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Plant essential oils as natural insecticides against *Callosobruchus maculatus*: Looking at the biochemistry of a greener alternative

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Abstract

Here we present a multi-session, investigative lab sequence that examines the effectiveness and biochemistry of plant essential oils as "natural" insecticides against the bean beetle *Callosobruchus maculatus*. Due to concerns about the effects of chemical insecticides on human health and non-target organisms as well as increasing levels of insecticide resistance observed in the field, there is interest in using "natural" insecticides to replace chemical insecticides. There is evidence that several plant essential oils are toxic to different insect pests and that their toxicity may be due to inhibition of various enzymes. Bean beetles are pests of legume seeds, and are easy to culture and use in a teaching laboratory. Students can use fumigant toxicity assays to determine effectiveness of commercially available essential oils from closely or distantly related plant species as natural insecticides against bean beetles. The students can study potential unintended consequences of using such "natural" insecticides by studying their effects on enzymes extracted from other organisms such as fish and mammals. Students can also study the effect of plant essential oils on several enzymes extracted from bean beetles as potential targets of the "natural" insecticide. This lab sequence relates enzyme activity to evolutionary processes in plant-animal interactions, ethnobotany, and agroecology.

Keywords: Plant essential oils, insecticides, acetylcholinesterase, butyrylcholinesterase, bean beetle, non-target organisms

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INTRODUCTION

Organophosphate insecticides are one of the largest applied classes of insecticides to control insect pests (Aker et al. 2008). There is great interest in replacing the use of these insecticides with more "natural" options due to concerns about the potential for contamination of soil and water, unintended effects on non-target organisms, as well as development of insecticide resistance in the target organisms. Research on plant essential oils as potential insecticides has suggested that they may be effective (Negahban et al. 2007, Ayvaz et al. 2009, Yazdgerdian et al. 2015, and Oboh et al. 2017) with some claiming that they may be less toxic to vertebrates (Rozman et al. 2007). Recently, insecticides claiming to use plant essential oils as their main and "natural" ingredients have become commonly available. Here are a few sample brands listing various plant essential oils as active ingredients in their products:

- Wondercide®: cedarwood oil, lemongrass oil, peppermint oil, and geraniol (derived from geraniums).
- Cedarcide: cedarwood oil, lemongrass oil, and soybean oil.
- Maggie's Farm™: canola oil, cedarwood oil, cinnamon oil, citronella oil, clove oil, cottonseed oil, geraniol (derived from geraniums), peppermint oil, rosemary oil, soybean oil, and thyme oil.
- Aunt Fannie's: cedarwood oil, citronella oil, clove oil, cottonseed oil, geranium oil, lemongrass oil, peppermint oil, rosemary oil, and soybean oil.

This investigative multi-week lab sequence explores the interest in using plant essential oils as alternative, "greener" insecticides. It is one of a series of multi-week lab sequences we have developed focusing on insecticide use against bean beetles. Students gain experience in designing a short-term research study using basic techniques such as toxicity assays and dose response curves, enzyme assays, colorimetric assays and spectrophotometry, and collecting, analyzing, and reporting data for such a study. They also learn about the life cycle of bean beetles (*Callosobruchus maculatus*) as a model organism and how to identify different sexes and how to culture the beetles. And they discuss the interplay between natural selection leading to the evolution of defensive chemicals in plants and insecticide resistance, ethnobotany, agroecology, and agribusiness. The exercise can be enhanced with an introduction to bioinformatics focusing on protein structure as well. Given the paucity of published data on toxicity of plant essential oils towards bean beetles, the data generated by the students will likely be novel data.

STUDENT OUTLINE

Objectives

Describe how and why organophosphate insecticides work to carry out their intended function and unintended consequences.

Design a study to investigate whether plant essential oils can be used as potential insecticides.

Explain why bean beetles are a good system for such studies.

Design a study to investigate whether commercial insecticides and plant essential oils inhibit the activity level of cholinesterases in vertebrates.

Collect, analyze, and present the data from such studies.

Discuss the limitations of the study and suggest future steps based on the results obtained.

Introduction

Bean beetles, in the genus *Callosobruchus*, are agricultural pests found in tropical and subtropical regions of Africa and Asia. They are pests of legume seeds (family Fabaceae) such as mung beans (*Vigna radiata*) and black-eyed peas (also called cowpeas, *Vigna unguiculata*). The adults do not require food or water and they spend their short lifespan (1-2 weeks) to mate and for the females to lay eggs on bean seeds. The larvae then feed on the bean embryo and endosperm and thus destroy the bean crop (Beck and Blumer, 2011). Hence, there is great interest in controlling or eliminating these pests and minimizing their effect on the bean harvest.

Organophosphate insecticides such as malaoxon (Figure 1) have been used to control insect pests such as bean beetles. These insecticides work through their effect on the enzyme acetylcholinesterase (AChE).

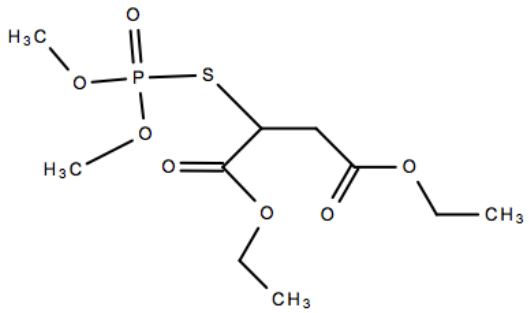


Figure 1. Malaoxon structure.

AChE is an important component of cell-cell signaling in the nervous system where it breaks down the neurotransmitter acetylcholine and helps to terminate the signal (see Figure 2). Inhibition of AChE by the insecticides interferes with this process, and thus with the insect's nervous system. This can cause the insect's death.

