Determining the Effects of Pinene on *Escherichia coli* Infected Epithelial Cells

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Abstract

The purpose of this experiment was to determine the inhibitory effects of pinene on *Escherichia coli* infected epithelial cells. This study was conducted in an attempt to discover a novel therapeutic and anti-bacterial approach to umbilical cord infections. Methods used included the MTT assay and scratch wound assay to test for cell metabolic activity and proliferation rates, respectively. All 96-well and 24-well plates were infected with *E. coli* (10^6 CFU/ml) and introduced to pinene ([10 µg/mL]), and three standard groups were set with equal concentrations. Contrary to predicted outcomes, pinene did not reduce *E. coli* proliferation rates, thus allowing the bacteria to flourish and kill cells. Conversely, in the cell standard, pinene allowed cells to grow and produced near tissue-like growth. Future studies could address the discrepancies with the pinene concentrations being too low to affect *E. coli* cells. In conclusion, we were unable to effectively use pinene as a therapeutic and anti-bacterial to mitigate the effects of *E. coli* infected epithelial cells.

Introduction

Escherichia coli (E. coli)

Escherichia coli (E. coli) is a gram-negative, facultative anaerobic bacterium, commonly found in the gastrointestinal tract of humans and acts as a harmless normal flora (Lim et al., 2010). Despite this, some strains can evolve to pathogenic *E. coli,* and is commonly associated with food poisoning, causing dysentery, urinary tract infections, and other related illnesses. According to the Canadian Encyclopedia (2018), approximately 470 Canadiens are infected with *E. coli* each year, mostly from food poisoning. In some instances, however, *E. coli* has been found to cause neonatal meningitis, mostly in underdeveloped countries, leading to low birth weights, premature babies and death (Ku et al., 2014). Furthermore, *E. coli* is one of the leading causes of bacterial umbilical cord infections, of which are the single most important cause of neonatal mortality (Mullany et al., 2005). While umbilical cord care would be the default line of defense to prevent such infections, it would be advantageous to investigate potential therapies and cures for post-infection scenarios.

Pinene

With the recent legalization of marijuana in Canada, many have argued its capacity to provide therapeutic and medical benefits. Moreover, studies pertaining to marijuana have been conducted for decades. Aromatic compounds, such as terpenes have been commonly found in cannabis plants, and have shown many medicinal properties such as anticoagulant, anti-inflammatory, antioxidant and antibiotic resistant effects (Park et al., 2021).

Pinene, a terpene most abundant in nature and smelling like pine, is commonly found in cannabis. Current research has been focused on cannabidiol (CBD) and tetrahydrocannabinol (THC) content in plants; however some studies have begun studying the effects of terpenes for medicinal uses (Green et al., 2021). Research has been conducted on the ‘entourage effect’ of

terpenes and cannabinoids, finding that an increased amount of terpenes in cannabis leads to greater activation of signaling pathways in human cells (Ferber et al., 2020). If terpene content was increased in cannabis products, it could provide greater therapeutic benefits to already existing components (Ferber et al., 2020).

While consuming cannabis products is discouraged when pregnant (Roncero et al., 2020), evidence on pinene's therapeutic benefits, and its ability to reduce infections, could be introduced to umbilical epithelial cells to reduce possible infections and prevent new *E. coli* growth. In this experimental study, we investigated the effects of pinene on the ability to mitigate *E. coli* infected epithelial cells, harvested from human umbilical cords. We chose this combination as there was current research on the effects of pinene reducing bacterial infections, as well as potential ways to reduce *E. coli* infections in epithelial cells.

