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Identifying Natural Inhibitors of Bacterial Efflux Pumps

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Abstract

Antibiotic resistance is a constantly progressing epidemic. Many strains of bacteria have developed a resistance to antibiotics, resulting in prolonged sickness and death. Resistance can be to a specific drug (single drug resistance) or to multiple drugs (multi-drug resistance). This resistance can be caused by a tripartite protein pump called an efflux pump that extends through the inner and outer membranes of the bacterium to pump antibiotics from the inside of the cell to the extracellular environment. In *E. coli* the efflux pump is called AcrAB-TolC. In the efforts to combat this problem, this experiment focuses on the inactivation of the AcrAB-TolC pump via organic compounds extracted from edible plants. Extracts from plants, nuts, and roots will be tested for their ability to inhibit the bacterial efflux pump. I hypothesize that naturally occurring compounds can block the activity of this pump and combat antibiotic resistance.

Acknowledgements

I would like to thank the University of Dayton Honors Department, as well as the Chemistry Department for funding my research and offering me the opportunity to further my education and scientific literacy. I would also like to thank the College of Arts and Science Deans Fellowship for allowing me the opportunity to work in collaboration with my peers on their research and provide outside of lab activities for us to enjoy. I especially want to thank Dr. Lopper and my fellow lab mates. We spent many hours together and each of them was better than the last. I look forward to working alongside you all one day. Lastly, I would like to thank life for giving me lemons. Without lemons I would not have made such significant strides in my scientific career or made lasting connections across multiple disciplines in the medical field.

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I. Introduction

Antibiotic Resistance

Antibiotic resistance is becoming the center of public health concerns worldwide (Swick). In the United States, 2 million people acquire an infection of resistant bacteria per year. At least 23,000 people die as a result of these resistant infections, and many more die due to complications (CDC). Bacteria can develop resistance to specific drugs (specific drug resistance, SDR) or to multiple drugs (multi-drug resistant, MDR). MDR bacteria are defined as being resistant to three or more classes of antibiotics (Swick). Resistance to antibiotics can be caused by many factors. One of the most significant factors is the over-prescribing of antibiotics. Prescribing antibiotics that are not necessary or not effective are some of the biggest contributing factors to resistance. Antibiotics used for livestock are also a major contributor (CDC). Livestock are given antibiotics in the efforts to prevent, control, or treat a disease, as well as promote growth. The Food and Drug Administration has taken steps to reduce the amount of antibiotics used in food production because they have not been found to be necessary (CDC). The safe and appropriate usage of antibiotics is important in combating this issue.

Bacterial Efflux Pumps

Multidrug resistance can be caused by many mechanisms, but a common mechanism is the bacterial efflux pump. The efflux pump is an energy-dependent transporter that spans from the inner to the outer membrane of gram-negative bacteria. These efflux pumps are not specific to the compounds they can transport, and they can therefore confer resistance to many different types of antibiotics (Marquez, 2005). These MDR pumps can be broken into two different types of transporters: Primary (or ABC) and Secondary (see **Figure 1**).

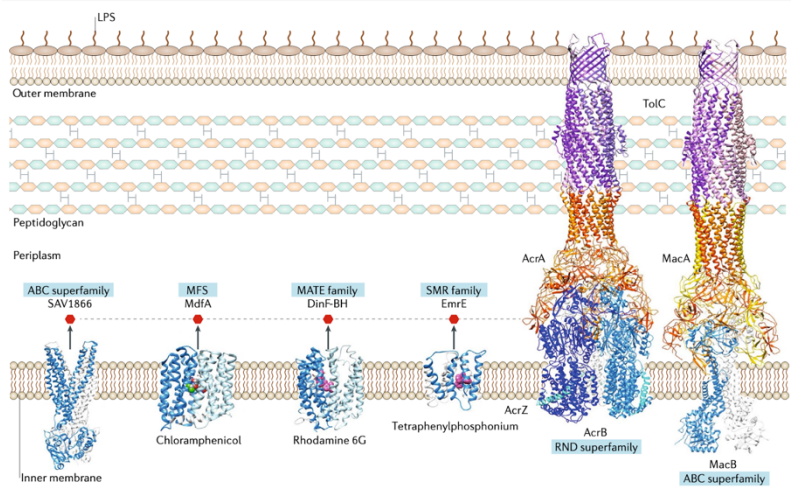

Figure 1: Figure reproduced from: Du *et. al*, 2018. Above is an illustration of the different types of efflux pumps present in gram negative bacteria. This shows the multiple types of mechanisms that can be used for MDR. The two pumps on the far right are the pumps of concern. For this experiment, the RND family was observed, specifically the AcrAB-TolC efflux pump (second from the right). A more detailed picture can be seen in **Figure 2**.

Primary transporters utilize ATP as their energy source, giving them the name ATP-binding cassette (ABC) transporters. Secondary transporters utilize an electrochemical gradient to generate energy to operate the pump. This type of pump is seen primarily with MDR transporters. Secondary transporters are broken into four different families: major facilitator superfamily (MFS), resistance nodulation and cell division (RND), small multidrug resistance (SMR), and multidrug and toxic compound extrusion (MATE) families (Marquez, 2005). The RND efflux systems are major contributors to antibiotic resistance and are particularly prevalent in *E. coli* bacteria (Marquez, 2005).

