

Screening for Biodynamic Plants

Introduction:

Since plants are non-motile and cannot flee from danger, they have evolved alternative strategies to protect themselves from predators. Some plants arm themselves with weapons such as thorns, hairs, or a thick cuticle. Other plants produce chemicals that render them toxic or unpalatable. The same compounds that have served plants so well in their chemical warfare against herbivores also provide many of our most important medicines. Various estimates suggest that about 40% of our prescription drugs contain a component derived from a natural source and that one quarter of all medicines contain an active ingredient derived from a flowering plant.

Plants that produce useful medicines are being actively sought by botanists and **pharmacognosists** (a scientist who studies drugs obtained from plants). One particularly ripe source of information about potentially-useful plants is traditional folklore, which accounts for the discovery of about 75% of all plant-derived drugs.

Once a potential medicinal plant has been identified, it is screened to determine whether it has enough biological activity to warrant further study. This initial screening is often done with a bioassay. If the bioassay results are promising, more exhaustive chemical, biological, and pharmacological studies are performed.

A **bioassay** examines the effect of a test chemical on a living system. In a typical experiment, the response of an organism is observed after treatment with the test substance. Obviously the test organism must be carefully selected since this test is based on the assumption that humans will respond in a similar fashion. The advantage of this tests is that they are quick, inexpensive and do not rely on using vertebrates (avoiding many ethical issues

There are a variety of bioassays that have been used to screen organisms for biological active ingredients. For example, the response of onion root cells to test chemicals is a commonly used test for chemicals causing chromosomal damage. The Ames test uses bacteria to screen for potential cancer causing agents. In other still other assays, plant extracts with anti-bacterial and/or anti-fungal activity can be readily identified by applying the extract to a filter paper disks and then placing it on the surface of a bacterial (*e.g.*, *Escherichia coli*, abbreviated *E. coli*) or yeast (*e.g.*, *Saccharomyces cerevisiae*) culture. If there are inhibitory chemicals in the extract they will inhibit growth around the disk leaving a zone of inhibition.

In this lab we will use the brine shrimp assay. Popularly known as sea monkeys, brine shrimp are crustaceans that live in saline environments. Their eggs (actually cysts), which can be inexpensively purchased from pet stores, hatch quickly and the larvae (termed a nauplius or plural, nauplii) are sensitive to small doses of biologically-active chemicals. One indicator of the toxicity of a substance is **LD₅₀**, which refers to the amount (*i.e.*, lethal dose or concentration) of a substance that kills half of the test organisms. Activities are considered significant if the LD₅₀ is less than 30 µg/ml (=0.03 mg/ml). In this bioassay, the mortality of brine shrimp that are incubated in the test solution is recorded.

Protocol

I. Extract Preparation

A. Commercial Product (*i.e.*, teas, medicines)

1. Grind your product finely with a mortar and pestle (if necessary). Weigh accurately approximately 500 mg (=0.5 g) of product.
2. Transfer the ground product to a baby food jar.
3. Add 50 mL of artificial seawater (ASW) to the jar.
4. Incubate for one hour at room temperature, shaking gently every few minutes.
5. Pour the extract through several layers of cheesecloth into another, labeled container. Squeeze out excess liquid
6. Measure the final volume of extract.

| Table 1: Summary of Extract Preparation from a commercial source | |
|---|--|
| Amount of Plant product (mg) | |
| Final Extract volume (mL) | |
| Extract Concentration (mg mL ⁻¹) | |

