Antibacterial Properties of Plants

Modified from: *STEM Transitions, a project of CORD, http://www.stemtransitions.org*

Objective: determine the susceptibility of *Staphylococcus aureus* to various plant extracts using the basic Kirby-Bauer disc diffusion method (the actual test has exact specifications which must be followed; these requirements are beyond the capability of work in this lab).

Prediction: If a bacterium is **susceptible** to an antibiotic, then a **zone of inhibition** of a certain diameter will form around an antibiotic disc placed on a nutrient agar plate. The size of the zone of inhibition is determined by the type of medium used, the solubility and rate of diffusion of the antibiotic, the amount of inoculum, as well as the effect of the antibiotic. If a bacterium is **resistant** to a particular antibiotic, then a smaller zone or no zone of inhibition will form around the antibiotic disc. If a bacterial colony is somewhat susceptible (intermediate susceptibility) to an antibiotic, then the zone of inhibition will measure in-between that of a susceptible and resistant bacterium.

Procedures: The lab is divided into three parts, starting with (1) extraction of the plant compounds, followed by (2) plating of the bacteria and the plant extracts on day 1, and finally (3) obtaining the results by measuring zones of inhibition on day 2 one week after the plating.

NOTE: The procedures listed on the following pages should be followed very carefully. In the event that you need to make any modifications to what is written, you **MUST RECORD** those changes. This way you will have an accurate record of exactly what you did.

(1) Plant extraction

Materials needed per extraction

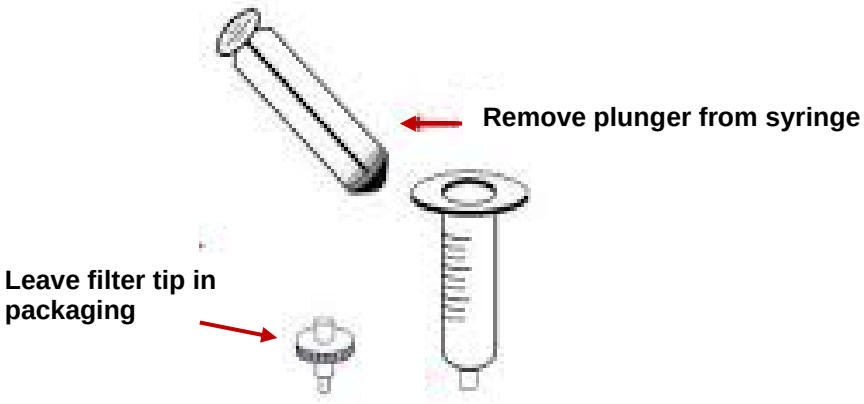
- Mortar and pestle (1)
- Graduated cylinder, 20ml (1)
- 50ml beakers (1)
- Permanent marker
- Plastic pipettes (2)
- 15ml centrifuge tube (2)
- Cheesecloth
- Knife / scissors
- Ethanol to disinfect
- Autoclaved dH_2O
- Weigh boats (1)
- Balance
- Centrifuge
- Small beaker (10ml) (1)