AcrAB-TolC Protein Complex

Gram-negative bacterial membranes are comprised of three layers. There is an inner membrane, periplasm, and an outer membrane. This membrane system provides the cell with a permeability barrier that contributes to the greater resistance found in gram-negative bacteria compared to gram-positive bacteria (Starvi). *E. coli*, a common gram-negative bacteria, utilizes the AcrAB-TolC system (Marquez, 2005). The membrane

transport provided by the AcrAB-TolC efflux pump is achieved by a three complex system, as illustrated below (**Figure 2**).

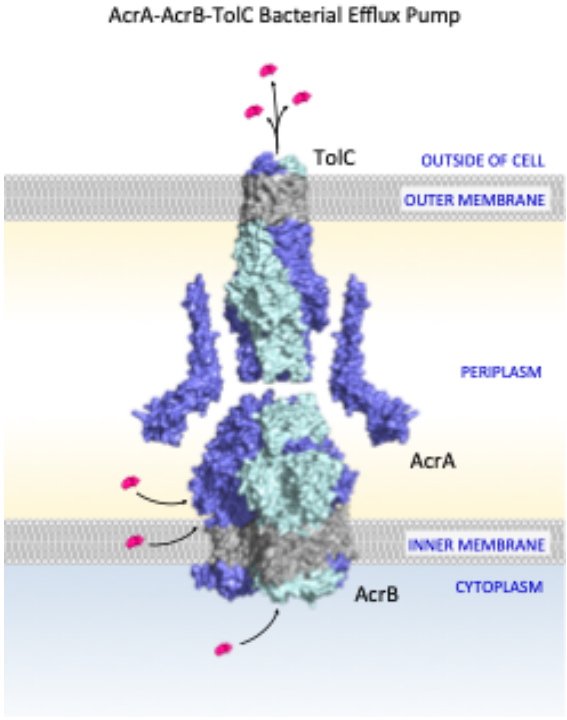

Figure 2 An illustration of the three-protein structure that spans the entire membrane and allows efflux out of the cell. This figure is an expanded view of the complex. *In vivo* the proteins would be in contact with one another. TolC is the exit protein that faces the extracellular matrix (long cylindrical protein). The inner protein, AcrB, collects compounds from the inside of the cell and the periplasm to be pumped out (bell shaped protein on the bottom). AcrA resides in the periplasm and serves as an adaptor protein that links AcrB to TolC.

The first complex is the inner membrane protein. The inner membrane protein, more widely recognized as AcrB, is energized by the proton motive force. The proton motive force is a charged gradient created at the membrane from the electron transfer chain. AcrB is followed by the second complex in the efflux system known as the membrane fusion protein, or AcrA. This protein changes conformation to create an iris-like mechanism that spans the periplasm and allows the outer membrane factor to open and close (Mikolosko, Bobyk, Zgurskaya, & Ghosh, 2006). The third and final component is the outer membrane factor. This protein, TolC, is a cylindrical channel leading to the extracellular space. The TolC protein is nondiscriminatory and can

transport anything to the extracellular space. These three complexes make up the RND membrane transport efflux pump used by Gram-negative bacteria for MDR.

Efflux Pump Inhibitors

Pump function can be altered by an efflux pump inhibitor. Inhibition can happen by a variety of mechanisms; disturbance of pump assembly, depletion of the energy source, inhibition of enzymes which maintain the proton motive force, blockage of the efflux channel, or alteration of genes (Prasch & Bucar, 2015). Any of these mechanisms can cause a decrease in the function of the pump. Efflux pump inhibitors (EPIs) can significantly decrease the minimum inhibitory concentration (MIC) of a cell. The MIC is the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism. An EPI should decrease the MIC of an antibiotic by decreasing drug efflux (Prasch & Bucar, 2015). MDR systems are not fully understood, but the EPIs are thought to bind directly to or act as substrates for the efflux pump. Through this action they can inhibit by ATP-binding, disturbing the proton motive force, or compete with antibiotics for pump access and result in antibiotic accumulation within the cell (Marquez, 2005).

I hypothesize that plants might produce compounds that can block bacterial efflux pumps and enhance the sensitization of bacterial cells to antibiotics. Plants have served as a pharmaceutical source since the beginning of medicine (Prasch & Bucar, 2015). Aspirin, a common anti-inflammatory drug, is derived from willow tree bark (Foster & Duke, 286). Native Americans discovered many plants, such as ginger, clover, or garlic, that served as interventions for various ailments at the time (Foster & Duke, 30). Many plants still serve medicinal purposes, including many antibacterial products. Plants have

to protect themselves from harmful bacteria and pathogens by providing self-produced antibacterial compounds to fend off infection and instill survival. To test my hypothesis, I will use plant extracts in a fluorescence indicated efflux assay to measure the accumulation of EtBr within the cell to determine inhibition of live *E. coli* cells.

