and your prediction here:

   _____
   _____

3. In your lab notebook, sketch a "map" of how you will arrange your replicates on your test plates. This map will be similar to the one you sketched previously. Once you have determined the number of replicates you will do for each mutant strain, mark the **bottom** of your plates accordingly, including the bacterial species, the date, your initials, and a label for each quarter section indicating what spice extract your mutant strain will be grown with.
4. Use the same aseptic technique you used previously (section III. C. 1-2) to prepare enough spice extract disks for your mutant strain experiment. Soak spice extract discs for the same amount of time (e.g. 20 minutes) that you used in the first experiment.

Wear gloves and use aseptic technique for steps 5 - 11.

5. Use a sterile 5 mL pipette to aseptically transfer 1 mL of bacteria in broth from the first test tube containing one of your mutant strains into a small microcentrifuge tube. Replace the broth tube back into the rack.
6. Using a 100µL pipette, transfer 25µL of bacteria in liquid from the microcentrifuge tube to *each* quadrant of the agar plate.
7. Then using a sterile bent glass/plastic rod spread the bacteria in each plate to create a bacteria lawn and then recover the plate. Be careful to not cross contaminate plates. Sterilize the bent glass/plastic rod after every use on a given plate with 70% ethanol solution.
8. Turn the plate upside down and let it sit for at least 5 minutes to dry.
9. Use ethanol-sterilized forceps to aseptically place the spice extract disks onto the surface of each plate, one on each quarter of the plate. Each plate should include one quarter section that contains a control disk. Gently, touch the surface of each disk with the sterile forceps once it is placed on the plate to ensure good contact between the disk and the medium. Re-sterilize the forceps after each disk is applied to prevent cross contamination, allowing the forceps to air dry each time so that ethanol does not drip onto your plates.
10. Repeat steps 5-9 with **each** of your broth cultures containing a different mutant strain, one strain at a time. **Use a *different 5 mL pipette for step 5 each time you begin a new broth culture.*** Discard empty or unused microcentrifuge tubes in a biohazard waste bag.
11. Once all of your inverted plates are dry and the disks are "holding", place the plates upside down in an incubator at 37° C for 24-48 hours (or at room temperature if that is the temperature used for the first experiment). Your instructor will advise when they should be removed, wrapped in parafilm around the edges, and placed in a refrigerator.

VIII. Lab 5: Results of Second Experiment Measuring Growth of Resistant Mutant Strains in the Presence of Spice Extract and/or Antibiotics

A. Identify Materials Needed for Measuring Zones of Inhibition

- a calculator
- small cm ruler to calibrate optical micrometer
- your "second generation" test plates (petri dishes)
- protective gloves and safety glasses or goggles

B. Measuring the Zone of Inhibition in Second Experiment Plates - Growth of Mutant Strains in the Presence of Spice Extract)

1. Measure the diameter of the zones of inhibition around each disk on each plate as you have done previously (refer to Section IV. B., Fig. 2).
2. Record your data in your lab notebook, and/or using a Table similar to Table 2.

C. Comparing the First and Second Experiments

1. Compare the mean zones of inhibition of the second experiment spice extract tests with the controls from the second experiment, using an independent samples t-test.
2. Although it appears logical to now compare the mean zones of inhibition from the first and second experiments (and what would be your working hypothesis for the outcome of that comparison?), there are other important variables to consider. For example, unless the bacterial broth cultures were grown in such a way that the concentration (density) of the bacteria was the same (e.g using an optical density measure), we cannot be certain that this is a fair comparison, since the stock culture used to inoculate the first experiment plates was most likely at a different bacterial density than the one you used the second time. Other variables, such as temperature, may have been different during the first and second week of experiments, and the extract itself was older during the second experiment. How might these factors influence the outcome of this comparison?

   _____
   _____

   How might you adjust and/or correct for these confounding variables to be able to directly compare the mean zones of inhibition from the first experiment with the mean zones of inhibition from the second experiment?

   _____
   _____

3. What other comparisons did you make? Do your results provide any evidence that bacteria are more sensitive to some spices than others? If so, which ones? Do your results provide any evidence that resistance to one compound is related to resistance to other compounds, or does it appear that mutations conferring resistance are completely independent?

   _____
   _____

4. Construct a bar graph for **each** of the comparisons you made in your experiments. Add these to your lab notebook, adding a few lines of written explanation below each graph.

Materials

Preparing Spice Extracts: Week 1

A. Spices

allspice
stick cinnamon
cumin seed
whole cloves
whole black peppercorns
yellow mustard seed
fenugreek seed
asafetida
coriander seed
fennel seed
thyme leaves
anise seed
whole nutmeg
rosemary leaves
turmeric
paprika
ginger root
garlic

Note: Whatever is available locally could be interesting to test! Spice stores, health food stores, etc. are more likely to have whole spices, not just ground. Fresh preparations could be compared to dried preparations. Spices can be frozen and used next semester or next year. You can also ask students to bring in spices from their own households. You may also want to consider lemons, limes, and oils and vinegars that are hypothesized to have antimicrobial properties. Some studies suggest that combinations of materials are more effective antimicrobial agents than single items.

