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Quorum Quenching Potential of Novel Phytochemicals on *Chromobacterium violaceum Quorum* Sensing

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ABSTRACT

The purpose of this project was to evaluate the antibacterial potential of phytochemicals rutin, syringic acid, and *Ziziphus mucronata* extract on *Chromobacterium violaceum quorum* sensing. *Z. mucronata* extracts were imported from South Africa, then extracted in the lab; a SEM imaging series was conducted to evaluate for contamination and further develop a portfolio on it. The null hypothesis was if plant compounds are tested individually, then they will have similar efficiency as current antibiotics; the alternative hypothesis was if plant compounds are tested individually, then they will have greater efficiency than current antibiotics. While the alternative hypothesis was supported by comparing averages, not all of the results had a significant difference to the positive control. The three assays used in the experiment were swarming motility, violacein inhibition, and biofilm inhibition. Throughout the assays, *Z. mucronata* leaf, and bark extract had the strongest antimicrobial results: both performed statistically stronger than the positive control in at least one assay. In the violacein inhibition assay, *Z. mucronata* leaf extract had a statistically significant difference to the gentamicin. Additionally, *Z. mucronata* bark extract had a statistically stronger antibacterial effect to the gentamicin in regards to biofilm inhibition. The results of this project suggest that all of the plant compounds tested had possessed antimicrobial potential as they performed better than the negative control in most assays, but they did not consistently perform stronger than the positive control. The results of this project support the case to further expand on these phytochemicals from an antimicrobial standpoint.

Introduction

Background

Quorum sensing is defined as the process through which bacteria communicate within a colony and with surrounding colonies. Molecules termed N-Acyl homoserine lactones, often referred to as AHLs, permit gram-negative bacterial species to coordinate immensely complex responses. The AHL system operates in a positive feedback loop through which bacteria release and receive AHL molecules; eventually, the AHLs in the bacterial environment achieve the required concentration the bacteria need in order to sustain synchronous actions, including the development of virulent factors leading to bacterial infection within a host (Asfour, 2018). Often, bacterial infections are treated through administering an antibiotic series targeting a specific species. However, due to the overuse of antibiotics, many infectious bacteria have evolved antibiotic-resistant mutations. One of the main attributing causes of this developing crisis is the intense selective pressure placed on bacteria when current antibiotics (gentamicin, ciprofloxacin, penicillin, etc.) inhibit bacterial growth to eliminate whole colonies (Li, Zhang, et.al, 2019). To counteract the growing problem of multi-drug resistant bacteria, the research of novel drugs termed "*quorum* quenchers" has become a rapidly expanding industry; these drugs aim to inhibit

bacterial pathways as opposed to inhibiting bacterial growth. Prior studies suggest this method exerts a lower selective pressure than current antibiotics, so the bacteria would take more time to develop antibiotic-resistant mutations (Mohabi, Kalantar- Neyestanaki, Mansouri, 2017).

The bacterium, *Chromobacterium violaceum,* is a gram negative bacteria often employed in *quorum* sensing studies due to its unique purple color once it reaches *quorum*. Through the CviR/CviI pathways, *C. Violaceum* produces a pigment termed violacein, which causes the bacteria to turn purple after achieving *quorum* (Harrison, Soby, 2020). The CviR/CviI pathway is the *quorum* sensing pathway used in *C. Violaceum,* often described as a homologous pathway to the commonly seen LuxR/LuxI pathway, which is also used in *quorum* sensing of other bacteria. In *C. Violaceum,* CviI is known to synthesize the autoinducer (AHL) C10-homoserine lactone; it then binds to a cytoplasmic protein coded by CviR that activates gene expression through controlling the promoter for enzyme employed in chitinase type VI secretion-related gene, a transcriptional regulator gene, a guanine deaminase gene, and CviI (Ravichandran, Zhong, Wang et.al., 2018). Depending on the concentration of CviI when binding to CviR, *quorum* sensing related genes will either be upregulated or downregulated (Soby, 2022). Often used in tandem with *C. Violaceum* is one of its mutants, CVO26, which is another valuable bacteria in *quorum* sensing experiments. As the CVO26 mutant lacks the CviI gene, it is unable to release AHLs and achieve pigmentation without an external source, so it can be used to evaluate the efficacy or lack thereof of chemicals on the CviI gene (Harrison & Soby, 2020).