Methods

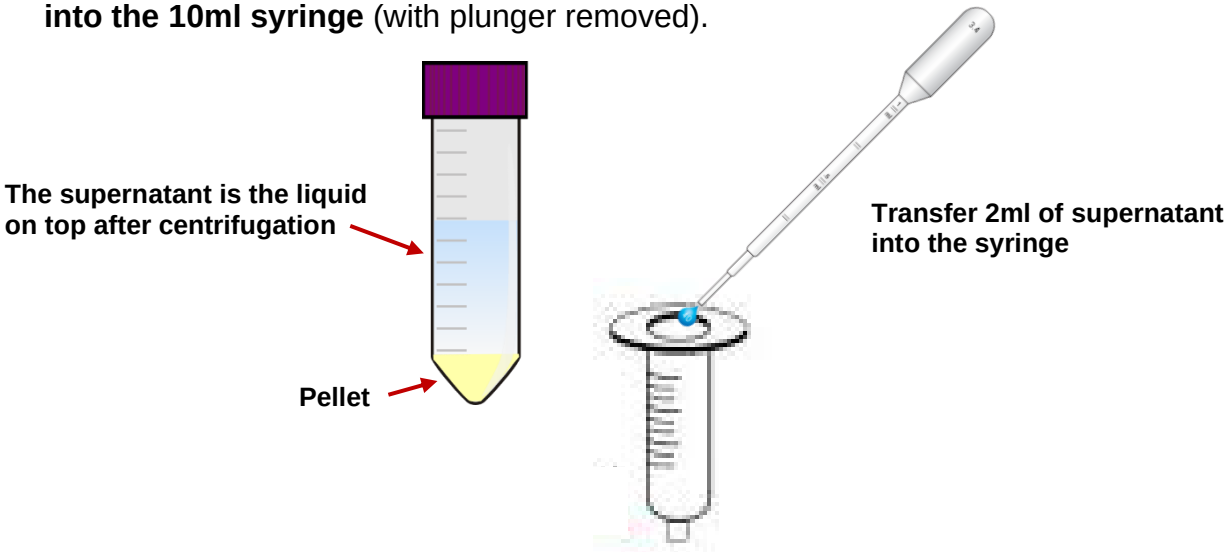
1. Weigh out **5g of the plant material** in a weight boat. Remember to tare (zero out) the balance after placing the weight boat on it. If you choose to use a different, remember to **record the starting weight**.
2. Cut the plant material into small pieces.
3. Place the cut pieces of plant material in the mortar and grind it with the pestle. If your plant material is dry, add **5 ml of autoclaved dH_2O.** Add 5ml more only if absolutely necessary. **Record how much water is added.**

4. Once the plant material has been ground up, **filter it through a cheesecloth** folded several times. Let it filter into a 50ml beaker.
5. With a permanent marker, label a 15ml centrifuge tube and a 10ml beaker with plant name.
6. Pour the filtrate into the labeled centrifuge tube. Use a second 15ml tube as a balance for the centrifuge by adding an **equal volume of H_2O** as there is filtrate in the other tube.
7. Add centrifuge tubes to the centrifuge. **Tubes must be balanced**. *Your instructor or lab technician will be in charge of running the centrifuge.* **Centrifuge for 10 min at 3000 rpm.**

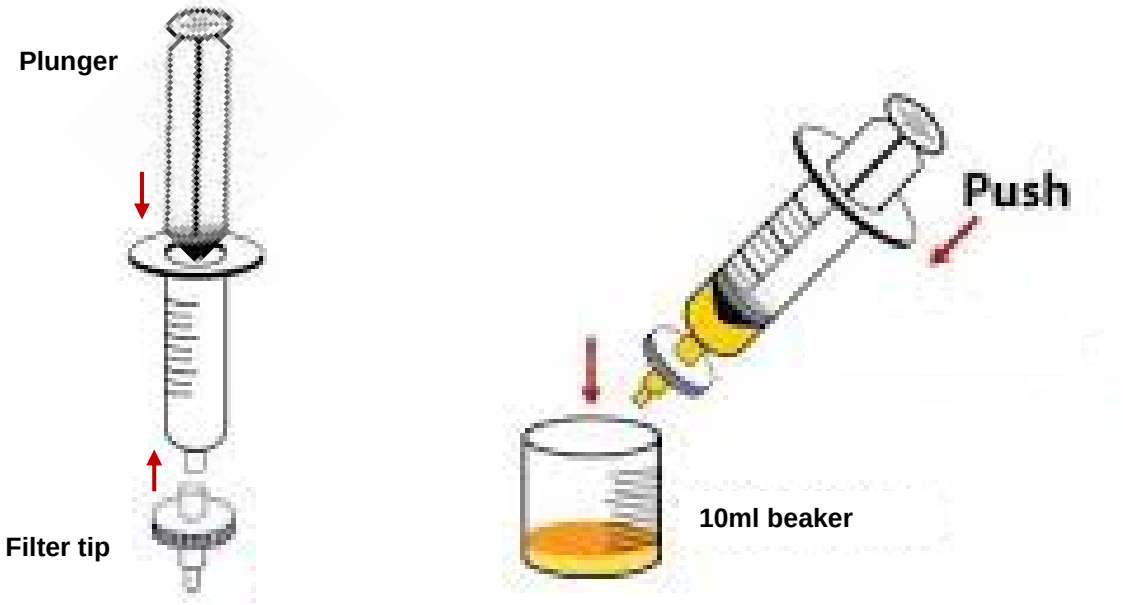
8. Collect the centrifuge tube with the extract. Be careful not to disturb the sediment pellet.
9. Remove the sterile 10ml syringe from packaging and remove the plunger. Leave the plunger and the filter tip in the packaging. **Do not let any items sit directly on the lab bench.**



10. Then using a plastic transfer pipet carefully **pipette approximately 2 ml of the supernatant into the 10ml syringe** (with plunger removed).