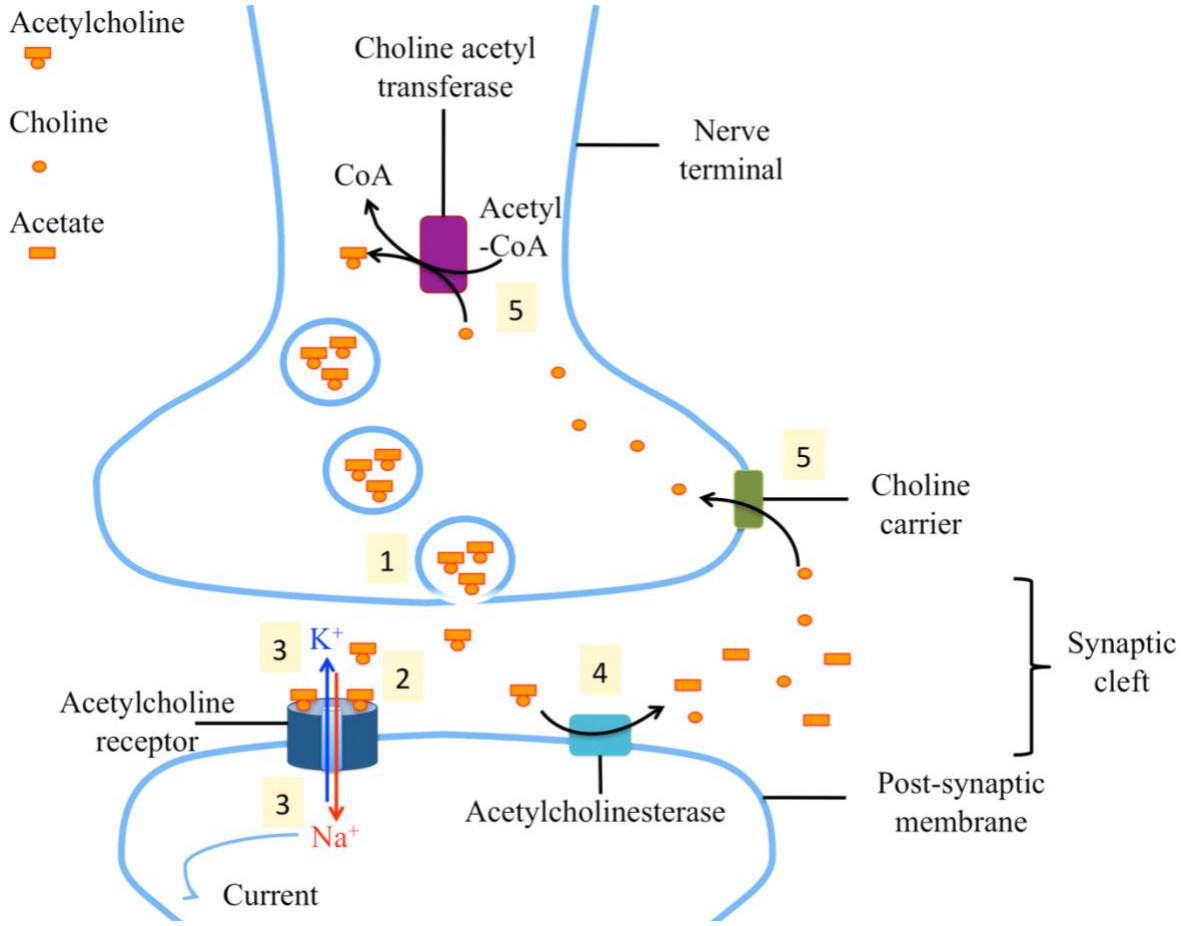


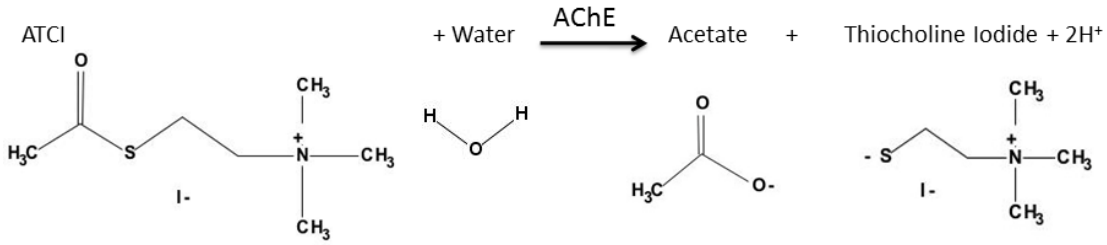
Figure 2. Synaptic signaling involving the neurotransmitter acetylcholine. 1. An action potential in the presynaptic cell triggers vesicle exocytosis at an active zone, releasing acetylcholine (ACh). 2. ACh diffuses rapidly across the synaptic cleft and binds to acetylcholine receptors at the postsynaptic membrane. 3. The receptor channel opens to allow Na^+ and K^+ ion flow, producing an excitatory postsynaptic potential. The action potential propagates to the rest of the cell. 4. Acetylcholinesterase (AChE) in the post-synaptic membrane hydrolyzes the acetylcholine into acetate and choline. 5. Choline is transported by choline carrier into the axon terminal to be resynthesized into ACh by choline acetyl transferase.

There is increased interest in using natural insecticides to replace chemicals such as malaoxon. The reasons are increasing levels of insecticide resistance observed in different pest species (e.g., Magaña et al., 2008, Reyes et al., 2011), as well as concerns about the effects of these chemicals on human health and unintended effects on non-target organisms (London et al., 2005, Patil and David, 2010, Sadeghi Hashjin et al., 2013). Natural insecticides are locally available, biodegradable, and presumably less toxic to vertebrates (Rozman et al. 2007). There have been reports of toxicity of essential oils from different plants to *Callosobruchus* species (Chaubey 2008) including bean beetles (Oboh et al. 2017). The observed toxicity may stem from inhibition of the Na^+/K^+ pump and acetylcholinesterase (Oboh et al. 2017). Essential oils from aromatic plant species contain varying combinations of different compounds; for example, 41 compounds in orange peel oil (Oboh et al. 2017) and 24 in *Artemisia sieberi* oil (Negahban et al., 2007). Due to their different compositions, essential oils from some plant species may be more potent inhibitors of the enzymes mentioned above than essential oils from other plant species. They may also inhibit or activate other enzymes not mentioned above or even considered so far. Multiple mechanisms of action by a natural insecticide could potentially reduce the possible development of resistance by the target insects against the insecticide. However, these mechanisms of action may also affect non-target organisms, including humans. For example, essential oils of several plants have been shown to affect activity levels of both acetylcholinesterase and butyrylcholinesterase extracted from vertebrates (Gomes da Rocha Voris et al. 2018, and Amir Rawa et al. 2019). In this exercise, we will study the potency of essential oils from different plant species to kill bean beetles. We will also study the effect of these oils on different cholinesterases extracted from vertebrates to determine what unintended consequences might result from the use of these natural insecticides.

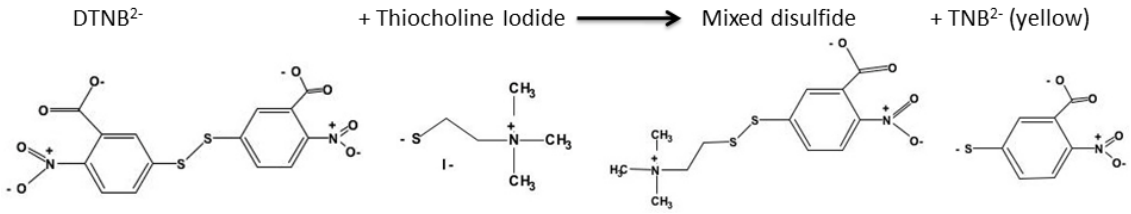
The enzyme assays

The enzyme assays we will use are based on the work done by Ellman et al. (1961), Ffrench-Constant and Bonning (1989), Spencer et al. (1998), Boga et al. (2011), Gbaye et al. (2012), and Gomes da Rocha Voris et al. (2018). Basically, we take advantage of the type of reaction carried out by the enzymes AChE and BChE by supplying substrates other than ACh (acetylcholine) and BCh (butyrylcholine) to the enzymes *in vitro*.

In the presence of the enzyme AChE, the compound ATCI (acetylthiocholine iodide) is hydrolyzed to produce acetate and thiocholine.



The thiol (R-SH, sulfhydryl) group of thiocholine can react with the indicator compound DTNB (5-5'-dithio-bis-2-nitrobenzoic acid) to form TNB^- (5-thio-2-nitrobenzoate), which ionizes to the TNB^{2-} dianion in water at mildly alkaline pH. This dianion is yellow and its presence can be detected using a spectrophotometer.



Similarly, in the presence of the enzyme BChE, the compound BTCI (butyrylthiocholine iodide) is hydrolyzed to generate butyrate and thiocholine iodide, which can then react with DTNB as above.

The more yellow TNB^{2-} products accumulate in the test tube, the higher the optical density (absorbance) of the solution in the test tube will be at a specific wavelength. If an inhibitor inhibits the enzyme, then the rate of reaction will be slower and the optical density (absorbance) of the solution in the test tube will be lower after a set amount of time compared to a similarly prepared test tube without such an inhibitor. Thus, we can use a simple assay using this indicator dye to determine whether the insecticide of interest inhibits either enzyme.

Timing

During lab 1 your lab instructor will review the life cycle of bean beetles and how to distinguish males and females. In addition, you will do some background research on the essential oils of the plant species assigned to you and any known effects on insects and on different enzymes in various species. You will set up a fumigant toxicity assay to determine how effective the essential oils from different plant species may be as potential insecticides against bean beetles.

During lab 2 you will carry out Parts B and C of the procedure using the essential oils from the species assigned to your group.

During lab 3 you will present your findings to the class. The class will then decide on the class question for further work. Everybody will carry out Part B of the procedure for the essential oils for the plant species chosen by the class.

During lab 4 the class performs Part C of the procedure for the essential oils for the plant species chosen by the class and analyzes the data to be included in the lab report.

Background research on the available plant essential oils

Your instructor will give you the list of the plant essential oils that are available for this research experiment. There is little, if any, information on the effectiveness of these plant essential oils as potential insecticides against bean beetles or their effects on the activity levels of the enzymes AChE or BChE. Therefore, you will be performing authentic research for which the answer is not well understood. As a first step, you should check through the published research and find out what information is available about these plant essential oils as potential natural insecticides, or their action as inhibitors of AChE/BChE or other critical enzymes in insects or other animals, including vertebrates. Each group will be using essential oils from one plant species. Your instructor will provide some suggestions on how to use the Library tools efficiently to find any

relevant published research on this particular subject.