Materials and Methods

Cell culturing

Ea.hy926 endothelial cells were obtained from a pre-prepped and subcultured T25 flask. 16 flasks were required to produce the desired number of cells for final testing. The initial T25 flask provided was subcultured into two new T25 flasks. Using an inverted microscope, the cells were viewed to ensure confluent growth and adhesion to the flask surface. Once it had been determined that the flask(s) had confluent growth, the media was removed and the cells were rinsed with PBS twice. 2 mL of trypsin was added. The flask was incubated for 2 minutes or until the cell lifted from the flask bottom (determined with inverted microscope). To deactivate the trypsin, 3 mL of complete L-15 media at 10% FBS was added to the solution. The solution was centrifuged at 300 rcf for 10 minutes or until a pellet had formed. The solution was poured

off and the pellet was resuspended in 1 mL of complete media. The pellet was completely dissolved in this media, and counted using a hemocytometer, to determine the number of flasks needed. 5 mL or 8 mL of complete media was added to the tube and mixed. The solution was divided into 2-3 new T25 flasks, with 3 mL media and incubated at 37℃. Cells were fed every 2 days by removing 1 mL of solution and replacing with 1-1.5ml of L-15 at 10% media. This process was repeated every 10-14 days depending on cell growth until 16 T25 flasks with confluent growth had been obtained.

Epithelial cell test preparation

The cells were trypsinized as in the previous sub-culturing process to lift and resuspend the cells. A count of the cells was taken by hemocytometer and 4 T25 flasks worth of cells were transferred into one 15 mL centrifuge. The endothelial cells were then serially diluted into 4 different dilutions of cells from 10^5, 10^4, 10^3 and 10^2 cells/mL.

E. coli bacterial cell preparation

An *E. coli* inoculum was taken from a provided culture plate and suspended in antibiotic free L-15 media. The *E. coli* was prepared to a standard of 10^6 CFU/mL for all tests (Menzies et al. 1998).

Pinene dilution

Pure pinene was diluted down to a cell viable concentration of 10 µg/mL (Hou et al. 2019). The pinene was diluted into standard L-15 at 10% FBS.

MTT assay

96 well plates in triplicate were used for this assay. Each 96 well plate contained 4 tests as follows, cell, cell + pinene, cell + *E. coli* and Cell+ *E. coli* + pinene. The four dilutions of

endothelial cells were plated out with clear separation for each test. After plating of endothelial cells, the plates were incubated for 24 hours at 37˚C. Following incubation all wells had media removed and were washed twice with 100 µL of PBS. The *E. coli* test wells had 100 µL of *E.coli* standard solution added to them while the other wells had 100 µL of antibiotic free L-15 media, the plates were incubated for 2 hours at 37˚C. All media was removed from the 16 wells per plate and lysozyme at 1 mg/ml for 20 minutes was used to lyse the remaining extracellular *E. coli* that was remaining. All wells were twice washed with 100 µL of PBS and then fed 200 µl of either L-15 media standard or L-15 media containing the diluted pinene. 4 wells also contained L-15 media only and no epithelial or *E. coli* cells. The plates were then incubated for 48 hours at 37˚C, due to the small volume of media in 96 well plates, 50 µL of L-15 was added after 24 hours to avoid drying out the wells. 10 µL of MTT reagent at 5 mg/mL was added to all wells and incubated at 37˚C for 4 hours. The reaction was halted by removing all media and washing the wells with PBS, then adding 100 µL of M199 clear Media. 24 hours later 100 µL of DMSO was added to each well and the plates were incubated at 37˚C for 30 minutes. Absorbance readings of 539 nm and 621 nm were taken in triplicate of each plate.

Scratch wound assay

Triplicate 24 well plates were prepared by scoring the underside of each well in a + shape. Each 24 well plate contained 4 tests as follows, cell, cell + pinene, cell + *E. coli* and Cell+ *E. coli* + pinene. The four dilutions of endothelial cells were plated out with clear separation for each test. After plating of endothelial cells, the plates were incubated for 24 hours at 37˚C. Following incubation all wells had media removed and were washed twice with 400 µL of PBS. The *E. coli* test wells had 400 µL of *E. coli* standard solution added to them while the other wells had 400 µL of antibiotic free L-15 media, the plates were incubated for 2 hours at 37˚C. All media was

removed from the wells and lysozyme at 1 mg/ml for 20 minutes was used to lyse the remaining extracellular *E. coli* that was remaining. All wells were twice washed with 400 µL of PBS and then fed 400µl of either L-15 media standard or L-15 media containing the diluted pinene. Following 48 hours of incubation at 37˚C a scratch in each well was done using a single stroke of a 200µL pipette tip. Using an inverted microscope an initial measurement was taken using the digital measuring tool provided by the microscope's tools. After 24 hours another measurement was taken using the same method for each well and dilution of endothelial cells.