II. Materials and Methods

A. Extracting natural compounds

Plant materials were purchased from Whole Foods (Miamisburg, OH), washed, cut into small pieces, and dried in plastic weigh dishes in an open incubator at room temperature with the incubator's fan running. Once dry, the foods were chopped further and ground with a mortar and pestle. The powders were weighed, placed in glass vials, and stored -80 degree Celsius.

Natural compounds were heat extracted using a Soxhlet extractor (**Figure 3**). Methanol was placed in the round bottom flask and secured to a ring holder. This was then attached to the extraction chamber. A paper thimble was filled with the dried and processed food before the condenser was attached. The heating mantle was adjusted to bring the methanol to a gentle boil and the mechanism was left untouched for 24 hours, or until the liquid filling the Soxhlet chamber was clear and the remaining liquid in the round bottom flask was colored. The Soxhlet chamber extracted the natural compounds by boiling the methanol in the round bottom flask and allowing the liquid to vaporize. This vapor travelled through the chamber and into the condenser. When it reached the condenser it condensed and dripped through the thimble in the chamber. This chamber filled up with warm methanol, allowing the thimble to steep in it until the arm released the liquid back into the round bottom flask and the whole process repeated. The

extraction was considered complete when the methanol filling the chamber had run colorless and the liquid in the round bottom flask was strongly pigmented. The compounds remaining in the round bottom flask were placed in a 50 mL centrifuge tube at room temperature and set under a steady stream of compressed air to dry. Once dried, the tubes were stored at -80 degree Celsius until testing.

The natural compounds were also extracted through a room temperature methanol extraction that did not involve the Soxhlet extractor, but instead used centrifuge tubes. The grams per milliliter concentrations were calculated from the heat extractions and used to create the same concentration of compound in the cold extractions. The proper amount of compound was added to 50mL of methanol and the tube was covered in parafilm. The centrifuge tubes were then placed on an orbital shaker for 24 hours. After 24 hours the solution was passed through filter paper and left to dry on large watch glasses. Once the solutions dried they were scraped using 3 mL of milliQ water and stored in a 15 mL centrifuge tube until testing.

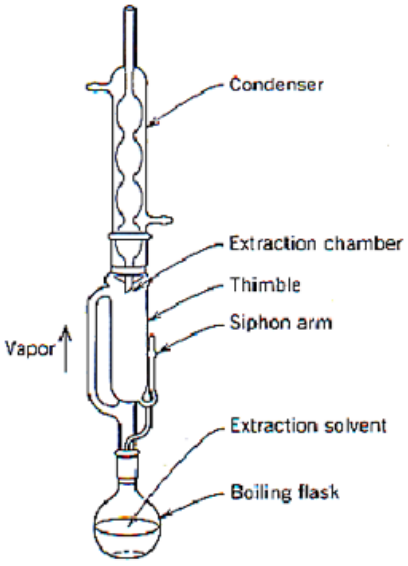

Figure 3: An illustration of the Soxhlet Extractor used to collect organic compounds from plant materials. Methanol was added to the boiling flask, the produce was added to the thimble in the Soxhlet chamber, and water was run through the condenser.

B. Room Temperature Methanol Extractions

The natural compounds were also extracted through a room temperature methanol extraction that did not involve the Soxhlet extractor, but instead used centrifuge tubes. The grams per milliliter concentrations were calculated from the heat extractions and used to create the same concentration of compound in the cold extractions.

The mass of the produce before extraction in hot methanol was taken and divided by 100. This provided a grams per mL measurement. I then tarred this much produce and placed it in a 50 mL centrifuge tube with 50 mL of methanol. The top was sealed with parafilm to secure the lid and prevent leaking. These tubes were secured in a bin and placed on the orbital shaker at maximum speed for 24 hours. The tubes were then removed and the contents were strained through a Kimwipe onto a large glass evaporating dish in the fume hood. This was left to dry. Once dried 3 mL of milliQ water was used to scrape the contents into a 15 mL centrifuge tube and stored at -80 degree Celsius until testing.

C. Ethidium bromide efflux assay

An efflux assay with the fluorescent indicator ethidium bromide (EtBr) was performed using AG100 *E. coli* cells. This assay allows the efflux of live cells to be measured by fluorescence measurements. This assay was derived from a method proposed by Coldham *et al* for an ethidium bromide accumulation assay (Coldham et al, 2010). To perform this assay, AG100 *E. coli* cells were inoculated into 3 mL of LB and incubated at 37 degrees Celsius overnight. The inoculated cells were then diluted in 25 mL of LB to and OD600 of 0.400. The cells were incubated at 37 degrees Celsius until they reached an OD600 of 0.600. Six centrifuge tubes were collected and filled with 1

mL of cells. These cells were centrifuged for 60 seconds at 15,000 rpm. The supernatant was removed and the cells were washed with phosphate buffered saline (PBS). This process was repeated once more. After the third and final centrifuge, the supernatant was removed and the cells were suspended in PBS at an OD600 of 0.100. Two milliliters of cells were removed and placed in a heat block set at 90 degrees Celsius for 10 minutes. These cells acted as a heat-killed positive control.