Plant species for your group:

After you perform your library research, state the null and alternative hypotheses you will be testing for the essential oil for your group:

Alternative:

Null:

Materials

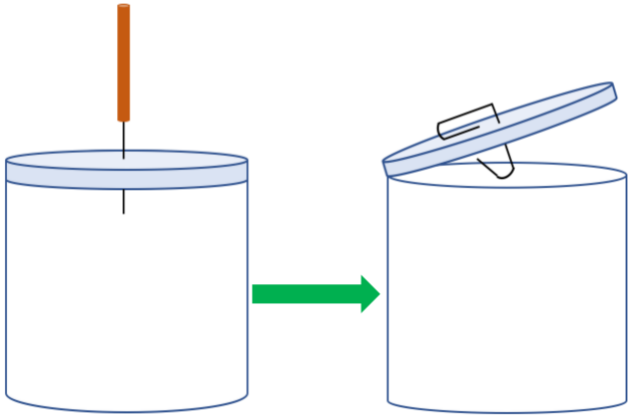
- Bean beetles, *Callosobruchus maculatus*, note the strain: _____ and the legume seed on which the bean beetles are grown: _____
- Small paint brushes
- Dissecting microscopes
- 300-mL snap-seal disposable plastic containers with pin holes in the lids
- Paper clips
- 2.5 cm glass fiber filter circles
- Lab tape
- Permanent markers
- Insect net cloth
- 20 mM Tris HCl buffer
- ATCI: 10 mM acetylthiocholine iodide in 20 mM Tris HCl buffer
- BTCI: 10 mM butyrylthiocholine iodide in 20 mM Tris HCl buffer
- Malaoxon stock: 5 mM solution in 20 mM Tris HCl buffer
- Plant essential oils
- Plant essential oils dissolved in 99.9% methanol (1% v/v)
- 99.9% methanol
- DTNB: 1.5 mM 5-5-dithio-bis(2-nitrobenzoic acid) in 20 mM Tris HCl buffer
- Purified acetylcholinesterase (AChE) enzyme from *Electrophorus electricus*
- Purified butyrylcholinesterase (BChE) enzyme from equine serum
- Spectrophotometer
- Spectrophotometer cuvettes
- Centrifuge
- Petri dishes
- Vortexer
- 1.5 mL microcentrifuge tubes
- Micropipettors and tips
- Water bath set at 30°C
- Ice buckets

Methods and Data Collection

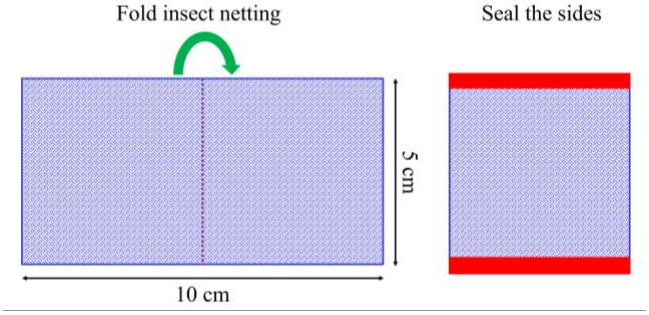
Part A: Fumigant Toxicity Assay

We will use a fumigant toxicity assay based on Oboh et al. (2017) to compare the mortality of the bean beetles exposed to the essential oils from different plants.

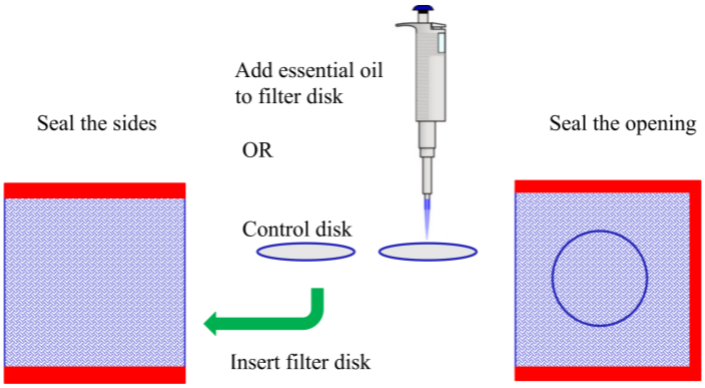
1. Each group should take 2 snap-seal plastic containers with lids. The lids should have no holes in them or at most one hole in the center of the lid.
2. If the lids have no holes, use the dissecting needles provided to make one hole in the center of the lids.
3. Partially open the paper clips provided. Insert half the paper clip through the lid for each container as shown below.



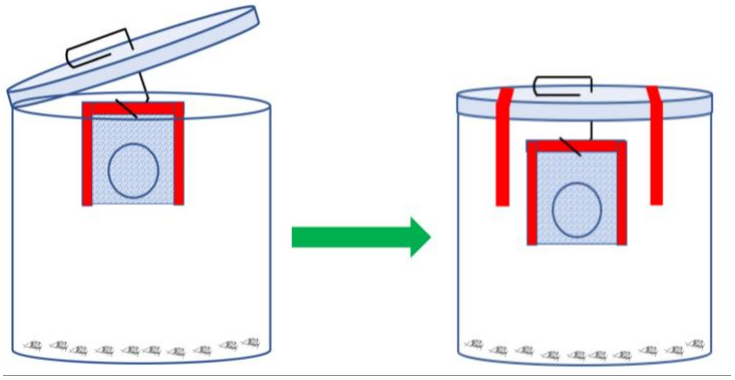
4. To each container add 10 bean beetles, preferably 5 males and 5 females. Close the lid to make sure the beetles do not escape. Label one container as the - control and the other one as the essential oil treatment.
5. Take a piece of the insect net cloth provided. Cut two pieces that are 5 cm x 10 cm each. Fold each piece along its length and tape the two sides to make a 5 cm x 5 cm pocket, as shown below.



6. Wear gloves. Take two filter circles. One will be the negative control and one will receive the essential oil. What will be done to the control? Why do we need the negative control treatment?
7. Place the one filter circle in a small Petri dish. Use the appropriate micropipettor with a correct disposable tip to add 25 µL of the plant essential oils for your group to the disk. Use forceps to place the filter circle in one of the pockets you made above. Tape the opening so that the filter circle stays in the pocket as shown below.



8. Hang this pocket (with the filter circle) from the paper clip inside the container for the essential oil treatment. Close the lid to make sure the beetles do not escape. Tape the container shut as shown below.



9. After you have decided what should be done to the control filter circle, insert the control filter circle into the other pocket you made above. Tape the opening so that the filter circle stays in the pocket.
10. Hang this pocket (with the control filter circle) from the paper clip inside the container for the control treatment. Close the lid to make sure the beetles do not escape. Tape the container shut.
11. Do not open the containers again until the next lab. Every day count the number of beetles that are alive versus dead in each container until all beetles have died or until the next lab, whichever comes first. Record your data in Table 1 below. Calculate % mortality for each treatment for each day.
12. Using Microsoft Excel, make a graph (X-Y scatter plot) of the data to show how % mortality changes over time for the two treatment levels. Include in your oral presentation.
13. Disposal: Place the sealed containers with the beetles in the freezer (-20°C). After 72 hours the lab technicians will discard the dead beetles in the biohazardous waste containers.

Table 1. Mortality of bean beetles exposed to essential oils from _____

Day	# Dead beetles		% Mortality	
	Control	Treatment	Control	Treatment
0				
1				
2				
3				
4				
5				
6				
7				

Part B: AChE Enzyme Assay

1. Wear gloves and goggles. You must follow all lab safety rules relating to use of hazardous substances in the lab.
2. Each group will set up a total of 18 tubes for the assay: A1-A5, B1-B5, C1-C5, and 3 blanks.
3. Follow the steps below and Table 2 and Figure 3 to set up the reaction tubes and the blanks for each enzyme assay.
4. Use a micropipettor to transfer 300 μL of the purified enzyme solution AChE to all A, B, and C tubes that are numbered (not the blanks, see below). Keep the tubes on ice.
5. Label three Blank tubes as A-BL (Blank no inhibitor), B-BL (Blank for malaoxon treatment), and C-BL (Blank for plant essential oils treatment).
6. Add 300 μL of Tris HCl buffer to each Blank tube.
7. Add 20 μL methanol to the A-BL (Blank no inhibitor) and A tubes.
8. Add 20 μL malaoxon (5 mM) to the B-BL (Blank with malaoxon) and B tubes. Note: we are using malaoxon as our + control.
9. Add 20 μL plant essential oils solution (already diluted in methanol) to the C-BL (Blank with essential oils) and C tubes.
10. Incubate the tubes at 4°C or keep on ice for 5 minutes.
11. Use a micropipettor to add 50 μL of the "reaction substrate" (ATCI) to all A, B, C, and Blank tubes. Keep the tubes on ice.

12. Use a micropipettor to add 150 μL of the indicator DTNB to all A, B, C, and Blank tubes. Keep the tubes on ice until you are ready for the incubation.
13. Why is it important to add the substrates after the insecticide or plant essential oils?