Statistical analysis

All data manually compiled on Excel.

Results

MTT Assay

There was a great deal of variation in the MTT assay results. As seen in Table 1, there is no trend between concentrations of cells for any of the 4 tests. Absorbance readings should have trended down with lower concentration of cells. The 10^4 cell concentration is the greatest cause of this disturbance in the expected absorbance trend. Cell + pinene test had the highest average absorbance over all of the concentrations of cells. Cells alone had the lowest average absorbance values in both 621 nm and 539 nm wavelengths. Figures 1 and 2 are difficult to interpret with the high degree of variation of the MTT absorbance values. It does, however, show that there are higher absorbance values when cells are grown with pinene and for cells that are infected with *E. coli.* When cells are infected with *E. coli* and then treated with pinene they perform similarly to our control of just cells without treatments. Some cells lived, and some cells died.

	Test Type	Number of endothelial cells			
621 nm absorbance value		10^5 cells	10^4 cells	10^3 cells	10^2 cells
	cell	0.088	0.093333	0.083	0.083
	cell + E.coli	0.113667	0.094	0.099333	0.088667
	cell+ pinene	0.135333	0.106667	0.110667	0.097667
	cell+pinene+ E.coli	0.097	0.087	0.093	0.109
539 nm	cell	0.163667	0.181667	0.158333	0.151333
	cell + E.coli	0.217333	0.164667	0.169	0.120333
	cell+ pinene	0.271667	0.192	0.195333	0.168333
	cell+pinene+ E.coli	0.181333	0.153	0.158	0.143667

Table 1. Average absorbance results for each test and cell count at 621 nm and 539 nm. This was done on triplicate plates with triplicate measurements resulting in 9 measurements per test at each wavelength.

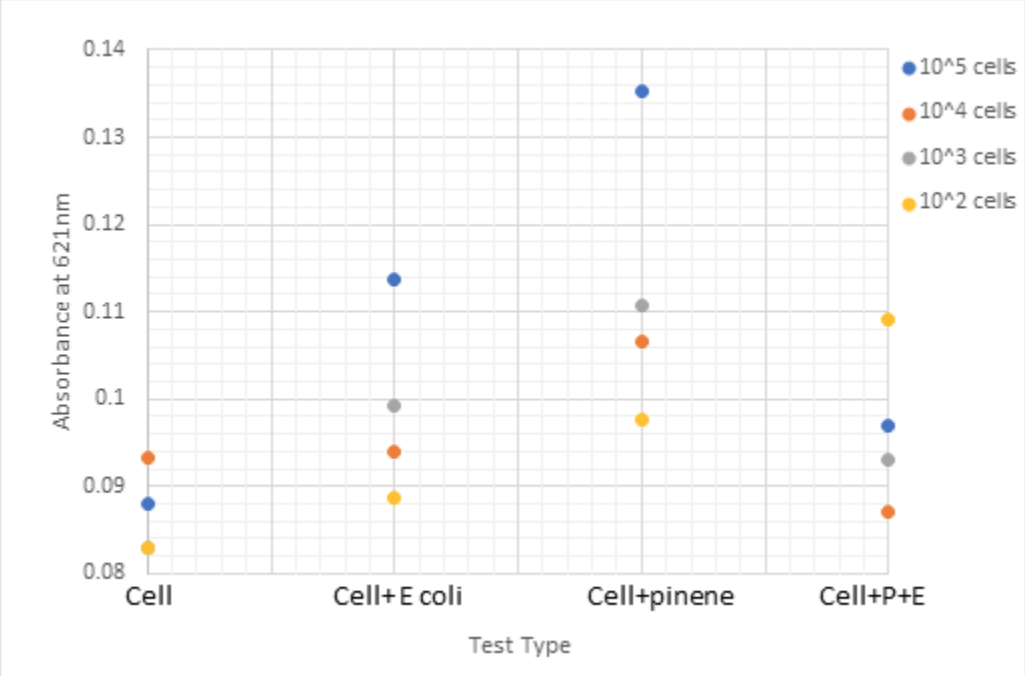

Figure 1. MTT average absorbance values at 621nm of E. coli infected endothelial cells