B. Additional Materials

Per Lab Group:

- mortar and pestles (or an old coffee grinder)
- 1 tsp measuring spoon (or plastic spoons)
- electronic scale (0.01g capable)
- distilled water
- 100% methanol
- *w*eigh boats
- 10mL graduated cylinder
- 2-10mL Buchner flask or filtration flask
- clear tubing to connect vacuum and filtration flask, one for each flask
- funnel that fits each Buchner flask or filtration flask
- *s*ample vials (2dram glass with lid); several per student team
- *s*ample vial rack
- nitrile gloves (1 pair per student)
- safety glasses (1 per student)
- any additional materials requested in group proposal

1. Preparation of Test Plates for First Inhibition Experiment: Week 2

A. General Supplies

- *v*ials of group's previously prepared extracts
- 70% ethanol; one dispenser bottle of about 100 mL per team
- forceps – 1 or 2 pairs per student team
- sterile blank paper disks – one container of 12-24 per team
- sterile LB (Luria) or MH(Mueller-Hinton) agar plates (100 x 15mm)
- TSA (tryptic soy agar) or NA (nutrient agar) plates, may also be used but growth may be slower
- Number of plates per team according to group research proposals
- Broth cultures of *E. coli* or *S. epidermis* - these may be prepared by students in advance or prepared by instructors in advance **BUT** *each student team must use the same culture tube for inoculation of all test plates since bacterial density will vary among culture tubes*.
- sterile 5mL pipets – several per student team
- 100µL pipettor and sterile tips – 1 pipettor and 1 box of tips per student group
- microcentrifuge tubes – several per student team
- biohazard waste containers – 1 per team or 1 per work area is desirable
- bent glass or plastic rod – 1 per student group
- Sharpies to label plates – 1 or 2 per student team
- 37° C incubator – 1 sufficient for class of 24.
- Parafilm to seal plates for storage – 1 roll per team or per student work area.

B. Antibiotic Disks

Optional: This information is provided for instructors who wish to include a comparison of inhibition of bacterial growth on spice extracts with inhibition of bacterial growth on standard antibiotic disks – please note that concentrations of standard antibiotic disks will not necessarily be comparable to concentrations of spice extracts prepared by students.

amoxicillin with clavulanic acid
chloramphenicol
ciprofloxacin
erythromycin
penicillin
streptomycin
sulfosoxazole
tetracycline
OR any others that you can acquire.

Note: these are available from Fisher, VWR etc. Check with your microbiology colleagues regarding any disks they may already have and for assistance with background on bacterial strains and help with media preparation.

2. Analysis of Results: Week 3

- mm rulers to measure zones of inhibition – 1 or 2 per student team
- nitrile gloves (one pair per student)
- biohazard bags for plate disposal – 1 per student team or 1 per student work area desirable
- access to computer statistical software or TI graphing calculators

See *Appendix B: Sources of Materials and Supplies* for ordering information.

Notes for the Instructor

A. Scheduling and Time Allotments.

There are a number of ways to schedule the components of this lab to work within the time constraints of a few 2- or 3-hour labs, to use "lecture" time in the same class for some of the shorter, transitional procedures between weekly 2- or 3- hour labs, or to be ideal for a class meeting for 90 minutes, two times per week. As a guideline, a typical lab section of 24 students requires the following time periods to complete each of the modular steps outlined in the Student Handout:

Lab 1 – 2 hours – preparation of spice extract; preparation of broth cultures of bacteria from stock cultures

Lab 2 – 1 hour – preparation of inoculated cultures with spice extract disks

Lab 3 – 2-3 hours – measure zones of inhibition, analysis, inoculate broth cultures with resistant colonies

Lab 4 – 1 hour – inoculate plates with resistant strains with spice extract disks

Lab 5 – 1 hour- measure zones of inhibition, analysis

The number of days between each set of labs (e.g. between Labs 3 and 4) can be easily adjusted if bacterial cultures in broth or on agar (which require 1-2 days to "grow out" in an incubator) are refrigerated in between the two steps. For example, the number of days between Labs 2 and 3 can be anywhere between 2 and several days if the culture plates are refrigerated after being allowed to grow for 2 days.

Labs 1 and 2 can be combined into the same 2 or 3 hour laboratory period if the instructor has previously prepared nutrient broth cultures (ready to be inoculated with bacteria by the students). Stock cultures of the test bacteria, e.g. *E. coli* and *S. epidermidis*, on agar plates, should be available.