Table 2. Chemical components of the A, B, C, and Blank tubes for the enzyme assay to determine the effect of the insecticide (malaoxon, + control) or plant essential oils on the activity of the AChE enzyme. Add in the order from left to right.

Tube	300 μL	20 μL		50 μL	150 μL
A tubes	Purified enzyme	Methanol	Incubate for 5 minutes	Reaction Substrate	DTNB
A-BL	Tris HCl buffer	Methanol		Reaction Substrate	DTNB
B tubes	Purified enzyme	Malaoxon		Reaction Substrate	DTNB
B-BL	Tris HCl buffer	Malaoxon		Reaction Substrate	DTNB
C tubes	Purified enzyme	Plant essential oils solution		Reaction Substrate	DTNB
C-BL	Tris HCl buffer	Plant essential oils solution		Reaction Substrate	DTNB

14. Invert each tube 5 times to mix the solution and then incubate all tubes in the water bath (your instructor will show you how) at 30°C for 10 minutes. After 10 minutes, transfer the tubes to ice to slow down the reaction.
15. While you are waiting, take 18 spectrophotometer cuvettes. Label them so that they correspond to the tubes A1-A5, B1-B5, C1-C5, and the three blanks. Make sure to label them on the sides that do not interfere with light absorption; your lab instructor will show you how to do this.
16. Transfer 500 μL of the solution from microcentrifuge tube A1 to the clean spectrophotometer cuvette A1. You can use a micropipettor with the correct tip. Do this very gently. When using a micropipettor, touch the tip of the pipette to the side of the cuvette to avoid forming any bubbles. Discard the tip. Repeat for the rest of the A tubes including the A-BL tube.
17. For each cuvette measure the absorbance at 412 nm using the spectrophotometer provided. Follow the instructions below:
    a. Turn on the spectrophotometer. Allow it to warm up for a few minutes.
    b. The lab instructor will show you how to program the spectrophotometer.
    c. Our spectrophotometers have 1 space for a blank cuvette and 5 spaces for samples. Place the A-BL (Blank with methanol) cuvette and your A cuvettes (A1-A5) in the correct slots in the spectrophotometer. A-BL goes into the slot marked B (blank) in the spectrophotometer. The other cuvettes go into the numbered slots (1-5).
    d. Record the absorbance readings at 412 nm in Table 3.
    e. Repeat steps 16 and 17 c-d for the B tubes. Record the absorbance readings as above.
    f. Repeat steps 16 and 17 c-d for the C tubes. Record the absorbance readings as above.
18. Your instructor will tell you how to discard your tubes and chemicals. The solutions should be disposed of in a Hazardous Waste bottle and the empty cuvettes and microcentrifuge tubes should be disposed of in a Biohazardous waste container.

Table 3: Measured absorbance values (at 412 nm) for AChE enzyme activity without (A) or with the potential insecticides (B = malaoxon, C = plant essential oils).

Tube	Absorbance (AU)
A1	
A2	
A3	
A4	
A5	
B1	
B2	

B3	
B4	
B5	
C1	
C2	
C3	
C4	
C5	

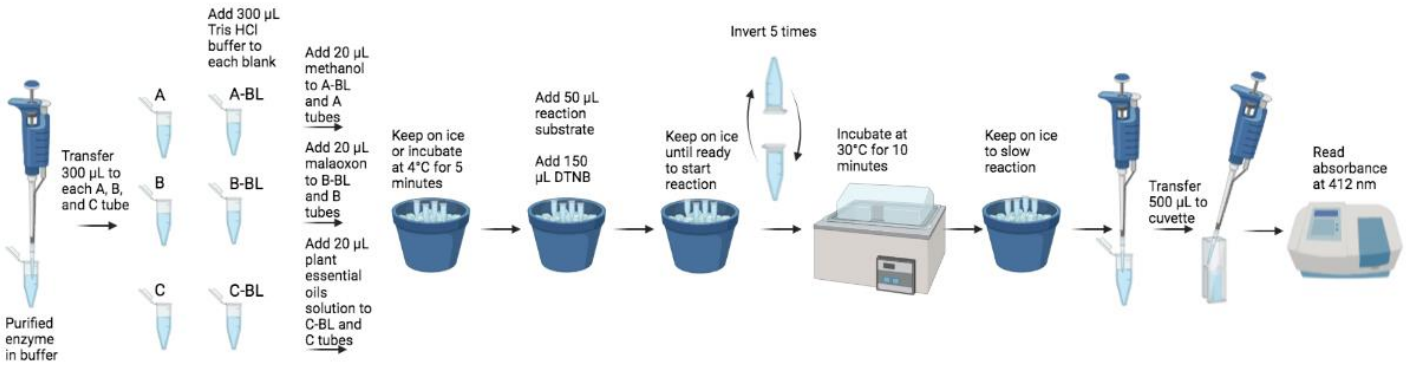


Figure 3. The procedure for the enzyme assay using purified enzyme dissolved in Tris HCl buffer. Figure created with BioRender.com.

Part C: BChE Enzyme Assay

You will follow the same procedure as part B with the following exceptions:

- Use BChE purified enzyme instead of AChE.
- Use BTCI as the reaction substrate instead of ATCI.

The rest of the procedure is exactly the same.

Data Analysis

The difference between the absorbance readings for the A and B tubes in each enzyme assay shows the effect of malaoxon (+ control), if any, on the purified enzyme. The difference between the absorbance readings for the A and C tubes in each enzyme assay shows the effect of the plant essential oils. We are interested in whether the plant essential oils affect the reaction at all.

Make a column graph of the means ± s for the absorbance values for the negative control treatment, the positive control treatment, and the experimental treatment. Include in your oral presentation. Do the means look different?

Perform a t-test to determine whether your hypothesis is supported. What type of t-test is appropriate for this analysis? Record the results of your t-test in Table 4. Do the results of the t-test support or reject your alternative hypothesis? How confident are you? Explain. Include in your oral presentation.

Table 4: The results of the t-test for the comparison of the effect of the plant essential oils on the enzyme.

D.F.	
t-critical for 95% confidence level	
|t-calculated|	
Confidence level	

Another way to look at the effect of the plant essential oils or malaoxon on the activity of the enzyme is to look at relative effects. You can use the average of the absorbance values for the A tubes as the baseline against which we consider the effect of the potential insecticides. Percent relative effect (RE) due to malaoxon or plant essential oils can be calculated as:

$$RE = \frac{100 \times (\text{Average absorbance for A tubes} - \text{Absorbance for each B (or C) tube})}{\text{Average absorbance for A tubes}}$$

Calculate the relative effect (RE) of the enzyme due to each potential insecticide (B = malaoxon, C = plant essential oils) for each tube. If you have a negative value, what does that mean? If you have a positive value, what does that mean?

Calculate the means and standard deviations for the relative effects. Record in Table 5. What effect does Malaoxon have on the enzyme? How about the plant essential oils?

Table 5: The relative effect (RE) of the enzyme activity caused by the insecticide malaoxon or by the plant essential oils used.

Potential insecticide used	Malaoxon	Plant Essential Oils
Mean (%)		
Standard deviation (%)		

Note: You need to analyze the data for both AChE and BChE following the steps above.

Oral Presentations

Each group will prepare a 10-minute oral presentation for the group experiment and results. The easiest platform to use will be Microsoft PowerPoint. Use bullets on the screen to highlight the points you would like to make and then orally present the details to your audience. Do not type everything you want to say on the slides you project. The language you use has to be concise and precise. Any word, phrase, sentence, or even paragraph that does not add to our understanding of your project should be removed from your presentation. In your presentation include the following:

- (1 point) Who you are.
- (3 points) Title: has to be informative. Provide a short title that tells us what is being investigated as the main point of the experiment. For example: "The effect of talking to geranium plants on their flowering time".
- (20 points) The background research leading to the justification for your hypothesis; state your hypothesis.
- Your experimental design:
  - (5 points) Specific methods used. Do not repeat details from the lab manual.
  - (20 points) A table listing the variables, treatment levels, replications and sample sizes, the species being used, your prediction.
- (30 points) Results: Your data (in table or graph form). Note: all graphs and tables must be numbered and have a short caption/title. **Do not present raw data!**
  - One graph of the data to show how % mortality changes over time for the two treatment levels. Do not present raw data!
  - Two graphs of means ± standard deviation of the absorbance values you calculated for your experiment: one for each enzyme. Do not present raw data!
  - (Two graphs of means ± standard deviation of the relative effects that you calculated for your experiment: one for each enzyme.)
  - Two tables containing the results of the statistical analysis, in this case, the t-tests (one for each enzyme).
  - Make sure to walk your audience through your data!
- (20 points) Discussion: Your conclusions and future experiments.
  - Restate your hypothesis. Do your results support your hypothesis? How confident are you about this conclusion?
  - What do the results tell you about the potential effectiveness of the plant essential oils you used to inhibit AChE or BChE? Or to be used as an insecticide?
  - If you were to take this experiment one step further **based on your results and conclusions**, what would be the next experiment you would try? Explain.
- (1 point) Who contributed what to the presentation. This is commonly included in scientific reports; for example, see Author Contributions at the end (p. 1397) of the following article published in a scientific journal: https://bsapubs.onlinelibrary.wiley.com/doi/epdf/10.1002/ajb2.1543
- Note about citing your sources: Any information used from external sources must be cited properly. This includes any figures or pictures you may use if not generated by you. See Citation Guidelines. Plagiarism will not be tolerated.
- Your lab instructor may ask you to evaluate each other's contributions to the experiment and the oral presentation.