The other positive control used was chlorpromazine (CPZ). This was made by adding 0.05329 grams (355.33 g/mol) to 1 mL milli Q water to create a concentration of 150 mM. This solution was suspended and 6 mL was removed and placed in a separate tube with 94 mL of milliQ water. This created a CPZ concentration of 9 mM.

Ethidium bromide was prepared by removing 31.45 mL of 50 mM stock solution and diluting it with 1 mL of milliQ water to create a stock solution of 50 mL. This solution was kept out of light and in the refrigerator to avoid decomposition.

All of the prepared solutions were then placed in a black, flat-bottom 96 well plate for reading. 180 mL of cells were added to columns 1, 3-12. In column 2 180 mL of heat killed cells were added. Next, 10 mL of crude extract was added to columns 4-12. To keep the volumes equal 10 mL of water was added to columns 1 and 2. In column 3 10 mL of CPZ was added. The plate was then inserted for a blank measurement when the reader reached 37 degrees Celsius. Measurements were taken at an excitation measurement of 515 nm and an emission measurement of 600 nm. (An illustration of this is pictured below, **Figure 4**) The plate reader then ejected the plate and 10 mL of EtBr solution was added to all wells. The plate reader was then run taking measurements every 2 minutes for 30 minutes. This process was repeated for all of the crude extracts.

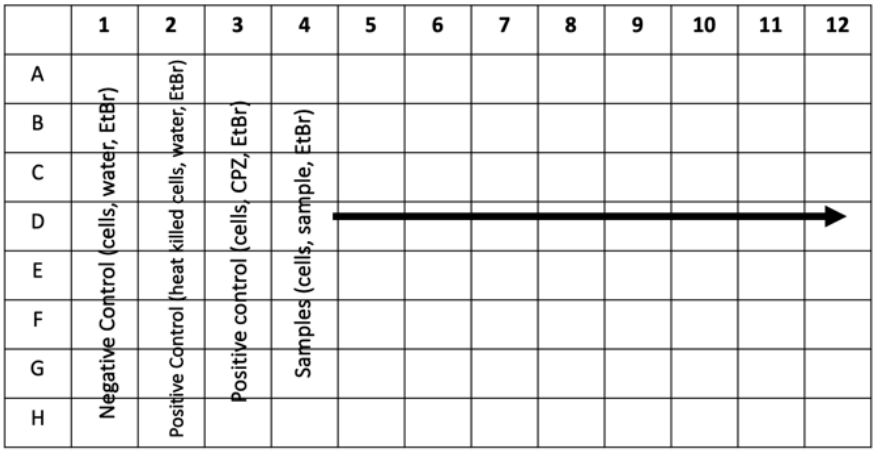

Figure 4: An illustration of the well plate used in the ethidium bromide efflux assay.

The same process as listed above (subheading C) was conducted for all 96 fractions. Instead of crude sample, 10 mL of each fraction was added. One well was done for each fraction instead of eight replications.

D. High Performance Liquid Chromatography (HPLC)

The crude extract was fractionated by high performance liquid chromatography (HPLC). A volume of 50 mL of extract was diluted by the addition of 450 mL of milliQ water, the solution was clarified by centrifugation at 15,000 rpm for 1 min at room temperature and the supernatant was loaded onto a (19 x 50mm) preparatory C18 column that had been pre-equilibrated with an aqueous solution of 0.1% trifluoroacetic acid (TFA). The column was resolved at 2.5 mL/min using a linear gradient of water plus 0.1% TFA to methanol plus 0.1% TFA over a period of four hours and 2.5 mL fractions were collected. Absorbance was measured at 280 nm. The fractions were placed on small glass evaporation dishes and allowed to dry at room temperature. The dried films were scraped into 500 mL of MilliQ water and stored in centrifuge tubes at -80 degree Celsius.

F. Liquid broth microdilution assay

A liquid broth microdilution assay was conducted to measure the clinical relevance of the efflux assay findings. AG100 *E. coli* was inoculated into 3 mL of MHB and incubated at 37 degrees Celsius overnight. The saturated cultures were used to inoculated 25 mL of MHB and the cells were grown to an OD600 of 0.400. The MHB used included 10.4 g of MHB powder, 0.0158 g $CaCl_2$, 0.0200 g $MgCl_2$. ML 342 cells The cells were incubated until a reading of 0.600 was achieved at OD600. The cells were then diluted to an OD600 of 0.100 with MHB (0.5 McFarland).

Carbenicillin with a starting concentration of 2.9 μg/mL was used to create a 64 μg/mL stock solution. This was done by adding 0.22 mL of 70 μg/mL carbenicillin to 978 mL of MilliQ water.

A 48 well plate was used for this experiment. The experimental wells contained 300 mL of 0.5 McFarland AG100 *E. coli* cells in MHB, 87.5 mL of MHB, 50 mL of the test compound, and 62.5 mL of 64 μg/mL carbenicillin. The control wells had 300 mL of 0.5 McFarland AG100 *E. coli* cells in MHB, 150 mL of MHB, and 50 mL of the test compound. Wells containing 137.8 mL of MHB and 62.5 mL of 64 μg/mL carbenicillin were also present to compare the extract samples to a normal 8 μg/mL carbenicillin growth curve. This plate was then placed in the plate reader at 37 degrees Celsius and an absorbance reading was taken at 625 nm every 5 minutes for 16 hours. Before each measurement the plate reader was set to shake for 30 seconds to avoid sedimentation.