Labs 4 and 5 may be omitted to reduce the total experiment time. This eliminates the part of the exercise in which students test the inhibition of bacterial growth for resistant colonies that were grown on a particular spice extract; hence, this is the component that is most pertinent to evolutionary biology. But for instructors having fewer lab periods that may be devoted to the exercise, components 1, 2, and 3 (which can be accomplished in two lab periods) will predictably demonstrate inhibition of bacteria by certain spice extracts. As noted in the Lab 5 instructions, the last experiment does not represent the best controlled method to directly compare zones of inhibition resulting from the growth of resistant colonies on the previously tested spice extract; we caution against having students compare with the initial results of Lab 3 (zones of inhibition resulting from the first test of spice extract with the test bacteria) since multiple factors could vary (e.g. the density/concentration of the bacterial cultures used for inoculating plates, environmental variables that may have been different during the first test and the second, and the age of the extract). All of these could influence the outcome as measured in changes in the zone of inhibition. Rather than including a statistical comparison for first test vs. second test zones of inhibition in this section, we have instead encouraged students (and instructors) to consider the importance of experimental design and to think about how other variables might have affected the outcome of this comparison? In addition, instructors may challenge students to develop an appropriate experimental design that would eliminate or minimize the effects of those variables.

B. The Group Research Proposal.

We have students complete the research proposal a week or two prior to the start of the Lab 1 component. The proposal will have been reviewed by the instructor, and the students will have received feedback from the instructor before beginning Lab 1. Having students complete the Group Research Proposal prior to beginning the exercise helps ensure that students are prepared. It also creates a certain level of "project ownership" and allows students to select spices to test that may be of personal interest. Students are encouraged to look up background information on the spices they choose, and may discover ethnic and cultural connections of interest. They may also find that the effectiveness of a spice as an antimicrobial agent may vary significantly depending on what part of the plant is used (roots, stems, leaves, seeds, fruits), how it is prepared (dried or wet, ground, heated, etc.), or even the country of origin (check packaging labels). These differences lend themselves to further experimental questions that students can easily test. For example, some commercial preparations contain expiration dates, and students may be interested in determining whether those expiration dates are related to

antimicrobial effectiveness, or not. The solvent used for extracting spice compounds is of utmost importance and can be used as an independent variable as well. For example, we have found that spices such as rosemary, garlic, cinnamon and cumin result in very different effects on bacterial growth when extracted in distilled water as compared with extraction in methanol. In some cases water as a solvent renders a spice extract more "potent", and in other cases, it is the methanol extract that appears to be more "potent". The effects of other solvents could also be investigated.

C. Comparing Spices with Commercial Antibiotic Disks – an Optional Extension

One of us has routinely included a comparison of the effectiveness of spice extracts in inhibiting bacterial growth with that of commercial preparations of common antibiotics available as pre-treated disks (Appendix B). Students find it very interesting to discover that certain spice extracts may appear nearly as effective as commercial antibiotics, and the patterns of growth around zones of inhibition for spices and antibiotic disks will demonstrate intriguing variability. Students will also discover that some antibiotics are more effective in limiting *E. coli* and others are more effective in limiting *S. epidermidis*, just as they may discover that different spices appear to have different levels of effectiveness against one bacteria or the other. For an instructor who chooses to include commercial antibiotic disks in potential comparisons; however, it is very important to consider that the *concentrations of the commercially treated disks are unlikely to be similar to the concentrations of crude spice extract* that students prepare in the lab, and so instructors should be prepared to augment instructions such that concentrations can be rendered similar for direct comparisons, and/or caution students in how they interpret results before they jump to a conclusion such as: "Garlic is more effective at limiting the growth of *E. coli* than erythromycin is!"

D. Analysis of the Results and Evaluation of Student Learning.

We ask our students to analyze results using independent samples t-tests, using the software SPSS 22 (*Appendix C: Sample Results*, Figs. 5 & 6). Since not all comparisons under all scenarios of student-generated hypotheses would be suitable to the comparison of means zones of inhibition for two treatments only, instructors who have some student teams proposing comparisons among more than two treatments (e.g. rosemary extracted in water, cumin extracted in water, and control disks) will want to be prepared to coach students in using ANOVA. For even more complex comparisons investigating possible synergistic effects among two sets of independent variables (e.g. both solvent and spice type are independent variables), a two-way ANOVA would be suitable. The bottom line is that instructors will want to be prepared to provide feedback on the group research proposals in such a way as to limit the scope to simple comparisons or to be equipped to coach statistical analyses appropriate to the comparisons constructed by student-generated hypotheses. We accomplish this according to the level of the course in which we are using this lab. For example, in a course for non-science majors with no science or math pre-requisites, we will coach students (via feedback on the group research proposals) to limit the scope of their comparisons to one or a few independent variables, and because this will be the first time most have been introduced to statistical analysis at all, we cover only the independent samples t-test. We may coach students with more than one independent variable to break down each of their "sub-questions" (extract used vs. spice type) into a one-variable comparison that can be treated as an independent samples t-test, repeated independently for each variable. Although this is not statistically ideal, it allows beginning students to become proficient with a basic statistical test and learn some foundations of statistical hypothesis testing. It would also be appropriate to use paired samples t-tests if replicates are constructed in a paired manner (e.g. by culture plate). Instructors can provide feedback in the experimental design necessary for a paired samples t-test during approval of the Group Research Proposal. In a second-semester course for majors, in which students have already covered independent samples t-test previously, they are "ready" to be introduced to ANOVA, and so proposals are approved which include broader comparisons among more treatment groups. To obtain multiple replicates for the t-tests, we encourage students, by way of feedback on their Group Research Proposals, to include multiple plates that each have replicates of each treatment to be tested (Figure 4, Appendix A).