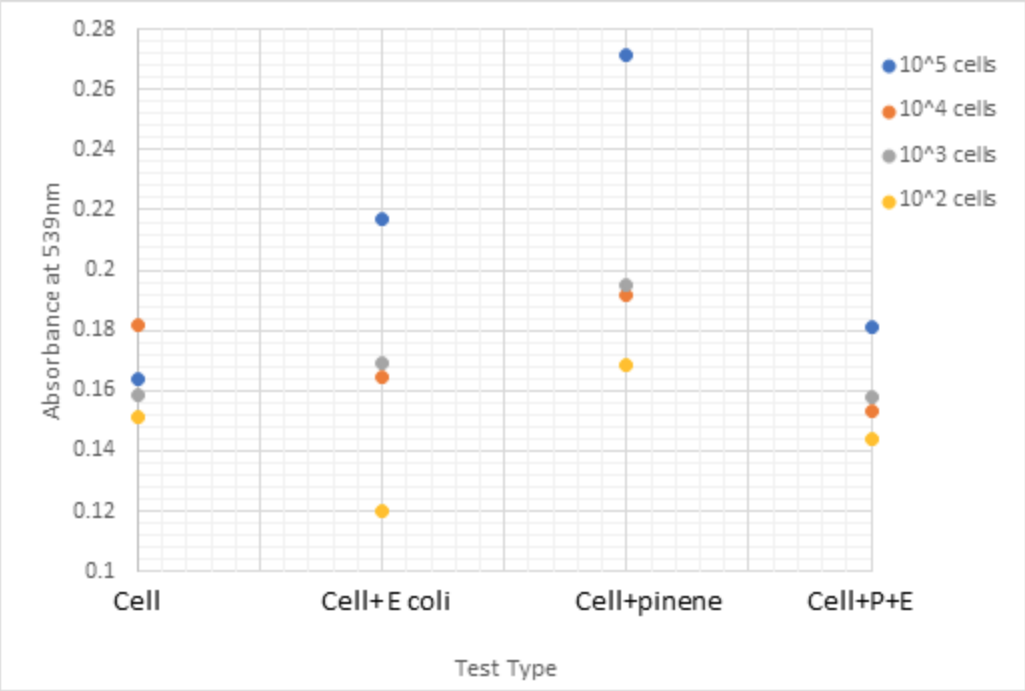

Figure 2. MTT average absorbance values at 539nm of E. coli infected endothelial cells.

Scratch test

The scratch test data is not valuable as none of our images contain points of measurement that are consistent from before and after scratching. Visually, they did show growth, but as shown in Figure 3 and 4 of the cell + pinene test, these before and after photos either do not have reference measurement scales or are not taken at the reference point. Without references being the same from before and after no assumption or measurements of cell recovery can be made.