The approach in testing phytochemicals as potential "*quorum* quenchers" is due to the millions of years of history between plants and bacteria. As plants lack a formal immune system, it is assumed that many of their bacterial-resistant properties are derived from their phytochemicals. Moreover, prior to the development of modern medicine, people treated medical ailments with traditional medicine created from plants in the form of pastes or liquids (Silva et.al., 2019). One of the most notable classes of phytochemicals studied is known as flavonoids, which is a subgroup of polyphenols known to contain high antioxidants. Syringic acid is a phenolic acid often found in many fruits and vegetables, and is shown to have potential antimicrobial effects (Cheemanapalli, et. al., 2018). Rutin is a flavonoid that is often found in plants from the family Rutaceae, and is shown to have antimicrobial properties; however, it is not as well studied as a "*quorum* quencher." In traditional medicine, *Ruta chalepensis L.* has been used to treat a variety of medical illnesses often relating to providing pain relief (Al-Majmaie, 2019). *Ziziphus mucronata,* commonly known as African Thorn Bush, is a plant native to South Africa and its bark and leaves are often used in traditional medicine throughout the country. Extracts of the bark of the plant have been previously tested in gram-negative bacteria, and have shown significant antimicrobial activities. However, due to its novelty, studies analyzing its antimicrobial activity are limited when compared to other medicinal plants. Some studies have found the presence of bioactive compounds (alkaloids, flavonoids, tannins, and phenolic compounds) in the bark extracts of the plant. (Mongalo, N. I., Mashele, S. S., & Makhafola, T. J., 2020).

Scientific Goals

To expand on these ideas, the designed project will attempt to evaluate the antimicrobial abilities of untested plant compounds (Syringic acid, Rutin, *Z.mucronata)* on the inhibition of *C. violaceum quorum* sensing. The concentrations for Syringic acid and Rutin extract being tested are 0.75 mg/mL and 1.00 mg/mL. The concentrations for *Z. mucronata* extract are 0.75 uL/mL and 1.00 uL/mL. These concentrations were determined by analyzing the concentrations that were strongly suggested to be effective in prior phytochemical studies. However, *Z. mucronata,* Rutin, and Syringic Acid have scarcely been tested in the antimicrobial field or with *C. Violaceum* so all data collected is novel. The positive control, gentamicin, will be tested at concentrations of 0.75uL/mL and 1.00uL/mL based on prior studies.

In order to analyze the dynamics of *C. Violaceum quorum* sensing, multiple assays evaluating various aspects of this process will be performed. As the motility of bacteria are indicative of its virulence, a swarming

assay will be used to evaluate the extent of reduced mobility caused by the phytochemicals. As violacein is the primary AHL used by *C. violaceum*, a microplate reader will be used in quantifying its absorbance to determine the extent of its production inhibition. As biofilm structural integrity is indicative of the strength of a bacterial colony, crystal violet stain and a microplate reader will be used to evaluate the quantity of biofilm produced.

Hypotheses

Null: If phytochemicals are tested individually, then they will have statistically similar success as current antibiotics in inhibiting bacterial *quorum* sensing.

Alternative: If the phytochemicals are tested individually, then at least one will have a statistically greater success than current antibiotics in inhibiting bacterial *quorum* sensing.

Materials and Procedures

Prior to experimentation, gloves, masks, aprons, and goggles will be worn to prevent contact with harmful substances. In addition, chemicals will be worked with in a fume hood to prevent inhalation. *Chromobacterium violaceum* and the mutant CV026 are the primary organisms used in this study. Throughout the course of this study, a general incubation period occurred on LB Agar plates and in LB medium at 30°C for 24h.