Upon completion of this laboratory module, students in our courses are required to complete a lab report in the format of a formal scientific research paper. We refer the reader to just a few of the many excellent resources on teaching the art of scientific writing that have been published in ABLE (Bohrer, 2004, Ferzli and Carter 2005, Howard and Gubanich, 2002).

Other methods of evaluating and assessing student learning appropriate to this exercise would include: evaluation of the lab notebook, on-going skills-based assessments (e.g. evaluating the quality of preparing a culture plate, evaluating accurate measurement of the zone of inhibition, evaluating correct apparatus set-up for the filtration procedure, etc.). Some of the authors have also included a "debate" format as a culminating evaluation and learning experience related to this lab. The resolution for this debate is: Should the U.S. government mandate the CDCs childhood vaccination schedule http://www.cdc.gov/vaccines/schedules/), compelling parents to have their children vaccinated? (Pro – Yes,

childhood vaccinations should be mandated by law. Con – No, childhood vaccinations should not be mandated by law). Although this topic has little (if anything) to do with antibiotic properties of spices, it relates vividly to the understanding of the evolution of antibiotic resistance in microbial populations and the consequences that this has for effective health care. Students groups become debate teams and must prepare, using *only primary scientific literature*, for arguing on both the Pro and Con side of the debate. About 10 minutes before the actual debate, each group "draws" for the side on which they will argue. Debate teams follow a prescribed format with defined preparation time, statement times, and rebuttal times. The format of this debate is deliberately designed to place the emphasis on student presentation, and the instructor evaluation of it, on student use of primary scientific literature to provide evidence for or against the resolution. One of the authors has used a different debate format Called Fishbowl: https://en.wikipedia.org/wiki/Fishbowl_(conversation), using the same debate topic.

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Acknowledgments

The original version of this lab had been done for many years in the GEMS 158 course (Biotechnology and You) taught by Maria Burnatowska-Hledin at Hope College. The authors received a Course-based Research Experience Development Award from the Hope College HHMI program directed by Dr. Cathy Mader and Dr. Karen Nordell Pearson to enhance research-related aspects of the lab. Sasha Balcazar worked on modifications and numerous test runs during 2014 and tested a modified version at Holland High School in 2014 while student teaching under the supervision of Ms. Eva Eveleth, who provided additional valuable suggestions. We are also indebted to the workshop participants at the 2015 annual meeting of ABLE at Boston University who provided several suggestions for improvements, and to the helpful reviewers of this ABLE manuscript.

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Citing This Article

Balcazar, S., Burnatowska-Hledin, M., Winnett-Murray, K., and L. Hertel. 2016. Antomicrobial Properties of Spices. Article 4 in *Tested Studies for Laboratory Teaching,* Volume 37 (K. McMahon, Editor). Proceedings of the 37th Conference of the Association for Biology Laboratory Education (ABLE). http://www.ableweb.org/volumes/vol-37/?art=4

Appendix A
Group Research Proposal – The Spice of Life Lab

What is a Research Proposal?

A research proposal is a request for support of sponsored research, instruction, or the extension of a project. A well-written research proposal should answer the following questions:

- What do you want to do? How much will it cost? How much time will it take?
- What difference will the project make to: your university, the discipline, and or concerned parties?
- What has already been done in the area of your project?
- How do you plan to do it?
- How will the results be evaluated?

To compose an actual research proposal to a scientific agency, the proposal must be typed and carefully reviewed by colleagues prior to submission, but for this mock proposal, please complete the instructions below to complete your group's research proposal. Please note that your research proposal must be approved by your instructor before proceeding to experiment.

Group Member Names:

_____
_____
_____
_____

1. **Question**. State the scientific question and then describe why it is worth investigating this question.