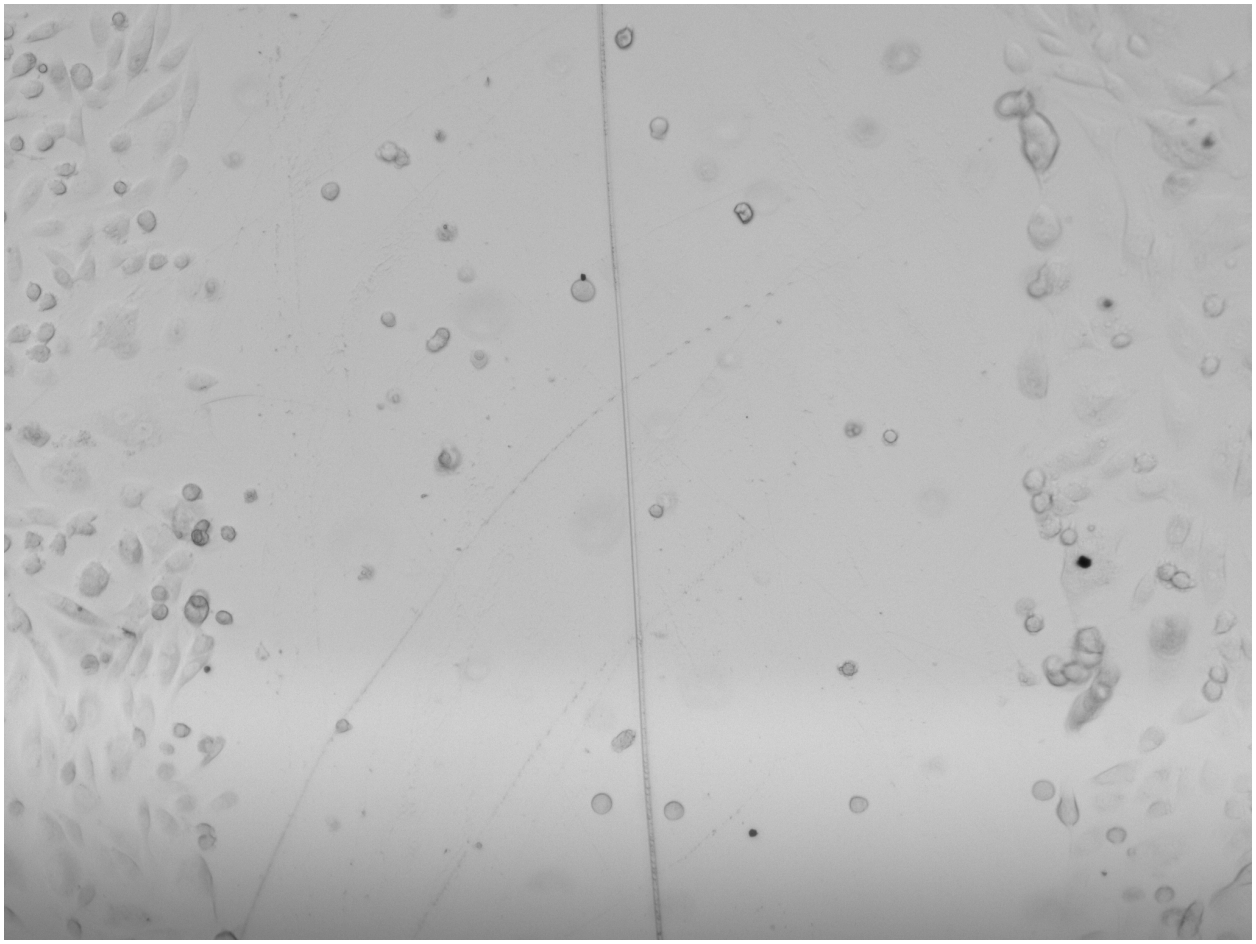

Figure 3. Initial scratch wound of cell + pinene treatment. The image shows a good scratch but with no measurement bars defined

