Development of a Screening Protocol for Antiprotozoal Activity Against Acanthamoeba Castellanii

Introduction
The area of emphasis of this research project was the study of phytochemistry. Phytochemistry involves the study of chemicals that are derived from plants. These chemicals are generally known as phytochemicals. Phytochemistry is an essential area within the field of chemistry because many phytochemicals possess characteristics that are beneficial to humans. An example phytochemical that is used today to treat pain is salicylic acid. Salicylic acid is the active ingredient in aspirin. This particular phytochemical was first documented by the Greek physician Hippocrates in the 5th century BC. Hippocrates recorded the use of a white, bitter tasting powder extracted from the bark of the Latin salix, a type of willow tree, to relieve pain.

In this particular experiment, a screening protocol was developed for the pathogenic protozoan, Acanthamoeba castellanii, in hopes of isolating a specific phytochemical from various plant extracts or essential oils that was able to inhibit the pathogenic activity of the protozoan. Acanthamoeba castellanii is one of the most common soil dwelling protozoa and is also frequently found in fresh water habitats as well. These protozoa consist of small, eukaryotic cells from 15 to 35 µm in length with a general oval shape, and they feed on bacteria such as E. coli. A. castellanii have two distinct phases including a metabolically active trophozoite phase and a metabolically inactive cyst phase. The cyst phase is resistant to fluctuations in temperature and pH along with most antimicrobial agents, making the protozoan a tough target for treatment.

After the cysts were detected on a given plate, the assay could then be performed. The plate on which the cysts were detected was washed with 5 mL of phosphate buffer solution (PBS). When the PBS was added to the plate, the inoculating loop was used to gently scrape the plate to free the protozoan from the agar. A. castellanii can be characterized by tracks made in the agar by the cyst that could be seen under the microscope. New plates were prepared as needed, before previous plates were overgrown. The sample stock culture, a subculture of the stock culture, was prepared on PYB agar. To prepare the stock culture, a subculture of the Acanthamoeba cells was prepared on PYB agar. To prepare the subculture of the Acanthamoeba, a PYB agar plate was obtained along with an E. coli culture. Each plate was prepared under the tissue culture hood to prevent contamination from spores. E. coli was first added to the plate by scraping the plate containing the E. coli culture with an inoculating loop and then swiping the loop onto the PYB plate. It is critical to note that the inoculating loop was flamed before, in-between, and after each use to ensure that stock cultures of the Acanthamoeba and E. coli were not contaminated. Once the E. coli was added to the plate, the inoculating loop was flamed and the Acanthamoeba was added to the plate in the same way as the E. coli. Two subcultures were initially prepared and stored at 25 °C so that the protozoan could continue to grow until the cyst phase was reached. The plates were periodically viewed under a light microscope to ensure the protozoan was developing and to determine when it had entered the cyst phase, which was characterized by tracks made in the agar by the cyst that could be seen under the microscope. New plates were prepared as needed, before previous plates were overgrown.

Following the addition of the extract or oil, the plate was allowed to sit for a time period of 48 hours at 25 °C. This time period was determined through a ‘guess and check’ method. In the first assay, the plate was allowed to sit for a time period of 5 days, but when the cells were counted the vast majority of the cells were found to be dead, even in the negative control solution. Another assay was performed and the plate was allowed to sit for a 24 hour period, but at this time period there were still live cells detected in the negative control. At 48 hours, however, the Acanthamoeba cells in the positive control appeared to be dead and those in the negative control were still alive, so this time period was chosen. Additionally, an agent that did not inhibit the activity of the Acanthamoeba 48 hours would not be the best treatment for an infection. Once the 48 hour time period elapsed, 200 µL of tryptan blue dead stain was added to each well. A drop of this solution from each well was subsequently added to a hemocytometer and the dead, stained cells found in each well were counted. The number of cells counted, if any, was compared to the number of dead cells found in the positive control to determine if the extract or oil possessed any antiprotozoal activity. Each assay was performed in triplicate.