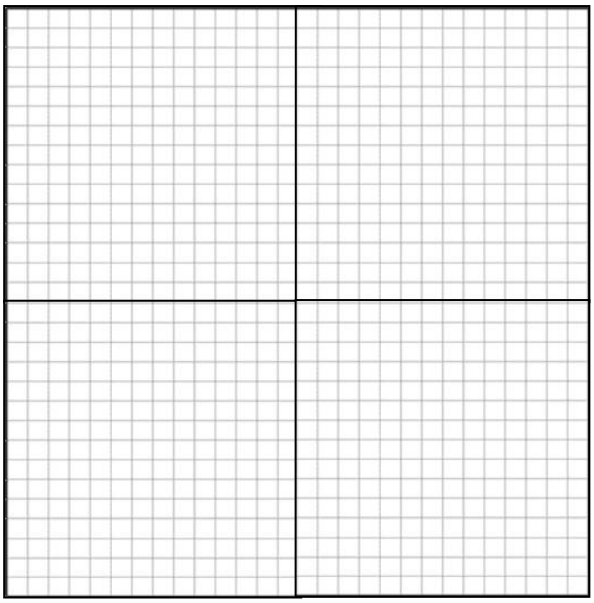
_____
_____
_____

2. **Working Hypothesis.** State your working hypothesis as a testable statement. Include the independent variable(s) and the dependent variable(s) in the statement, e.g. the independent variables might be cinnamon concentration at two treatment levels (5 g/L and 500 g/L) and the dependent variable might be the width of the zone of inhibition for bacterial growth. For this lab, select only two treatment levels for your independent variable. A wide range of choices are available. You can select two types of spices or two types of antibiotics to compare. Or you can compare the effectiveness of a spice against a known antibiotic. Alternatively, your treatments could be two different ways of preparing a spice (water extracted vs. methanol extracted) or two different preparations of a spice (store-bought ground cumin vs. freshly ground cumin from seeds). You may even compare the effectiveness of a single spice or antibiotic on the growth of two different bacterial species. Based on your question, what will constitute the two treatments for your independent variable?

_____
_____

3. **Sketch the Primary Prediction.** This is derived from your working hypothesis as a graph in the space below. Label the independent variable on the x-axis and the dependent variable on the y axis. (For example, if your working hypothesis states that "*Cinnamon inhibits bacterial growth; when the concentration of cinnamon is greater, there will be greater inhibition of bacterial growth."* you would label your test concentrations of cinnamon on the x axis and your measure of inhibition along the y axis. What relationship would be indicated on this sample graph?)

_____
_____
_____
_____
_____
_____
_____
_____



4. **Background and Rationale for your Working Hypothesis**. Write one detailed and well-researched paragraph describing why you proposed the working hypothesis that you did. What is currently known about the antibiotic (or other biochemical) properties of the spice you are testing? This may include the chemical compounds present, and known functions or biological effects of similar compounds; it may include other scientific studies that investigated inhibition of microbes by this spice, or other studies that suggest a link based on cultural traditions, properties of similar compounds, or ecological attributes of the plant in the wild (e.g. perhaps this plant is known to produce secondary compounds to inhibit herbivores). In your paragraph, provide the scientific name of the plant from which the spice is derived, the native geographic distribution of this plant, and a few examples of its culinary and/or medicinal use in human culture(s). Cite all of your sources according to the example at the bottom of this handout.

Citations:

5. Prediction. A prediction is a re-wording of your hypothesis as an if-then statement. Use the graph you sketched in item #2 above to write your prediction for this experiment as an if-then statement. Include the names of the independent and dependent variables, as you did on the graph.

6. Test of the Working Hypothesis = Protocol for your Experimental Design.

a. Which bacterial species will be used to test your spice(s) or antibiotic(s)?

b. Explain what your spice (or antibiotic) treatments will be (precisely, giving concentrations, from what part of the plant you will make extracts, and what type of extract is used (water vs. methanol)).

c. Describe how you will control for other, non-test variables that could influence bacterial growth as well (e.g. incubation temperature). What is the control treatment?

d. You will be required to replicate your experiment. This means that for each treatment level you have specified, including the control treatment, you will have redundant samples set up in precisely the same way. For this lab, you should try to include 3-9 replicates of each treatment level. Your instructor will advise you according to materials available. Why is it important to replicate each treatment level (essentially this is running the same experiment multiple times, simultaneously).

e. You will be using petri dish plates to grow bacteria. Each petri dish can be divided into thirds (or quarters) to allow running more than one replicated test at a time. One section is always reserved for a control (in our case this will be a plain, sterile paper disc that has no antibiotic or spice on it) and the other sections can be used for discs that have been soaked in your spice extracts (or have been prepared commercially with known antibiotics). On the following page, sketch the number of petri dishes you anticipate using, divide them into sections, and indicate which treatment will be applied to each section. Include one control treatment in one section on each plate.

Group Research Proposal Design

Sketch the number of bacterial growth plates (petri dishes) you will use and show how they will be divided for each treatment and the controls (Fig. 4).