B. Wild Plants

1. Collect about 10 - 20 grams of fresh plant material in a small paper bag. Keep the parts of the plants separate because they could have different activities. In addition, collect a sample to prepare a herbarium voucher specimen that will serve to document the identity of the plant that you are studying. Press, dry, identify, label, and mount this specimen according to the directions provided by your instructor. The voucher will be deposited in the CSB/SJU Bailey Herbarium for permanent reference.
2. Weigh the fresh leaf sample (see Table 2)
3. Dry the sample in an oven at 60 C until thoroughly dry. Calculate the water content of the leaves (Table 2).
4. Weigh, in a weigh boat, approximately 500 mg of dried plant material (record the exact weight in the table below) and then powder in a mortar with pestle.
5. Transfer the powdered sample to a baby food jar and add 50 mL of ASW (= Instant Ocean; 120.96 g/gallon) to the jar.
6. Incubate for one hour at room temperature, shaking gently every few minutes.
7. Pour the extract through several layers of cheesecloth into another, labeled container. Squeeze out excess liquid.
8. Measure the final volume of extract.

| Table 2: Summary of Extract Preparation from a plant source | |
|--|--|
| fresh weight of leaf sample (g) | |
| dry weight of leaf sample (g) | |
| % water in tissue $[(fw - dw)/fw * 100]$ | |
| weight tissue extracted (mg) | |

| | |
|--|--|
| final volume extract (ml) | |
| extract concentration (mg ml ⁻¹) | |

II. Dilution Series

In order to determine the biological activity of the extract we will need to see how the brine shrimp respond to different concentrations of the extract. Thus, we need to prepare a dilution series.

1. Obtain 6 test tubes and label them 1-6 (note – always prepare your labels on a piece of tape).
2. Using a disposable pipet, put about 10 mL (don't measure) of the plant extract into tube #1.
3. Accurately pipet 9 mL of ASW into each of the other tubes (2-6).
4. Pipet 1 mL of the extract from tube #1 into tube #2 and mix well. This represents a 1:10 dilution of the extract. Or in other words, this solution now is 10% as concentrated as the original extract.
5. Remove 1 mL from tube #2 and place it in tube #3 (a 1:100 dilution; 1%) and mix.
6. Remove 1 mL from tube #3 and put it in tube #4 (a 1:1000 dilution; 0.1%) and mix.
7. Remove 1 mL from tube #4 and put it in tube #5 (a 1:10,000 dilution; 0.01%) and mix.
8. Finally put 1 mL from tube #5 into tube #6 (a 1:100,000 dilution; 0.001%) and mix.
9. Complete Table 3 by calculating the concentration of the extract (mg ml⁻¹) in each tube

| Tube | ASW to add (mL) | Amount (& source) of extract to add | Dilution (relative to original extract) | Concentration (% of original) | Concentration (mg ml ⁻¹ ; from table 1 or 2) | Concentration (µg ml ⁻¹) |
|------|-----------------|-------------------------------------|---|-------------------------------|---|--------------------------------------|
| 1 | 0 | 10 ml of original extract | 1 | 100 | | |
| 2 | 9 | 1 ml from tube 1 | 1:10 | 10 | | |
| 3 | 9 | 1 ml from tube 2 | 1:100 | 1 | | |
| 4 | 9 | 1 ml from tube 3 | 1:1000 | 0.1 | | |
| 5 | 9 | 1 ml from tube 4 | 1:10000 | 0.01 | | |
| 6 | 9 | 1 ml from tube 4 | 1:100000 | 0.001 | | |

III . Brine Shrimp Bioassay

A. Rearing Brine Shrimp

1. Obtain a brine shrimp hatchery made from a plastic soap dish.
2. Fill the hatchery with artificial sea water (ASW)
3. Sprinkle brine shrimp eggs on the surface of the seawater in the larger compartment
4. Place the hatchery in the light. The brine shrimp should hatch within 24 - 48 h.
5. You can feed them a commercial food or a yeast extract (3 mg yeast in 5 ml seawater). For short periods, food is unnecessary.