11. Attach the filter tip to the syringe and insert the plunger. Avoid touching the filter tip. **Filter the supernatant through the filter tip to sterilize it into the labeled 10 ml beaker.** This is your final extract.

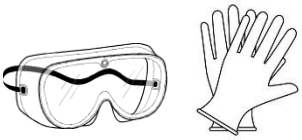


(2) Plating

Materials needed per group

- Mueller-Hinton nutrient agar plates (2)
- *Staphylococcus aureus* broth culture
- Penicillin disc (instructor demo)
- Blank discs (6)
- Plant extracts in 10ml beakers (4)
- Permanent marker
- Two sterile pipettes
- L-shaped glass rod
- Ethanol in a 250 ml beaker
- Forceps
- Bunsen burner
- Parafilm

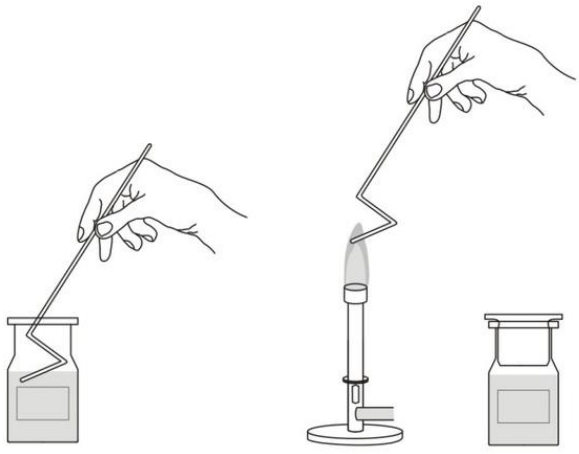
Methods



1. Clean your work area and disinfect with table disinfectant. Wear gloves and safety googles.
2. Sterilize the forceps by dipping in ethanol followed by passing the tip through the flame of the Bunsen burner. Then using the sterilized forceps, place a filter disc in each of the 10ml beakers with plant extracts. There should be enough extract to cover the discs.
3. With a permanent marker, label the bottom of two nutrient agar plates with your name, date, and name of bacterium (*S. aureus*). Draw lines on the bottom of the plate to divide it into **three equal sectors**. Label two sectors with the name of two of the plants and label the third sector negative control. On the second plate, label two sectors with the name of the two other plants and label the third sector negative control.
4. Your instructor will demonstrate how to inoculate a nutrient agar plate with *S. aureus* and add a **penicillin disc in the middle of the plate**. This will serve as a **positive control** for the entire class. Alternatively, a video may be shown and a pre-plated penicillin disc will be available as a positive control.

   While performing the following steps, follow these general aseptic technique guidelines:

   - *Start the operations only when all equipment and materials are within immediate reach.*
   - *Complete all operations as quickly as possible, but without hurry (read ahead so you know what to do).*
   - *Do not talk as this can spread bacteria.*
   - *Work close to the flame where air currents are drawn upwards.*
5. Dip the L-shaped glass rod in the ethanol. Make sure the entire shorter end is fully submerged. Then pass it through the flame to sterilize it. Let the glass rod cool before proceeding with step 7. Do not set it down on the lab bench - one lab partner can hold it while another proceeds with the next step.