III. Results and Discussion

A. Ethidium Bromide efflux assay of crude extract

I tested my plant extracts for their ability to block bacterial efflux pumps using an ethidium bromide-based efflux assay. EtBr has strong fluorescence properties when bound to DNA and it is known to use the efflux pump to exit the cell. A normal, uninhibited bacterial cell would efflux the EtBr, which would result in a low fluorescence measurement because the EtBr would not accumulate inside the cell. A cell with inhibited efflux pumps would be unable to efflux the EtBr out, causing an increased fluorescence within the cell. In **Figure 5**, the water control represents uninhibited cells that pumped the EtBr out. CPZ was the positive control that should inhibit efflux pumps. The CPZ control shows an increase in inhibition over time. The heat-killed cells should be unable to efflux, thus trapping the EtBr in the cell and showing a high level of fluorescence. The lemon peel extract shows an increase in fluorescence that mimics that seen by CPZ, indicating a significant inhibition of the efflux. All of the other plant extracts that I tested failed to cause an increase in fluorescence (data not shown).

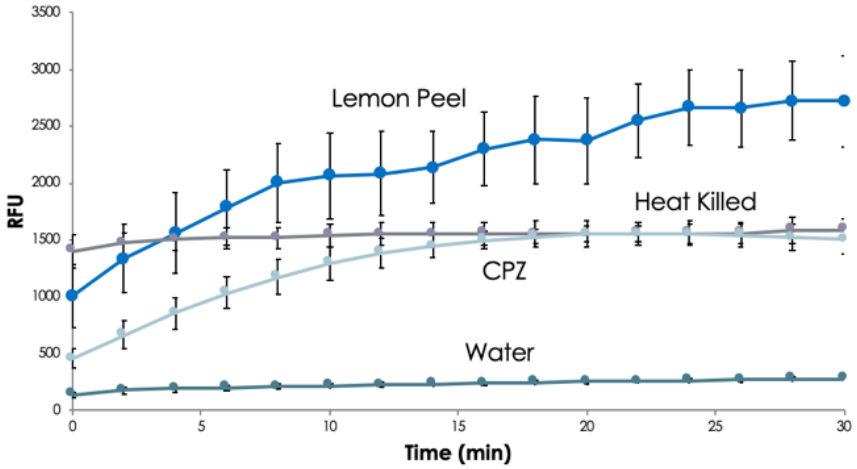

Figure 5: The line on the bottom is the water control. This flat line near zero indicates efflux by a normal healthy cell. The flat line on the top is the heat killed cell. This is showing a cell unable to efflux, therefore the EtBr was trapped in the cell and caused an increase in fluorescence. The sloped line in the middle is the CPZ control. This line shows inhibition, and an increase in fluorescence over time. The line that slopes towards the top is the lemon peel extract. It follows the same shape of the CPZ, showing increased inhibition over time.

B. HPLC of crude extract

Several experiments were ran but the crude extract proved to complicate the results. The crude extract of lemon peel is a complex mixture that likely contains many compounds. My goal was to find out which compound inhibited efflux pump activity. I fractionated the crude extract using the HPLC so the proper compounds could be identified. The extract was run through the HPLC to separate the compounds to allow for accurate readings. The separation resulted in 95 tubes with 2.5 mL of clear solution in them. The HPLC took readings of the sample at 280 nm as it was leaving the column. The chromatogram below (**Figure 6**) shows the HPLC results.

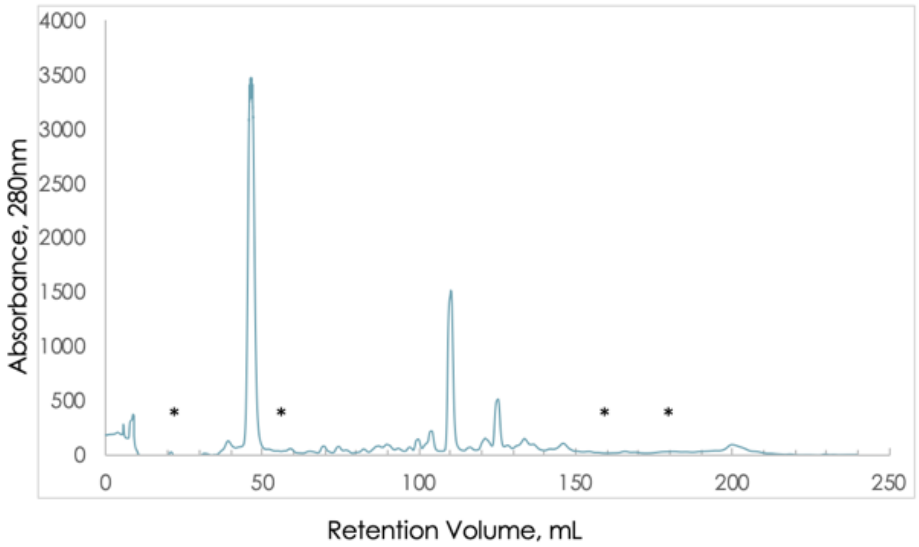

Figure 6: The HPLC chromatogram shows peak measured at 280 nm. The asterisks represent the active fractions found in the crude lemon peel extract.