Class experiment

Although all groups are working on the same big question, each group has used the essential oils from a different plant

species. Based on the presentations, the class will decide to carry out a large-scale experiment using the essential oils from one or more plant species. If the class chooses to study the essential oils from more than 1 species, then consider whether to choose species from the same plant family or different families and justify your choice.

Plant species chosen for further study:

Alternative hypothesis:

Null hypothesis:

Procedure for class experiment

Perform procedure parts B and C using the plant essential oils chosen by the class. Each group should use a minimum of 3 tubes for each treatment level for each enzyme. Record your group data in a table similar to Table 3. Share your data with the class. Use the class data and the appropriate tests to determine whether the class hypothesis is supported. What type of statistical test will you use?

Present your data in your lab report. Do the data support the class hypothesis? Make sure to include the appropriate graphs and tables.

Note that you have used enzymes that were purified from a fish (AChE) and a mammal (BChE). If these enzymes are affected by the insecticide malaoxon or by the plant essential oils you studied, what can you claim about potential, unintended consequences of using these compounds to control the population of an insect pest such as bean beetles? Can you claim the insecticide or the essential oils will affect human farm workers? Other animals that might be exposed to these compounds? Are the proteins you studied in vitro identical to the ones that might be affected in the field?

Your Lab Report

Each person will hand in a short report for the experiment using the class data. Note that the focus of the lab report is on the class experiment, not the group experiments or the mortality data; you already presented those in your oral presentations. Your report is due on the date listed on the syllabus. The report must be typed, double-spaced, font size 12, 1" margins on all sides, and must include the following information in appropriately labeled sections. Also see the course policy on lab assignments in the syllabus.

- (1 point) Checklist, properly checked and attached to the report
- (2 points) Your name and the name of the other members of your group
- (2 points) Title: has to be informative. Provide a short title that tells us what to expect as the main point of the experiment.
- (20 points) Class hypotheses with justification (1-2 paragraphs)
  - (15 points) Provide the background information that led to the hypothesis. Presumably the hypothesis developed from the first set of experiments. Do not repeat the details of the first set of experiments since those were already presented to the class during the oral presentations. You should summarize and reference them. Provide background information to justify the hypothesis for the class experiment = focus of this report; you may need additional library research.
  - (5 points) Clearly state what the hypothesis is.
- (20 points) Experimental Design.
  - (10 points) List the variables, treatment levels, replications and sample sizes, and the species being used. You may include a table but MUST describe in paragraph form.
  - (5 points) Specific methods used. You need not repeat all of the procedural steps in the lab handout. You may simply state that you followed the steps described in the lab manual to determine x and y, but you must describe anything you did differently.
  - (2 points) Include the experimental prediction.
  - (3 points) State what method was used to analyze the data. Do not include the analysis here.
- (30 points) Results and Data Analysis:
  - Do not include raw data.
  - (5 points) A couple of sentences stating what is presented in which figure or table and summarizing the data with reference to the figures and tables. Do not discuss the implications of your results here.

  - (25 points) Analysis of the data
    - Graphs of means ± standard deviation of the absorbance values you calculated for your experiment: one for each enzyme. Do not present raw data!
    - (Graphs of means ± standard deviation of the relative effects that you calculated for your experiment: one for each enzyme.)
    - Tables containing the results of the statistical analysis for the class investigation. Note that you need a different table for each enzyme studied.
    - Note the formatting and the required elements for a properly presented figure or table. Note that the table number and caption are placed above the table, whereas the figure number and caption are placed below the figure.
- (15 points) Discussion and Conclusions:
  - (1 point) Remind the reader what the class hypothesis was.
  - (2 points) State whether the hypothesis is supported by the data you presented in the results section.
  - (7 points) Your conclusion based on your data analysis presented in one or two short paragraphs; note: conclusions are not the same as summary of data!
    - What do the results tell you about the way the plant essential oils studied affect the two cholinesterases? About the potential for using the selected plant essential oils to control bean beetle populations? About any unintended consequences for different species?
  - (5 points) Future Directions: What the next step of the research should be, based on your results and conclusions, stated in one or two short paragraphs. You should not propose future experiments that either test unrelated hypotheses or have no relation to your results.
- (5 points) Correctly formatted citations within the text and a Literature Cited section at the end of the report.
- Note: Plagiarism will not be tolerated. Any information used from any source other than your own thoughts must be cited properly. This includes any figures or pictures included that you did not generate yourself. See Citation Guidelines.
- (5 points) Overall organization and grammar.
  - There must be consistency in tense used and subject-verb agreement.
  - Complete sentences, not just fragments ☺
  - Text must be divided into paragraphs, not just one long run-on report.
  - Note about your writing: Avoid superfluous language that does not add to our understanding of the experiment. Your writing must be concise and precise. Any word, phrase, sentence, or even paragraph that does not add to our understanding of your project should be deleted from your report.

After your instructor returns your graded report to you, you have the option to rewrite the report if you would like to improve your grade. In that case, the optional second draft (rewrite) will be due on the date listed on the syllabus. The final grade for the report will be calculated as 40% original grade + 60% rewrite grade.

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MATERIALS

Here is a list of materials for a class of 24 students (6 groups of 4):

Fumigant Toxicity Assay	**Per Group**	**For a class of 24 (6 groups)**
Adult Bean beetles (*Callosubruchus maculatus*) (Biohazardous waste)	20 (10 male, 10 female)	120
Small paint brushes	2	12
Dissecting microscopes	1	6
Corning 300-mL Snap-Seal disposable plastic sample containers	2	12
Small paper clips	2	12
2.5-cm glass fiber filter circles (or similar size filter disks)	2	12
Insect net cloth (5cm x 10cm)	2	12
Plant essential oils	30 μL	200 μL
Dissecting needles	1	6
Forceps	1	6
Fine-point permanent markers	2	12
Lab tape	1	6
100-mm Petri dishes	1	6
Enzyme assays in Parts B&C		
Solutions		
Tris HCl buffer: 20 mM, pH 7.5 (Hazardous)	2 mL	12 mL
ATCI: 10 mM acetylthiocholine iodide in Tris HCl buffer (Hazardous)	1 mL	6 mL
BTCI: 10 mM butyrylthiocholine iodide in Tris HCl buffer (Hazardous)	1 mL	6 mL
Malaoxon stock: 5 mM solution in Tris HCl buffer (Hazardous)	300 μL	2 mL
DTNB: 1.5 mM 5-5-dithio-bis(2-nitrobenzoic acid) in Tris HCl buffer (Hazardous)	7.5 mL	45 mL
Purified acetylcholinesterase (AChE) enzyme from *Electrophorus electricus* dissolved and diluted in Tris HCl buffer (Biohazard)	5 mL	30 mL
Purified butyrylcholinesterase (BChE) enzyme from equine serum dissolved and diluted in Tris HCl buffer (Biohazard)	5 mL	30 mL
Plant essential oils dissolved in 99.9% methanol (1% v/v) (Hazardous)	600 μL (of each plant essential oils used)	4 mL (if using the same oils for the whole class)
99.9% Methanol (Hazardous)	240 μL	1.5 mL
Instruments		
Spectrophotometer	Preferably 1 per group	3-6
Microcentrifuge (10,000-14,000 rpm)	-	3
p-100-1000 micropipettors and tips	2	12
p-20-200 micropipettors and tips	2	12
Vortexers	1	6
Incubator or fridge at 4°C (or just keep on ice)	-	1-2 (Depends on size)

Water bath set at 30°C	-	1-2 (Depends on size)
1.5-mL spectrophotometer microcuvettes	36	216
1.5-mL microcentrifuge tubes	36	216
Microcentrifuge tube rack	1	6
Ice buckets	1	6
Hazardous waste solution container	1	1
Biohazardous waste disposal bag / container	-	1

Recipe for Making Stock Solutions

Note: to make the solutions, you need basic PPE: gloves, goggles, and lab coat. No hood is needed to prepare the solutions.