6. Swirl the contents of the *S. aureus* broth culture gently (avoid moistening the cap) until it is equally murky throughout. Using aseptic technique, follow the steps A) through E) (see diagram below). Holding the ***S. aureus* culture tube** with your **left hand** (if you are right handed):

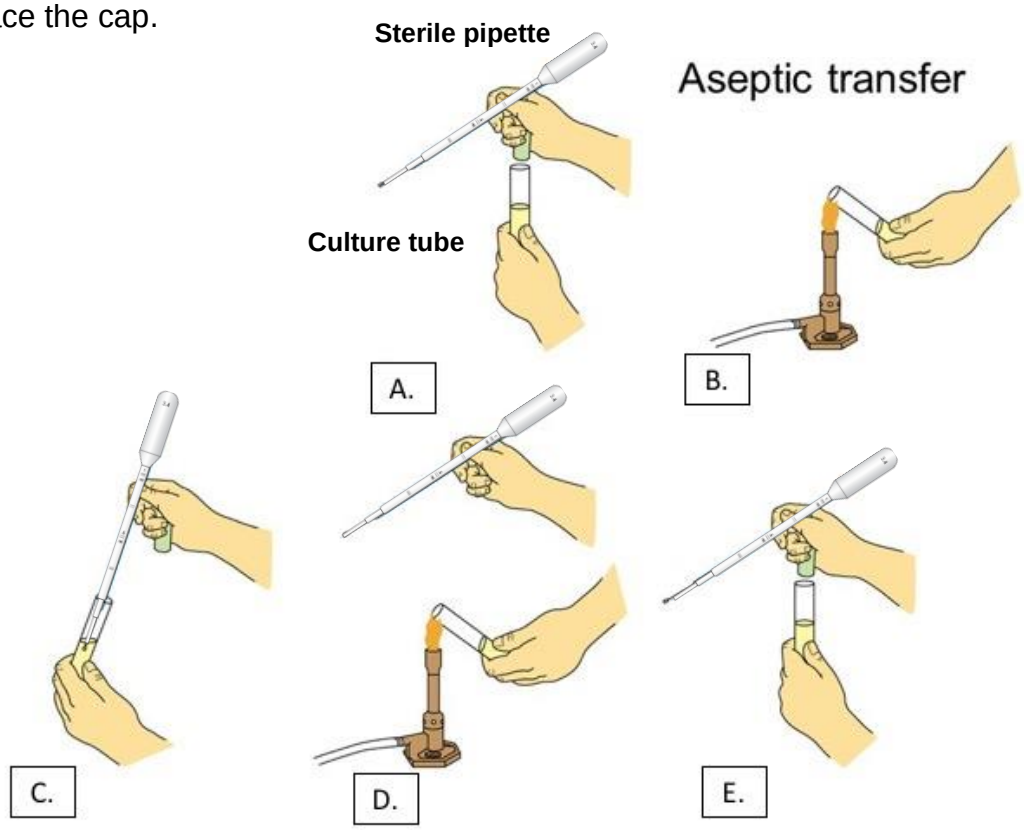
   A. **Remove** a **sterile pipette** from the packaging by opening **at the bulb end** – not the tip end. **Hold** the sterile pipette **with your right hand**. **Remove** the **plastic cap** from the culture tube **by curling the little finger of your right hand** around it and **pulling it out**. Do not put it down because that could introduce unknown bacteria from the environment into the culture tube or vice versa.

   B. **Pass** the **mouth** of the open culture **tube through the flame** to sterilize it.

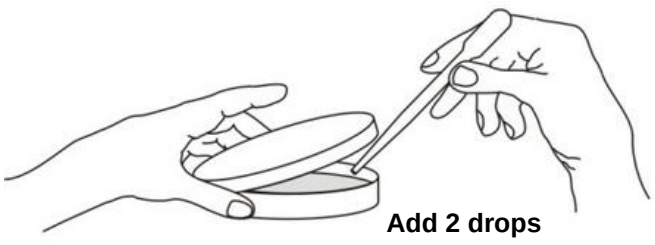
   C. Use the **pipette** to **withdraw** a small amount of the **broth** from the culture tube.

   D. **Pass** the **mouth** of the culture **tube through the flame again**.

   E. Replace the cap.

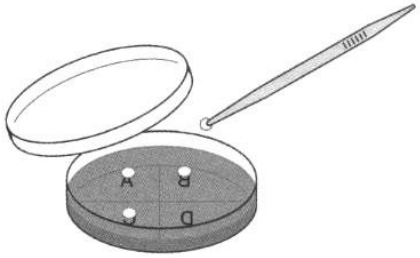


7. Partially remove the lid of the agar plate - do not set it down on the lab bench but hold it over the agar plate to minimize risk of contamination. **Add two drops** of the broth culture with the pipette to the **surface of the agar**. Dispose of the pipette in the **biohazard bag**.