Violacein Inhibition Assay

Grow cultures of *C. violaceum* for 24h in LB broth with respective conditions. After incubation, transfer 500 uL of *C. violaceum* and LB Broth to a microcentrifuge tube then centrifuge the mixture at 10,000 rpm for ten minutes to generate a cell free supernatant. Add 500 uL of glacial ethyl acetate to each tube in order to extract violacein from the supernatant; then, vortex and centrifuge the mixture to separate phases. After separation, isolate 200 uL of the upper phase and quantify absorbance in the microplate reader at 575 nm. Later, convert absorbance to concentration using the standard curve of violacein.

Biofilm Inhibition Assay

Grow cultures of *C. violaceum* and CV026 for 24h in LB broth with respective conditions. Transfer 80uL of the culture and 20uL of fresh medium per well in a microtiter plate, then incubate for 24h at 30°C. After incubation, wash out the bacteria with distilled water to reduce background staining when adding 125 uL of 1% crystal violet to each well. After 15 minutes of incubation at room temperature, rinse the plate up to four times then flip the plate down to dry overnight. After the plate is dry, add 125 uL of 30% acetic acid to each well to solubilize the crystal violet. Transfer 125 uL from each well to a new microtiter plate, then scan it at 550 nm using the microplate reader for quantification. Later, convert absorbance to concentration using the standard curve of crystal violet.

Swarming Motility Inhibition Assay

Grow cultures of *C. violaceum* for 24h in LB broth with respective conditions. Place 2 uL from each culture on the center of a LB Agar plate then let the plates incubate for 24h at 30°C. After incubation, measure the diameter of the motility zone.

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SEM Imaging Series for *Ziziphus mucronata*

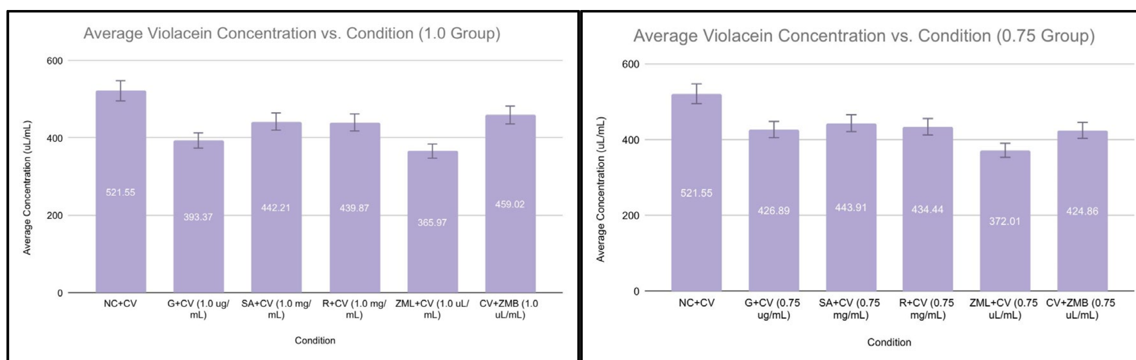
Let dried leaf and bark samples soak in 100% ethanol for 24h. After drying, place the leaf and bark samples in an oven at 65°C for 5 minutes. For transport to the scanning electron microscope facility, store samples in microcentrifuge tubes. Prior to scanning, prepare the samples by placing them on a SEM stub then spraying with a gold coat. Later, analyze the images for any signs of contamination.

Results and Conclusions

Shorthand was used throughout this section: CV-C.Violaceum, CV026-Mutant, NC-Negative Control, G/PC-Gentamicin, SA-Syringic Acid, R-Rutin, ZML- Z. mucronata Leaf Extract, ZMB- Z. mucronata Bark Extract.

Violacein Inhibition Assay

Results and Statistical Analysis



Graph 1. and Graph 2. Average violacein concentration in the 0.75 and the 1.0 groups, in which a lower concentration suggests stronger antibacterial activity.

T-Test of Violacein Concentration (uL/mL) vs. Positive and Negative Control (0.75 group).

Table 1. T-Test statistical analysis of the 0.75 group (P-value<0.05). ZML has a significant difference from the positive control. All have a significant difference from the negative control.