- Tris HCl buffer 20 mM
  - Make 1 M Stock: Dissolve 6.05 g of Tris base in 30 mL of H_2O, adjust pH to 7.5 with 5 M HCl, bring volume up to 50 mL.
  - Dilute 1 M stock 1:50 with water to make the 20 mM solution as needed.

- Acetylthiocholine Iodide (ATCI), 10 mM
  Dissolve 0.318 g in 110 mL of 20 mM Tris HCl buffer. Store at 4°C.

- Butyrylthiocholine Iodide (BTCI), 10 mM
  Dissolve 0.349 g in 110 mL of 20 mM Tris HCl buffer. Store at 4°C.

- Malaoxon Stock, 5 mM
  Add 32.05 μL of 3.9 M solution (Millipore-Sigma Product #: 36142) to 25 mL of 20 mM Tris HCl buffer. Store at 4°C.

- 5-5-diothio-bis(2-nitrobenzoic acid) (DTNB), 1.5 mM
  Dissolve 0.059 g of DTNB in 100 mL of 20 mM Tris HCl buffer. Store at 4°C in the dark.

- Purified Acetylcholinesterase (AChE) Enzyme from *Electrophorus electricus*
  (Millipore-Sigma Product #: C3389-2KU, 200-1000 U/mg protein)
  - Arrives in powder form.
  - Dissolve the entire content of the vial, e.g., 9 mg with 222 U/mg, in 10 mL of 20 mM Tris HCl buffer to achieve a stock concentration of approximately 200 U/mL. The enzyme concentration in the powder can range from 200 to 1000 U/mg; check the concentration on the purchased bottle and adjust accordingly. Suspend in buffer with gentle mixing: no shaking or vortexing or pipetting up and down; just inverting the tube a few times.
  - Aliquot 15 μL of stock into microcentrifuge tubes and store at -20°C (Freezer).
  - On day of experiment, dilute 1:1000 with 20 mM Tris HCl buffer.

- Purified Butyrylcholinesterase (BChE) Enzyme from Equine Serum
  (Millipore-Sigma Product #: C7512-1.2KU, 10 U/mg protein)
  - Dissolve 2 mg in 10 mL of 20 mM Tris HCl buffer (concentration: 2 U/mL) by suspending in buffer with gentle mixing: no shaking or vortexing or pipetting up and down; just inverting the tube a few times.
  - Aliquot 25 μL of stock into microcentrifuge tubes and store at -20°C.
  - On day of experiment, dilute 1:50 with 20 mM Tris HCl buffer.

General Supplies Shopping List

- Corning™ Snap-Seal Disposable Plastic Sample Containers, Fisher Scientific Catalog No. 02-540-23
- No-See-Um Polyester Netting, BioQuip Products Part# 7250NSW, 54" wide, minimum purchase 5 yards
- Plant essential oils: many are available from Millipore-Sigma. Here are some examples:

Plant Essential Oil	Product No.
Cupressaceae	
Juniper berry oil (*Juniperus communis*)	W260401
Pinaceae	

Pine needle oil (*Pinus* ssp.)	W290500
Rutaceae	
Lime oil (*Citrus aurantifolia*)	W263109
Orange oil (*Citrus sinensis*)	W282510
Lamiaceae	
Lavender oil (*Lavandula angustifolia*)	61718
Rosemary oil (*Rosmarinus officinalis*)	W299200
Thyme oil (*Thymus vulgaris* and/or *Thymus zygis*)	W306509
Myrtaceae	
Clove oil (*Eugenia* ssp.)	C8392

NOTES FOR THE INSTRUCTOR

This laboratory exercise was designed for an Introductory Biology class. Our class typically has 200+ students with multiple lab sections with 24 students per lab. By the time this set of experiments begins our students have done multiple case studies of parsing experiments, and have performed a multi-week lab sequence in which they have designed experiments, carried out the experiments, analyzed the data with descriptive and inferential statistics (simple t-tests and sometimes chi-squared tests too), and written a lab report with the option to rewrite.

The biggest problem we have encountered during the lab is incorrect pipetting, which may lead to unequal volumes, cross-contamination, and negative absorbance readings. The other problem is incorrect citation of references as many introductory students are unsure of where they need to include citations in oral presentations and in lab reports, despite the citation guidelines and examples we provide.

Introducing the Students to the Lab

The students learn about the life cycle of the beetles, observe them under the dissecting scope, and learn to distinguish males and females. The latter part takes a bit of practice and requires help from the lab instructors. Some lab instructors have resorted to pre-sorting male and female beetles to help the students.

The essential oils that we use for the fumigant toxicity assay are too concentrated to use as they are for the enzyme assay. If the insect is exposed to the essential oils as potential insecticides, the concentration reaching the enzymes in the nervous system will be much lower than the exposure concentration. We are using purified enzymes in the enzyme assay, so we need to lower the concentration by dilution. We leave the reasoning behind the dilution for discussion during the introduction to the lab as opposed to writing it in the student handout up front.

The Student handout presented here focuses on unintended consequences since the purified enzymes being used are extracted from fish and mammals. Many insecticides target AChE in insects, and insects lack BChE. BChE is from vertebrates only, the result of a gene duplication and divergence. So, we want to see whether both AChE and BChE in vertebrates are affected by these compounds (Pezzementi et al. 2011). During different semesters we have run different versions of this lab sequence in addition to the example for the student handout provided: a) extracting AChE from bean beetles (following Fermin et al., 2014) and looking at the effect of the essential oils on the extracted enzyme as a potential mode of action, b) extracting defensive esterase enzymes from bean beetles (following Firooznia et al., 2019) and looking at the effect of the essential oils on the extracted defensive enzymes, and c) looking at the effect of the essential oils on total ATPase activity in extracts from bean beetles, using ATPase assay kits purchased from Millipore-Sigma. We have also added a bioinformatics section to this lab sequence to compare the protein sequences for the two purified enzymes (from fish and mammals) with those of other species as students ponder the unintended consequences of using insecticides that may

affect the activity of these enzymes in more than the target species. The lab sequence could be designed to involve more independent work by allowing the students to pick a focus for further studies: differences in insecticidal potential of the plant essential oils for different strains of beetles, determination of minimum lethal concentrations or LC50 values, age dependent toxicity of essential oils in post-emergence adults, different enzymes identified through literature searches, the effects of the plant essential oils on enzyme kinetics, determining the minimum inhibitory concentrations for the essential oils, dose-response curves, comparison of plant essential oils purchased from Millipore-Sigma with those available commercially at grocery stores, etc.

In the handout provided here, we ask the students to incubate the reactions for a set period of time after adding the substrate and then measure an end-point absorbance value. If you want the students to look at enzyme kinetics and have spectrophotometers that allow you to take continuous absorbance measurements, you can have the students transfer the mixture to a cuvette as soon as they add the substrate and indicator dye to start taking absorbance measurements immediately. That way the students can look at the change in absorbance over time and calculate reaction rates from the slopes. We have sample data to show for this type of approach as well.

Pre-Laboratory Preparation of Material

You need to have a large population of adult beetles for each investigation. We have multiple cultures started at different times in our incubator and maintain a large population. You may choose to have students start their own cultures early in the semester so that they may be able to use adults from their own cultures as well. With our incubator, 5 weeks is the correct time interval to have adults. You should try growing the cultures in your lab in your incubator to get an idea of what the time interval between adult generations will be for you.

As listed above, some of the solutions can be made ahead of time and stored at room temperature or in the refrigerator. Some have to be diluted at the beginning of lab. Before you dilute the enzymes for the experiments on the day of the lab, you may wish to try a couple of different dilutions. For example, for AChE, we have found that 1:1000 dilution as listed above works well. You may wish to dilute 1:500 first and run an assay yourself to see whether the enzyme activity is in an acceptable range. If the activity is too high and out of range for your spectrophotometers, then dilute 1:1000.