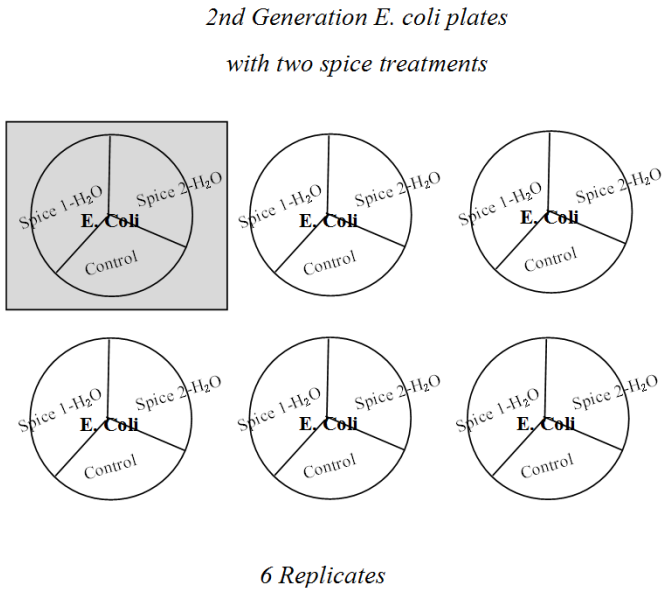


Figure 4. Sample sketch of replicated culture plates for an experiment testing the effect of spice 1 vs. spice 2 on the bacterial test species *E. coli*.

7. Evaluation of Results. Explain how you will evaluate your data (results) and how you will know if the spice extract affected bacterial growth? How has your experimental design excluded other factors that might affect bacterial growth? If you will test the significance of your results with an inferential statistical test, indicate what test will be used and how it reflects the type of question you began with.

8. Materials. Write a careful list of what you will need on each day you will be carrying out your experiment. You need to specify estimated amounts, concentrations, and identification of all materials you will need for your investigation.

Example of inadequate description: “beakers”. Example of adequate description: “twelve 50 mL beakers”. Example of inadequate description: “some cinnamon”. Adequate description: “4 sticks of commercially prepared cinnamon from a retail store”.

9. Sample Citation.

a. In-text Citation: When referring to the work of others in your scientific writing, state their results or finding and then at the end of that sentence provide the author’s last name(s) and the date of publication. For example: *Growth of several species of Bacillus bacteria is inhibited by essential oils from cinnamon, cumin, and coriander (Ozcan et al. 2006)*. Note that the “place-holder” *et al.* is used in the in-text citation only when there are more than two authors; this is done simply for brevity. If there are one or two authors, the last names of both are included, e.g. (*Ozcan and Sousa 2006)*.

b. End-of-text Literature Cited section. At the end of each proposal or report, you should include a Literature Cited section with the full citation for each article or source that you have cited. List references in your literature cited section alphabetically, by the first author’s last name. An example of proper citation format for this section is:

Ozcan, N.M., O. Sagdic, and G. Ozcan. 2006. Inhibitory effects of spice essential oils on the growth of *Bacillus* species. *Journal of Medicinal Food.* 9: 418-421.

Note that no urls are included, even if you referenced the article “on-line”, and that *et al*. is not used in the *full* citation. All of this information must be included to render your citation “archivable”, so that other scientists and readers can always find the source of information that you cite.

10. Instructor Approval._____

Appendix B
Sources of Materials and Supplies

Appendix B						
Ordering Information and Supplies Used						
	Item	**Manufacturer's**	**Size**	**Quantity**	**VWR Cat #**	**Fisher CAT #**
Autoclave Supplies		#				
	Autoclave bags orange		25" x 35"	pk of 200		01-814C
	Autoclave gloves, long			1		11-394-298
	Autoclave tape		1/2 " x 500"	1	36432-188	11-889-13
Chemicals and Reagents						
	Ethanol, 70%		4L	1	34172-000	23 794203
	Mueller-Hinton medium		500g	1		DF0252-17-6
	Nutrient agar		2kg	1		DF0001-07-2
	LB broth(Luria broth)					DF 0402-17-0
	LB agar(Luria agar)					DF 0401-17
	The next 4 items are to make your own LB media					
	NaCl		2.5kg	1		BP 358 212
	Yeast-Extract					DF 0127-17-9
	Bacto Agar					DF 0140-01-0
	Bacto-Tryptone					DF 0123-17-3
	ddH2O					
	Methanol					
	Amoxicillin with clavulanic disks			1		B31629
	Chloramphenicol disks		30mcg	1		B31274
	Ciprofloxacin disks		5mcg	1		B31658
	Erythromycin disks		15mcg	1		B31290
	Penicillin disks			1		B31321
	Streptomycin disks		10mcg	1		B31328
	Sulfisoxazole disks		0.25mg	1		B31296
	Tetracycline disks		30mcg	1		B30998
Spice Extract and Disk Prepatation						
	Balance	O Haus explorer	410 x 0.001gm	1	11379-178	01-915-28 (14557424)
	Weigh boats		medium			02 202 101
	Blank sterile disks			6		B31039
	Filter paper to fit your funnels					
	Funnel, glass, 75 mm dia.					
	Mortar and pestle					
	Rubber tubing for Vaccum Filtration System					
	Vial with closure, 2 dram		17 x 60mm	24 pk of 144	66011-085	03-339-22D
Spices						
Bacteria Prepartion						
	Stock Culture of *S. epidermidis* and *E. coli*					
	Petri Dishes, Stackable	NA	100 x 15mm	1cs/500	WLS26026-	FB 08-757-12
	Parafilm, American National Can Co.	PM999	4" x 250'	4	52858-032	13-374-12
	Pipet Pump, green	F37898	10 mL	10	53502-233	13-683C
	Disposable pipets,sterile polystyrene, indiv		5ml	1cs/500	29442-422	13-678-11D (NC0479383)
	Pipet Tips, Blue Racked	NA	200-1000 uL	1pk	83007-376	02-707-508 (NC0479420)
	100-1000 µl adjustable volume pipettor			6		
	Blue 1.5ml mc tubes		1.5 ml	1pk	20170-026	02-681-282
	Inoculating loop					22-363-602
	Sterile 15 ml test tubes					
	Sterile toothpicks					
	Sterile bent plastic rods (spreaders)			1pk/500		14-665-230
General Supplies						
	Bench paper			1	52857-120	14-206-30
	Eyepiece Micrometer, Leica	1406A	10mm x .01mm	6	41723-042	NC9345851
	White labeling tape		3/4" x 60yd	1	89098-024	15-901-20A
	Fine Point Sharpie					
	Safeskin Nitrile powderfree gloves	HC250N	Small (7)	1	82026-424	19-149-863A
	Safeskin Nitrile powderfree gloves	HC350N	Medium (8)	1	82026-426	19-149-863B
	Safeskin Nitrile powderfree gloves	HC450N	Large (9)	1	82026-428	19-149-863C
	Forceps, Extra Fine		5 1/2"	2 cs of 4	25607-856	13-812-42
	10 ml graduated cylinder					
	2-10 ml Büchner flask					