B. Bioassay

1. Obtain a clean and dry exposure chamber with six cells.
2. Label (on tape) the cells 1-6 and include your name and date.
3. Pour the extract dilutions into the appropriate cell in the exposure chamber.
4. With a wide-mouth pipet, gently transfer 10 brine shrimp into each cell. Add as little of the original culture water as possible. Record the actual number of brine shrimp in each cell in Table 5.
5. Record your initial data in Tables 4 & 5
6. Count the number of nauplii that are alive (# surviving) in each cell 24 hours later. Dead shrimp have their front appendages extended and are motionless. Record these data in Table 5.

| Table 4: Experiment Start/End | |
|--------------------------------|--|
| date experiment started | |
| time experiment started | |
| date results collected | |
| time results collected | |
| duration of experiment (hours) | |

| Table 5. Summary of brine shrimp data | | | | | | | |
|---------------------------------------|--|---------------------------|-----------------|----------------------------------|-------------------|--------|--------------------------------|
| Tube | Conc. of extract ($\mu\text{g ml}^{-1}$) | Log conc. (% of original) | Initial nauplii | Nauplii surviving after 24 hours | Deaths after 24 h | % died | % died (corrected for control) |
| 1 | | | | | | | |
| 2 | | | | | | | |
| 3 | | | | | | | |
| 4 | | | | | | | |
| 5 | | | | | | | |
| 6 | 0 (control) | | | | | | |

Data Analysis:

1. Convert the extract concentration to the log (to make the wide range of values easier to deal with)
2. Calculate the number of nauplii that died during the test period (initial - final).
3. Calculate the % died ($\# \text{ deaths}/\text{initial} \times 100$).
4. Correct the % died for any control deaths ($= \% \text{ death test} - \% \text{ death control}$).
5. Plot (if using Excel, plot XY scatter, don't plot the line, and just plot the points): % died corrected vs. log extract conc ($\mu\text{g}/\text{mL}$).
6. Determine the LD_{50} from the graph by:
 - drawing the best fit line (in Excel, right click on one of your data points, choose add trendline/linear. Under options, check box for 'display equation on chart')
 - Use the equation for the line to calculate "X" (LD_{50}) after substituting 50 for Y.
 - When you get your answer, you need to take the antilog or in Excel use the power function. To do so, put the cursor in an empty cell, click on Insert/function/power. Then, plug in 10, followed by a comma, and the answer from the above step.

Post-Lab#1

1. Prepare an herbarium label for your plant specimen. Put the label in the newsprint with your specimen.
2. Prepare a typed report about the plant that includes, at a minimum, the following information. Some potential references are provided below.
 - common name(s)
 - scientific name
 - family name
 - brief description of plant (*append an image of plant*)
 - indication of where plant grows
 - to where is the plant native?
 - which parts of plant, if any, have been used for medicine?
 - how has the plant been prepared for medicine?
 - who, if anyone, has used this plant and for what treatments?
 - chemistry of plant; identify any known chemicals/active ingredients in plant
 - other miscellaneous

Post-Lab #2

1. Complete, on separate sheets of paper, tables 1, 2, 3, 4, and 5.
2. Plot % died (corrected) vs. log extract conc ($\mu\text{g mL}^{-1}$).
3. Determine the LD_{50} from the graph. (*show your work*)
4. Prepare a written lab report documenting this experiment.

I. References

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Internet Resources

- [James Duke - Medical Botany Course Syllabus](#)
- [Society for Economic Botany](#)
- [James Duke - Phytochemical and Ethnobotanical Databases](#)
- [Virtual Library of Botany](#)
- [Internet Directory for Botany](#)

VII. Partial Supply List

A. Student Work Table (basket with following items; assume 2 pairs of students per table):

- flask with artificial sea water (100 mL) and pasteur pipet labeled ASW
- flask with dH₂O (100 mL)
- marker
- tape
- 1 mL pipet (3; label one water) and a pipet blue pump
- 10 mL pipet labeled water and green pump
- exposure cells (2 or 1/pair)
- transfer pipet with wide mouth (cut off tip) (2 or 1/pair)
- test tube rack (2 or 1/pair)
- dissecting scope/light

B. At center table

- pipet for extract (transfer pipet fine)
- petri plates with brine shrimp (remove shrimp from incubator and put into dishes)
- box of test tubes large enough to hold at least 15 mL