8. Place the nutrient agar plate on the **turn table (lazy Susan).** Partially remove the lid and **spread the bacteria on the entire surface of the agar** with the **cooled and flamed L-shaped glass rod** by holding it to the surface of the agar while spinning the turn table. ***Important*: the glass rod must be cooled to avoid killing the bacteria.** Move the rod back and forth to ensure the bacteria are spread to the edges of the plate. After inoculating the plate, return the glass rod to the beaker with ethanol.

9. Allow the surface to **dry for about 5 minutes** before placing discs on the agar.
10. With **sterile forceps** (dipped in alcohol and flamed three times), **apply the discs to a separate sector on each plate.** Place a **blank disc** on the sector labeled **negative control.** Once a disc touches the agar, *do not move it.* Sterilize the forceps between disc applications.
11. Press each disc gently with sterile forceps so that it makes full contact with the agar surface.



12. Using a **new sterile pipette and the second nutrient agar plate, repeat steps 5-11.**
13. Use **parafilm to seal the plates**. Invert only the plates with discs, not the plates with wells! Place the plates in the green bin in the front of the room. Plates will be incubated at 37^{o}C for 24 hours.
14. After 24 hours, the lab staff will remove the plates from the incubator and place them in the refrigerator until the next lab period to prevent overgrowth.

<u>Clean-up</u>

- Clean up all materials at your station.
- Scrub mortar and pestle thoroughly with sponge and soap. Rinse with ethanol when done.
- Any items that were in contact with bacteria should be disposed of in the biohazard bag.
- Wipe down lab bench with disinfectant spray.
- Follow additional clean-up instructions by your instructor.

Post Lab Questions:

1. After incubation, a clear area around each disc is called _____ and what does it represent?

2. Generally, the larger the zone of inhibition, the more _____ the bacterium is to that antibiotic.

3. What is the role of the Penicillin disc?

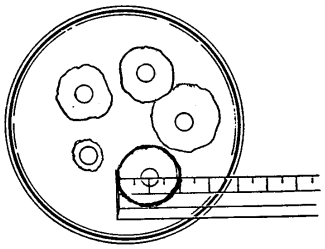
4. If there is no zone of inhibition around the Penicillin disc, what can you conclude? And if there is?

5. What is the role of the blank disc?

6. If there is a zone of inhibition around the blank disc, what can you conclude? And if there isn't?

7. Which plant extract do you predict will have the largest and smallest zones of inhibition? And why?

(3) Measuring zones of inhibition



Materials needed per group

- Metric ruler
- Plates from last week

Methods

1. Retrieve your plates, and look for the presence of antibiotic activity—***zones of inhibition*** surrounding a filter disc. On the bottom of the Petri dish (do not remove the lid), place a metric ruler across the zone of inhibition, at the widest diameter. Measure the diameter of the zone of inhibition in **millimeters** from one edge of the zone to the other edge. See diagram. Also do this for the positive control plate.
2. The disc/well diameter will be part of the zone of inhibition measurement. If there is no zone at all, report it as 0 even though the disc/well itself is about 7 mm in diameter.
3. Record inhibition zone diameters in millimeters in the table below and report the results as:
4. Take good **quality pictures** of your plates including the positive control plate.
5. Dispose of your plates in the biohazard bag.

Data Table

Test compound	Zone diameter (mm)	Plate
Penicillin (+ control)		Demo
Blank (- control)		1
		1
		1
Blank (- control)		2
		2
		2

Analysis Questions:

8. What is the clear area around each disc called and what does it represent?

9. Generally, the larger the zone of inhibition, the more _____ the bacterium is to that antibiotic.

10. Why is it necessary to include a positive and negative control?

11. How did the plant extracts compare to the activity of penicillin?

12. How did the plant extracts compare to each other?

13. How did the activity of your plant extracts compare to what has been published in the primary literature?

14. Do the plants tested have any traditional medicinal or other uses?

15. How might a bacterium become resistant to penicillin? Give two ways.

16. What would you do differently if you could do this experiment again? How might you expand or continue this experiment? What might work better?

These questions must all be addressed in your final presentation!