NOTE: Many of these items do not need to be purchased to do the lab. Check with your colleagues in Microbiology and Chemistry to see what can be borrowed.

Appendix C
Sample Results

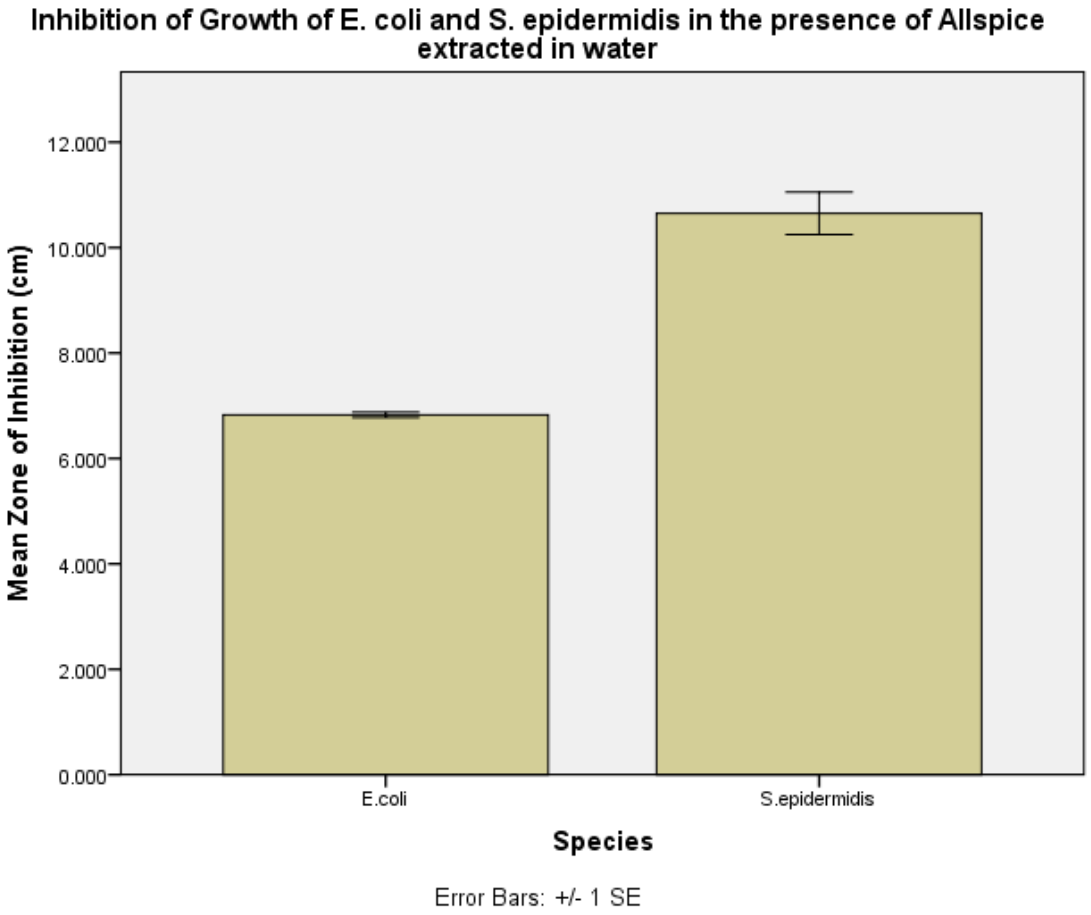


Figure 5. The mean zone of inhibition of *S. epidermidis* grown in the presence of allspice extracted in water is significantly greater than the mean zone of inhibition for *E. coli* grown in the presence of the same extract ($t = 9.427$, $df = 10$, $P < .001$). (Sample student-generated data by Sasha Balcazar, May 2014.)