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About the Authors

Fardad Firooznia received his BS in Biology from Yale University and his PhD in plant physiology from Cornell University. He has been an instructor at The City College of New York (CCNY) since 2010, where he teaches large introductory biology courses as well as upper-level seminar courses in ethnobotany, ecophysiology, and plant physiology.

Jhunior Morillo received his BS in Biology and MS in Entomology and Ecology from the City College of New York (CCNY). After receiving his MS, Jhunior was hired as a Senior Laboratory Technician at CCNY, where he continues to work very closely with students and professors. He is currently responsible for preparing various biology laboratories.

APPENDIX A
Sample Data

Sample data are presented below. So far, no lab has chosen to compare multiple plant essential oils. Therefore, the data lend themselves to simple t-tests. Depending on the level of the course in which this exercise is used, multiple comparisons and more advanced statistical analysis can be used. We introduce descriptive statistics and t-tests early in the semester and by the time of this lab sequence the students have already performed t-tests and written lab reports that include the results of the t-tests.

Table 6: Mortality of bean beetles exposed to essential oils from Tea Tree (*Melaleuca alternifolia*)

Day	# Dead beetles		% Mortality	
	Control	Tea Tree Oil	Control	Tea Tree Oil
0	0	0	0	0
1	0	6	0	30
2	0	14	0	70
3	1	20	5	100
4	1	20	5	100
5	1	20	5	100
6	4	20	20	100
7	8	20	40	100

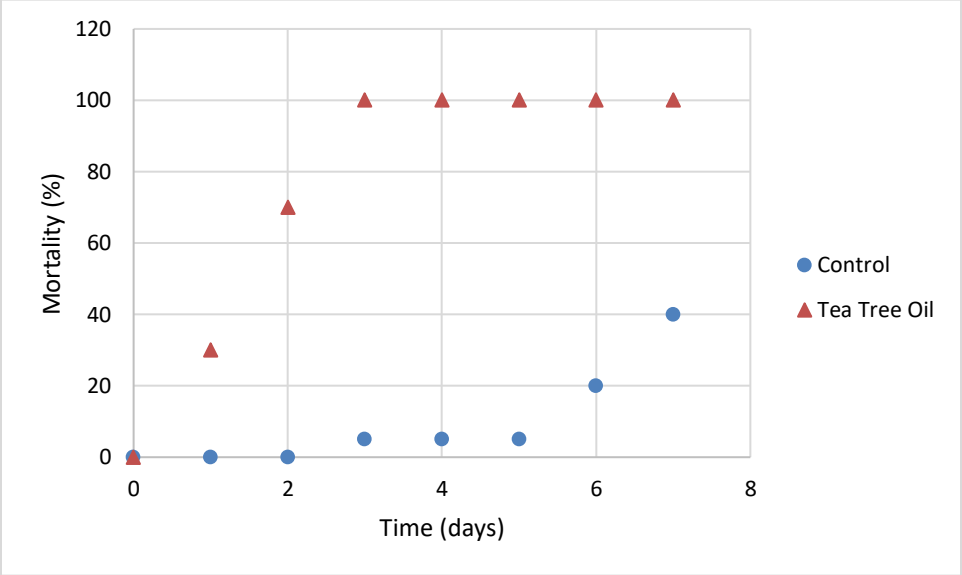

Figure 4: Mortality of bean beetles exposed to essential oils from Tea Tree (*Melaleuca alternifolia*) compared to negative control.

Table 7: Measured absorbance values (at 412 nm) and relative effect (RE) of malaoxon (tube B) and rosemary (*Rosmarinus officinalis*) oil (tube C) on the AChE enzyme activity.

Tube	Absorbance (AU)	RE (%)
A1	1.395	
A2	1.814	
A3	1.87	
Average of A tubes	1.693	
B1	0.371	78.1
B2	0.39	77.0
B3	0.394	76.7
B4	0.485	71.4

B5	0.406	76.0
C1	0.025	98.5
C2	0.043	97.5
C3	0.042	97.5
C4	0.045	97.3
C5	0.047	97.2

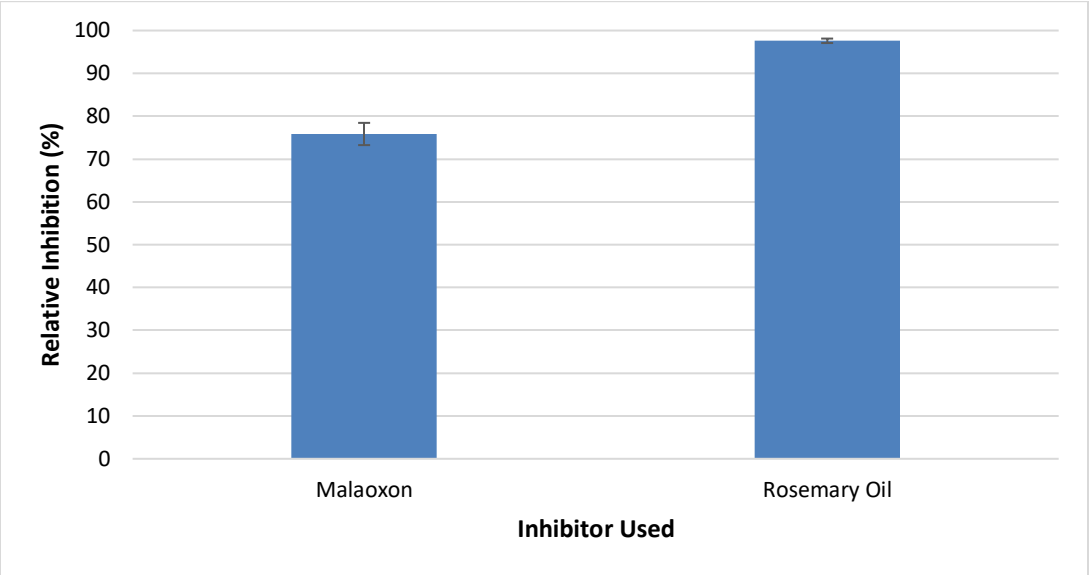

Figure 5: The relative inhibition of the enzyme AChE due to malaoxon (+ control) versus rosemary (*Rosmarinus officinalis*) oil. The columns represent the means, and the error bars represent standard deviations. The sample size was 5 for each treatment.

Table 8: Measured absorbance values (at 412 nm) and relative effect (RE) of malaoxon (tube B) and wintergreen (*Gaultheria* ssp.) oil (tube C) on the BChE enzyme activity.

Tube	Absorbance (AU)	RE (%)
A1	1.464	
A2	1.350	
A3	1.612	
Average of A tubes	1.475	
B1	0.053	96.4
B2	0.056	96.2
B3	0.052	96.5
B4	0.055	96.3
B5	0.061	95.9
C1	1.028	30.3
C2	1.078	26.9
C3	0.947	35.8
C4	1.058	28.3
C5	0.905	38.6

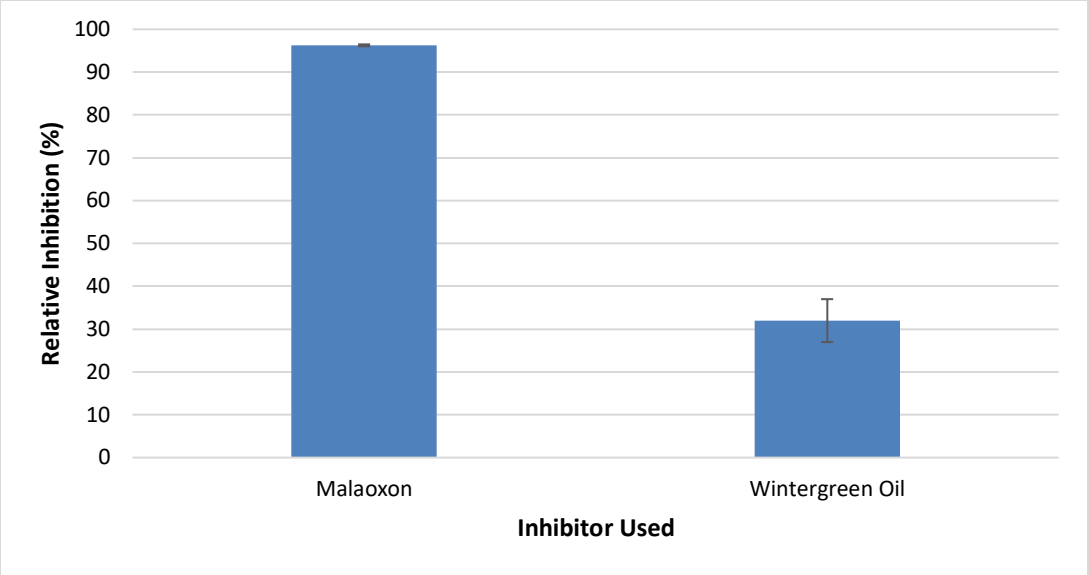

Figure 6: The relative inhibition of the enzyme BChE due to malaoxon (+ control) versus wintergreen (*Gaultheria* ssp.) oil. The columns represent the means, and the error bars represent standard deviations. The sample size was 5 for each treatment.