C. Ethidium Bromide efflux assay of HPLC separated compounds

Each HPLC fraction was tested in the ethidium bromide-based efflux assay. The same controls as before were used. The overall difference in fluorescence from $t = 0$ to t

= 30 min for each compound is shown in a bar graph below (**Figure 7**). An increase of fluorescence, as indicated before, is a way of showing inhibition of the efflux pump. As shown, some lines barely increased in RFU and were excluded from the remainder of experiments. HPLC fractions 9, 24, 68, and 72 produce an increase in fluorescence that appeared to be greater than that of the other fractions so they were chosen for further study.

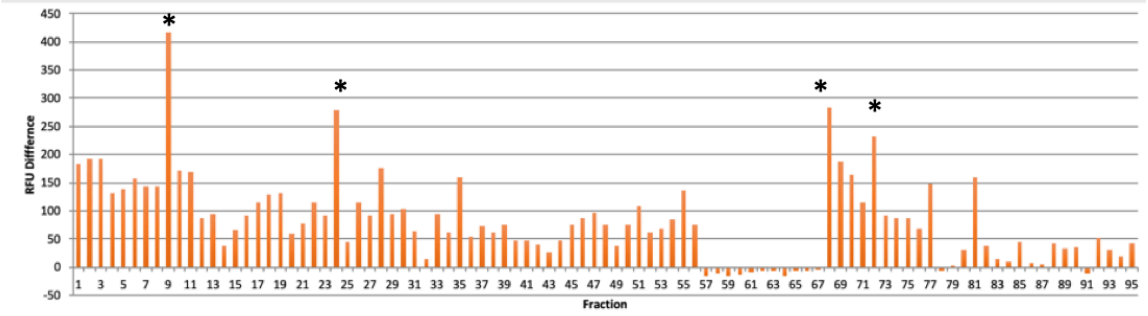

Figure 7: This bar graph shows the difference in fluorescence measurement from start to finish of the EtBr efflux assay for each compound. The compounds with the greatest increase indicate the greatest inhibition and were used for future experiments.

D. Broth microdilution assay results

The EtBr efflux assay provided information on the extract's ability to visualize efflux activity, but does not provide much insight on the clinical relevance of this methodology. The liquid broth microdilution assays allowed for the testing of antibiotic accumulation in the cell to test the clinical relevance of the extract in sensitizing the cell to antibiotic. Concentrations of the antibiotic carbenicillin were measured for use in the experiment. A growth curve that most mimicked an "S"-shaped cell growth curve was selected for. In the graph below (**Figure 8**), the concentration of carbenicillin can be observed. A concentration of 24 µg/mL resulted in a flat line from start to finish (bottom curve in Figure 8). The absence of carbenicillin results in a normal, uninterrupted cell growth curve (top curve in Figure 8). For my experiment, I was looking for a growth curve that showed cell death by antibiotic, but also mimicked a normal curve so that a

change in cell proliferation could be observed. A concentration of 8 µg/mL carbenicillin was chosen because of its effect on cell death as well as its ability to show a change in cell growth curve with differences in inhibition.

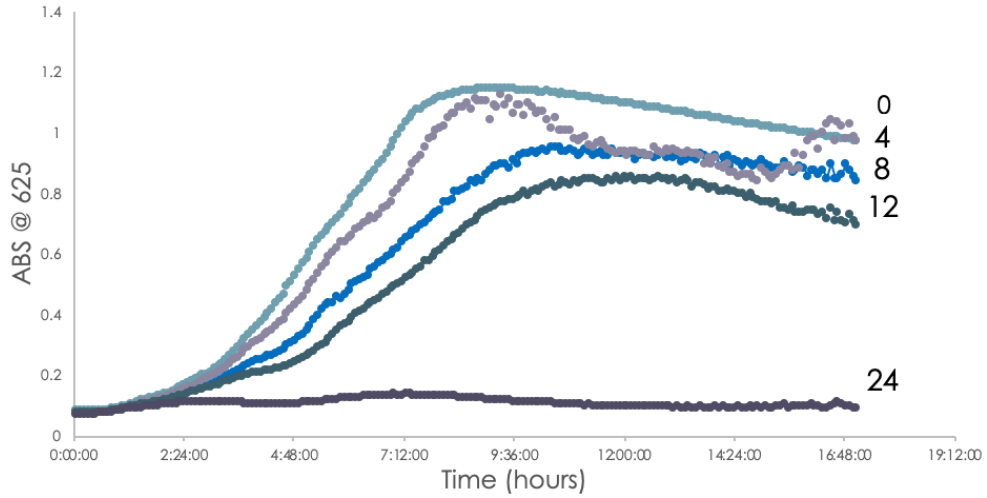

Figure 8: This graph shows the experimental testing of concentrations of carbenicillin in the presence of *E. coli* cells. The line on the bottom is a concentration of 24 µg/mL and shows complete cell death. The line on the top is a concentration of 0 µg/mL of carbenicillin, this shows a regular cell growth curve. For my experiments I used the 8 µg/mL concentration. This concentration of carbenicillin mimics a regular cell growth curve, as well as allows for measurement of inhibition and the increase or decrease of cell death in the presence of the lemon peel compounds.