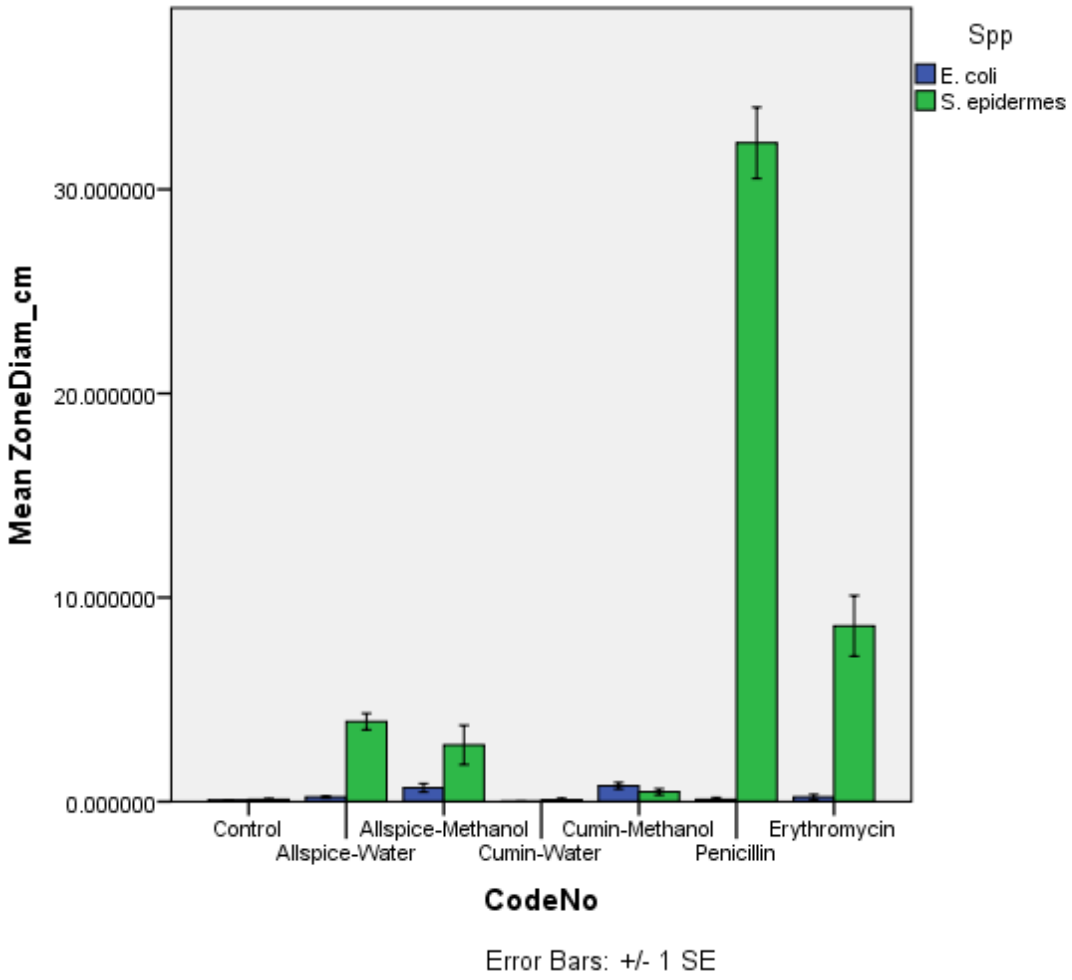


Figure 6. Student-generated figure comparing the effectiveness of commercially prepared antibiotic disks (penicillin, erythromycin) and various spices extracted in water (e.g. Allspice-Water) or in methanol (e.g. allspice-methanol),on the inhibition of growth in cultures of *E. coli* and *S. epidermidis.* No statistical comparisons were carried out, because concentrations of antibiotic disks and various spices were not controlled. However, the results demonstrate the great variety of responses investigators can expect in similar experiments. Zones of Inhibition are likely to vary with spice type (e.g. compare allspice in methanol with cumin in methanol), solvent used for extraction (e.g. compare allspice in water with allspice in methanol), and with bacterial test species (e.g. compare results of *E. coli* and *S. epidermidis* when both were tested on allspice-water). (Figure from Sasha Balcazar's lab notebook, May 2014).