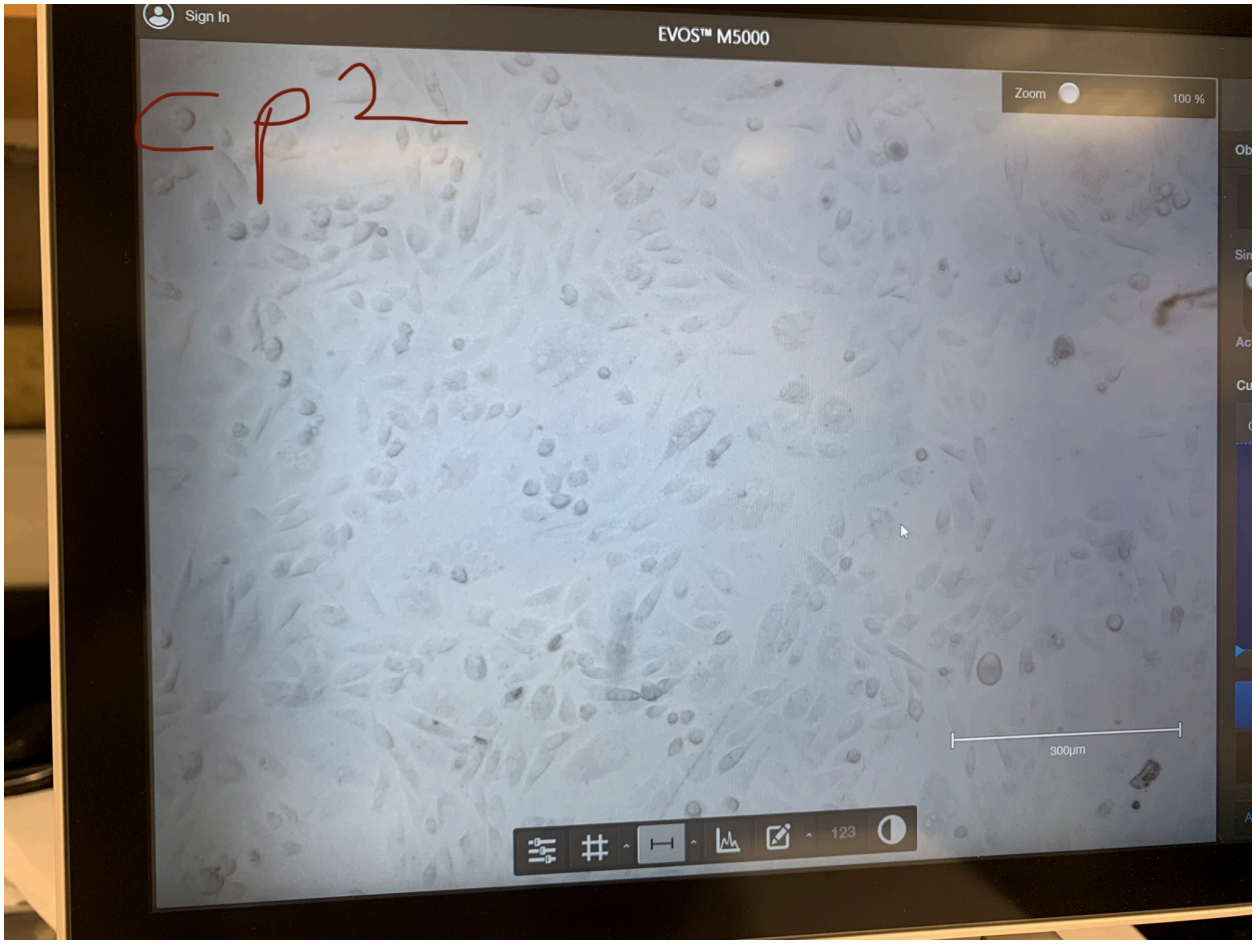

Figure 4. After scratch image of cells + pinene treatment. Image taken not in reference to scratch but with measurement bars.

Discussion

We did not observe the results we expected in this experiment. Originally, we had proposed to test the effects of pinene on *Candida albicans,* as there was primary research conducted by Nóbrega et al., suggesting the antifungal actions of pinene against *Candida* strains. Upon further discussions, we determined that *Staphylococcus aureus* would provide better invasion and have a better survival rate within epithelial cells, as per Menzies & Kourteva. Ultimately, we chose *Escherichia coli* as our bacteria as we did not have lysostaphin to kill the *Staph aureus*. We did have access to lysozyme, which lyse *E. coli*, but not that of *Staph aureus*, which primarily drove our experiment to use *E. coli.*

After running multiple tests, as seen above, the experiment demonstrated pinene did not induce *E. coli* apoptosis, and it had the opposite effect - increasing cell and *E. coli* proliferation. The small concentration of pinene showed increased cell proliferation rates, especially in plates with only cells and pinene. In reference plates with cells and *E.coli,* we saw a decrease of cells as the *E. coli* invaded and induced cell apoptosis. This is primarily due to the concentration of pinene being too low. Basing our concentrations off a 2019 study by Hou et al., we determined that using a lower concentration showed the most cell viability percentage after 0-24 hours.

This current study would need to be revised in order to be conducted again, and to provide any therapeutic benefits for those suffering from umbilical epithelial infections. Future work could be conducted in order to determine the appropriate levels of pinene required to reduce infections. Further studies are also required to determine the viability of this experiment, and whether it would provide therapeutic benefits.

As cannabis is a relatively new introduction to the medical field, further work would be required in order to comprehend the terpenes present and how they could possibly contribute to lower infection rates. Proper dosage, as well as the best method to combat said infections, would also need to be determined.

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Notes

Nicole: Abstract, Introduction and Discussion
Matt: Materials & Methods and Results

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