This concentration, alongside lemon peel compounds, was tested in a liquid broth microdilution test. Each compound was tested alone with *E. coli*, as well as alongside 8 µg/mL carbenicillin to show a shift in growth curve. With successful inhibition, the growth curve of the cells in antibiotic and extract would be shifted right, indicating decreased cell growth.

In Figure 9, it can be seen that the growth curve of the *E. coli* was shifted right, indicating increased inhibition in the presence of this compound. This graph shows the inhibition of efflux in the cells, causing the cells to become more sensitive to the antibiotic, resulting in cell death. This result further indicates the success of lemon peel extract in inhibiting the efflux of *E. coli* cells, implying further the clinical relevance of these compounds in combating antibiotic resistance.

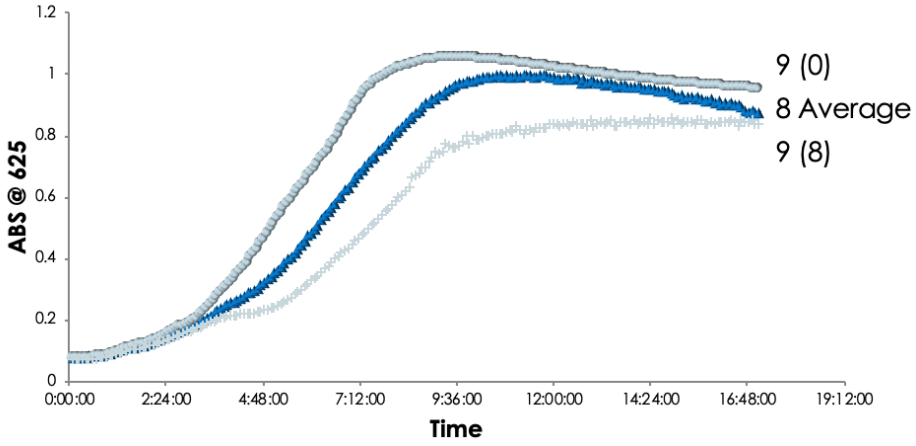

Figure 9: This graph shows the cell growth curves of *E. coli*. The top curve is cells with compound 9. This should be a regular cell exhibiting a regular cell growth curve. The middle line is the cells with 8 µg/mL carbenicillin. This is the standard curve used to compare the effects of the compounds alongside antibiotic. The bottom line shows this. The curve shows a rightward shift, indicating increased sensitization to the antibiotic, and cell death.

The same result as before can be seen in the following graphs (**Figure 10**, **Figure 11**, **Figure 12**). Compound 24 caused the cell to become more sensitized to the antibiotic and resulted in an increase in cell death.

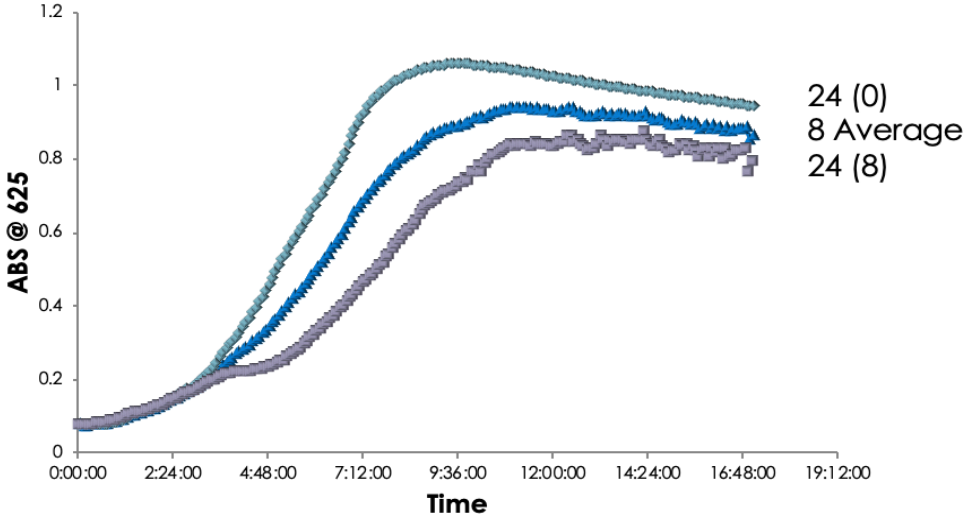

Figure 10: This graph shows the cell growth curves of *E. coli*. The top curve is cells with compound 24. This should be a regular cell exhibiting a regular cell growth curve. The middle line is the cells with 8 µg/mL carbenicillin. This is the standard curve used to compare the effects of the compounds alongside antibiotic. The bottom line shows this. The curve shows a rightward shift, indicating increased sensitization to the antibiotic, and cell death.