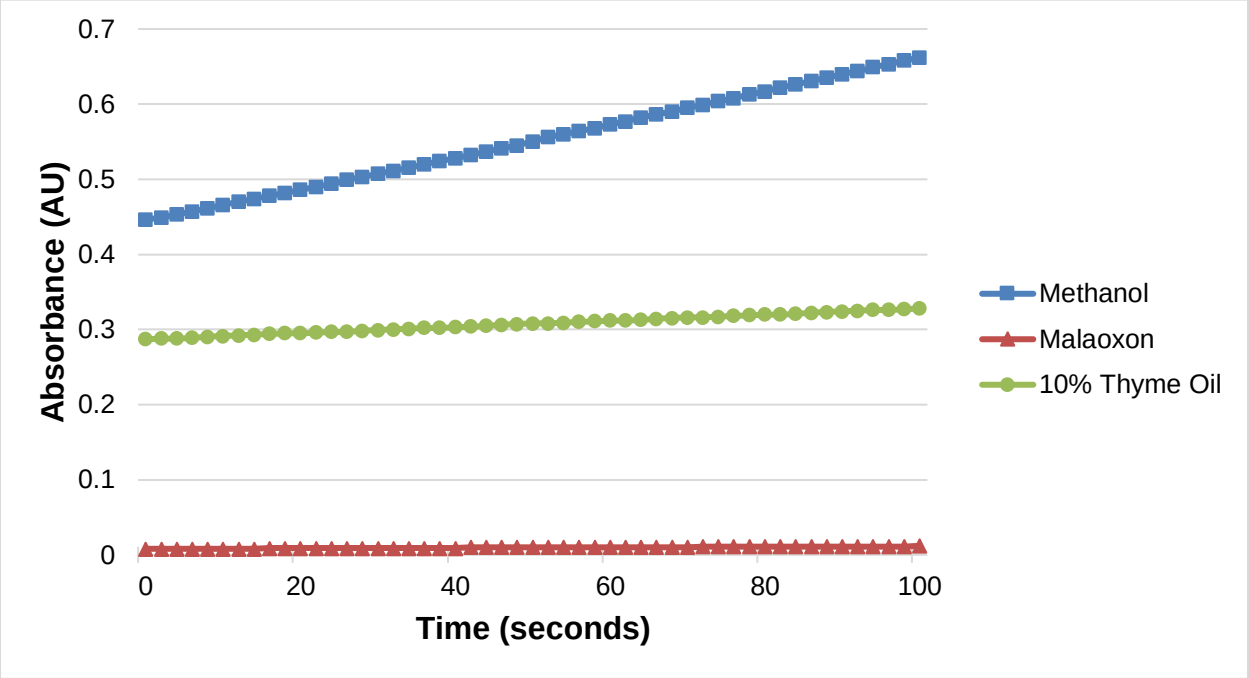

Figure 7: The change in absorbance at 412 nm over time for the enzyme AChE subjected to methanol (- control), malaoxon (+ control), and 10% thyme (*Thymus sp.*) oil. AChE enzyme concentration was 0.125 U/mL.

APPENDIX B

Notes for Lab TAs

These are the notes given to the lab instructors before we perform the procedure during our weekly prep sessions for this lab sequence:

This is a 4-lab sequence:

1. Lab 1: Introduce the bean beetles and the experiment. Building up on the basic Library Instruction earlier in the semester, help students to do background research on the plant essential oils assigned to them. Each group sets up a fumigant toxicity assay using essential oils of the assigned plant species.
2. Lab 2: Students perform parts B and C of the procedure using essential oils from one plant species. Each group prepares a presentation for next lab.
3. Lab 3: Each group presents their data to the class. The class will decide on essential oils from one (or more) plant species for further work. Everybody will do part B for the essential oils for the plant species chosen by the class.
4. Lab 4: Students perform part C of the procedure for the essential oils for the plant species chosen by the class and analyze the data to be included in their lab reports. (May also include some bioinformatics exercises).

What students should already know at this point in the semester:

- Basic structure of proteins
- What enzymes do
- What inhibitors and activators are, modes of inhibition

Lab 1:

Introduction to Bean Beetles (30 minutes)

- Remind the students about information on dissecting scopes from the microscopy lab.
- 10 minutes: Review the life cycle of the bean beetles (Figure in lab manual, and poster on the wall). Review the morphological differences between males and females (poster on the wall). Show the eggs and "window" in seed coats and emergence holes in the cultures.
- 15 minutes: Give each group 1 male and 1 female beetle. Allow the students to look at males and females under the dissecting scope to be able to distinguish the sexes.
- 5 minutes: Sing "A Bug's Life".

Introduction to the enzyme lab (maximum 30 minutes)

- 30 minutes: Introduction to this week's lab.
    - Why insecticides are used to kill these beetles.
    - Review Figure 2 for the role of AChE enzyme in the synapse (terminating the signal), the normal reaction carried out by the enzyme (break down acetylcholine), and why inhibiting the enzyme could lead to death of the insect.
    - Why there is interest in minimizing insecticide use, and in resistance to insecticides, which may mean more insecticide use!
    - Why the interest in "natural" insecticides.
    - Why our interest in plant essential oils.

Library instruction (45 minutes)

- 15 minutes: Building up on the introduction to using the library resources from earlier in the semester, help the students to focus their research on plant essential oils and their potential insecticidal activity and mode of action.
- 30 minutes: Allow the groups to search for sources related to the essential oils their group will be using.

Fumigant Toxicity Assay (the rest of the time)

- 10 minutes: Show the students the materials they need to set up the assay
    - Making the mesh pocket and placing the filter disk inside
    - Hanging the filter pocket from the paper clip inside the container
    - Control versus treatment: discussion about the appropriate control
- The rest of the time: the students set up their containers.
    - Note the beetle strain and the legume seed on which the beetles are reared.

- Note that they must check these containers once a day. Either they keep them in the incubator and come to check them on campus every day, or one person per group can take them home. If they do that, the containers should remain taped shut and kept in a warm place away from direct light, and they must return the containers with all the live and dead beetles the next lab. If they do not, they will get an F for this lab.

Lab 2:

- The students will be using purified enzymes. Note the sources of the enzymes.
- 20 minutes: Review the lab procedure.
    a. Safety rules: goggles and gloves, long sleeves, covered legs and toes. Anybody not dressed appropriately, **not allowed to stay in the lab!**
    b. Review how to use micropipettors correctly. Refer them back to the Introduction to Micropipetting exercise.
    c. Keep track of A, B, and C tubes. Review the order in which we add the solutions and the incubation steps (Table 2).
    d. Review why we need blanks.
    e. A group of 4 students could divide into two pairs, one pair doing the assay for AChE and one pair doing the assay for BChE.
- 2 hours: The students carry out the procedure.
- 20 minutes: Review how to analyze the data. Remind the students that they are presenting their experiments to the class during the next lab; review the oral presentation instructions.

Lab 3

- 60 minutes: Oral presentations by the groups.
- 15 minutes: The class decides on essential oils from one or more plants for the class experiment.
- The rest of the time, the students carry out Part B of the procedure for the class experiment.

Lab 4

- 90 minutes: The students carry out Part C of the procedure for the class experiment.
- 30 minutes: The class analyzes the class data for the class experiment for both AChE and BChE. Remind the class of the questions at the end about the relevance of the fish and mammal enzymes used in this lab.
- (60 minutes: if the Bioinformatics exercise is included. Each group should have at least one laptop, preferably two. You should do the exercise yourself ahead of time so that you know what to expect).
- Review what should be included in the lab report.
- Remind them to be careful about citing their sources.
- Remind them that jointly written lab reports are not accepted. Remind them that any form of plagiarism will not be tolerated.

Return formal feedback on oral presentations to the groups.

Mission, Review Process & Disclaimer

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