Screening Protocol
In order to screen the protozoan, it must first be allowed to grow until the cyst phase is reached. For this to occur, a culture of Acanthamoeba castellanii was obtained from the ATCC biological resource center. From this stock cells, a subculture of the Acanthamoeba was prepared on PYB agar. To prepare the subculture of the Acanthamoeba, a PYB agar plate was obtained along with an E. coli culture. Each plate was prepared under the tissue culture hood to prevent contamination from spores. E. coli was first added to the plate by scraping the plate containing the E. coli culture with an inoculating loop and then swiping the loop onto the PYB plate. It is critical to note that the inoculating loop was flamed before, in-between, and after each use to ensure that stock cultures of the Acanthamoeba and E. coli were not contaminated. Once the E. coli was added to the plate, the inoculating loop was flamed and the Acanthamoeba was added to the plate in the same way as the E. coli. Two subcultures were initially prepared and stored at 25 °C so that the protozoan could continue to grow until the cyst phase was reached. The plates were periodically viewed under a light microscope to ensure the protozoan was developing and to determine when it had entered the cyst phase, which was characterized by tracks made in the agar by the cyst that could be seen under the microscope. New plates were prepared as needed, before previous plates were overgrown.

After the cysts were detected on a given plate, the assay could then be performed. The plate on which the cysts were detected was washed with 5 mL of phosphate buffer solution (PBS). When the PBS was added to the plate, the inoculating loop was used to gently scrape the plate to free the protozoan from the agar. Subsequently, the PBS was removed from the plate, 1 mL at a time, adding each mL to a 1.5 mL centrifuge tube. Four tubes were obtained from on plate. Next, the centrifuge tubes obtained from the plate were placed in the centrifuge and were centrifuged on highest setting for 10 minutes in order to obtain a cell pellet of the protozoan. After the centrifuge period was complete, the tubes were removed from the centrifuge and the PBS solution above the pellet was removed and replaced with new PBS solution. Once the new PBS solution was added to the tubes, they were placed back into the centrifuge to spin for another 10 minute period. This process was repeated a total of four times with the purpose of centrifuging off the less dense bacterial cells. After the centrifugation process was completed, the supernatant was again removed and the pellets obtained were resuspended in 1 mL of PBS. All pellet solutions were combined following resuspension. Then, 5 µL of the pellet solution was obtained and 10 µL of the living stain, 4% methylene blue, was added to it. A drop of this stained solution was added to hemocytometer so that the number of Acanthamoeba cells in the solution could be standardized. Once the pellet solution was standardized, 100 µL was added into the wells of a 96 well plate, allowing the solution to sit in the wells for approximately 1 hour at room temperature to ensure the protozoan cells were able to adhere to the bottom of the wells. When this time period was complete, 100 µL of the specific plant extract or essential oil was added to a particular well on the plate, along with a positive control (active against the protozoan), 0.02% chlorhexidine gluconate (CHG), and a negative control (not active), PBS solution.

The results of the assay are given in the figure below:

Results
After standardization, the cell pellet solution used in the first assay contained 1.32 x 10^6 Acanthamoeba cells. The plant extracts used in the experiment were labeled with numbers 25 – 48, and each extract was tested in triplicate. A schematic diagram of the 96 well plate is given below with positive and negative controls also shown:

Extract 23, Erythrina lanceolata, was the only extract that appeared to obtain a degree of antiprotozoal activity, killing approximately 6.6 protozoa per 100 µL of cell solution compared to approximately 9 protozoa per 100 µL of cell solution in the known killer, the positive control.

In the second assay, only 5 essential oils were tested and they were labeled respectively. The 96 well plate was constructed like the schematic diagram in Figure 7, only using the 5 essential oils and testing them in triplicate. The result of this assay are depicted in the figure below:

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The Alabama Space Grant Consortium

Chelsey Stewart
Department of Chemistry
John Wright Dr.
Huntsville, AL 35899

The results of the assay are given in the figure below:

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None of the essential oils appeared to contain any antiprotozoal activity.

The cell counts in the pellet solution were significantly low, and the results obtained may be incorrect due to the low number of Acanthamoeba cells in the solution. Therefore, the concentration of the PBS solution needs to be adjusted in future assays to ensure a higher cell count in the pellet solution.