Compound 68, as seen in **Figure 11**, caused the cell to become more sensitized to the antibiotic and resulted in an increase in cell death.

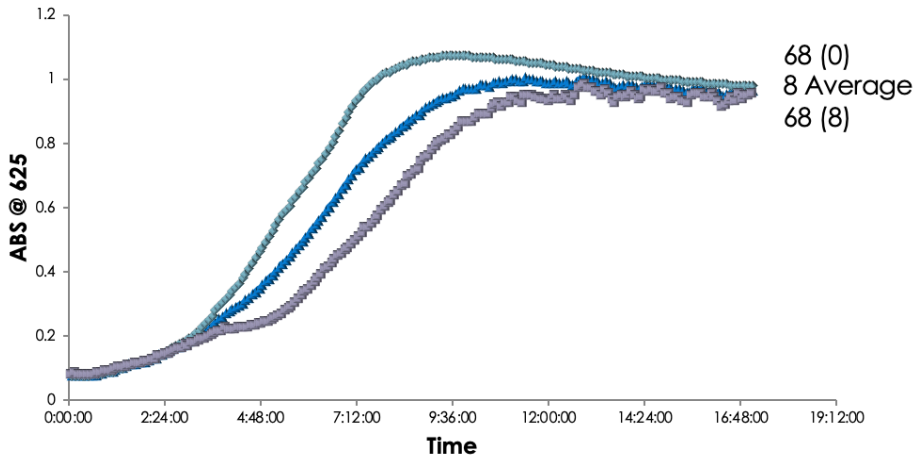

Figure 11: This graph shows the cell growth curves of *E. coli*. The top curve is cells with compound 68. This should be a regular cell exhibiting a regular cell growth curve. The middle line is the cells with 8 µg/mL carbenicillin. This is the standard curve used to compare the effects of the compounds alongside antibiotic. The bottom line shows this. The curve shows a rightward shift, indicating increased sensitization to the antibiotic, and cell death.

Compound 72, as shown in **Figure 12**, caused the cell to become more sensitized to the antibiotic and resulted in an increase in cell death.

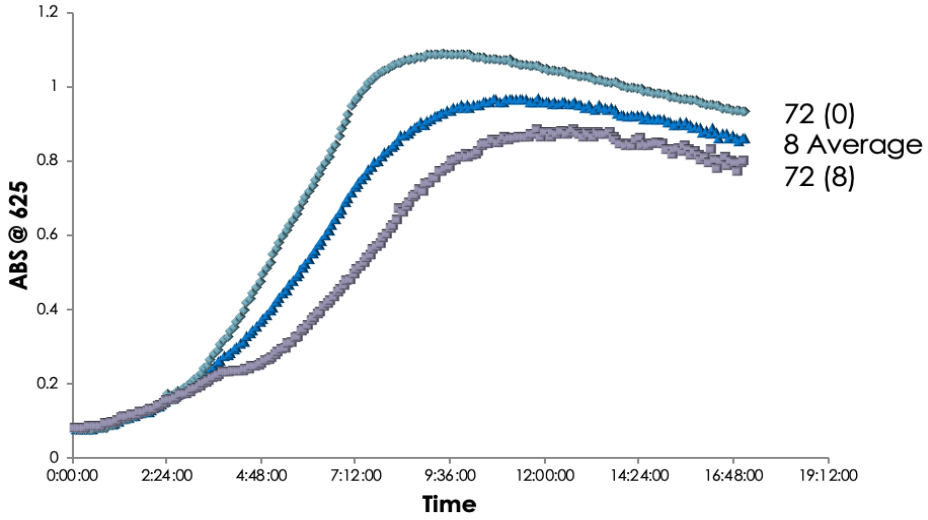

Figure 12: This graph shows the cell growth curves of *E. coli.* The top curve is cells with compound 72. This should be a regular cell exhibiting a regular cell growth curve. The middle line is the cells with 8 µg/mL carbenicillin. This is the standard curve used to compare the effects of the compounds alongside antibiotic. The bottom line shows this. The curve shows a rightward shift, indicating increased sensitization to the antibiotic, and cell death.

E. Conclusions

The results of my experiment show the ability of lemon peel extract to inhibit the efflux pump of *E. coli* bacteria. Using this information, the compounds of lemon peel

extract were tested for clinical relevance. The results of utilizing lemon peel to inhibit the pump, as well as antibiotic to interfere with cell proliferation were successful. The results of these tests indicate the ability of the compounds in lemon peel to sensitize the cell to antibiotic and aid in combating antibiotic resistance.

Future research should be aimed at identifying the compounds in HPLC fractions 9, 24, 68, and 72. I could be the case that these are four different compounds, or potentially the same parent compound that may have several variant forms. Research in the future could also be done to determine the cytotoxicity of the lemon peel extract in crude form, as well as each of the individual compounds from HPLC fractionation. Future research should also be done to determine the overall mechanism of action that lemon peel extract compounds use in increasing the sensitivity of bacterial cells to antibiotics. This can be done by testing cells without TolC, one of the proteins involved in efflux, to determine if the lemon peel extract inhibits that pump directly.

IV. References

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