	CV+SA (0.75 mg/mL)	CV+R (0.75 mg/mL)	CV+ZML (0.75 uL/mL)	CV+ZMB (0.75 uL/mL)
T-Test vs PC	0.4020228981	0.6831018731	0.02120716785	0.7543083986
T-Test vs NC	0.0008625439753	0.0001602703976	0.0000005383641027	0.0004558424675

T-Test of Violacein Concentration (uL/mL) vs. Positive and Negative Control (1.00 group).

Table 2. T-Test statistical analysis of the 1.00 group (P-value<0.05). All except ZMB have a significant difference from the negative control.

	CV+SA (1.0 mg/mL)	CV+R (1.0 mg/mL)	CV+ZML (1.0 uL/mL)	CV+ZMB (0.75 uL/mL)
T-Test vs PC	0.05775975286	0.07140863883	0.2745823325	0.06028885539
T-Test vs NC	0.001012413821	0.000904188243	0.00000040587809	0.05311467587

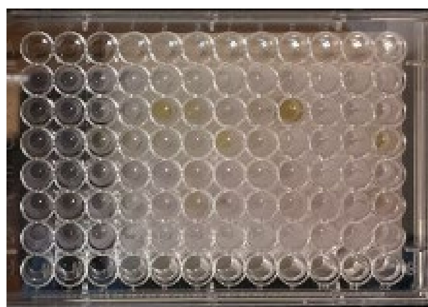


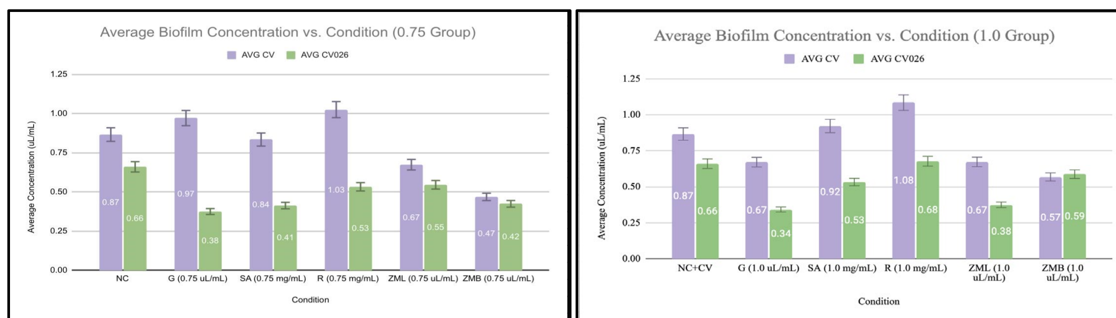
Figure 1. Extracted violacein prepared for microplate reader scan.

Analysis

When comparing the averages of each concentration, it is suggested that ZML has the greatest antibacterial potential in regard to violacein inhibition in the analyzed trials. In the 0.75 group, the p-values of all of the variables are significantly different from the negative control, suggesting that all of the variables at that concentration possess antibacterial potential. Further, only ZML exhibited a statistically significant difference from the PC, supporting the alternative hypothesis. In the 1.0 group, all of the variables except ZMB are significantly different from the NC, once again suggesting antibacterial potential. However, all of the variables aside from ZML have a statistically significant difference from the PC, thus rejecting both hypotheses as they are significantly weaker than the PC.

Biofilm Inhibition Assay

Results and Statistical Analysis



Graph 3. and Graph 4. Average biofilm concentration in the 0.75 and the 1.0 groups, in which a lower concentration suggests stronger antibacterial activity.

T-Test of CV Biofilm Concentration (uL/mL) vs. Positive and Negative Control (0.75 group)

Table 3. T-Test statistical analysis of the *C. violaceum*-0.75 group (P-value<0.05). ZMB has a significant difference to the positive control.

	CV+SA (0.75 mg/mL)	CV+R (0.75 mg/mL)	CV+ZML (0.75 uL/mL)	CV+ZMB (0.75 uL/mL)
T-Test vs PC	0.5804164405	0.868777946	0.212231239	0.04179945058
T-Test vs NC	0.8618471615	0.5764992126	0.2430206856	0.01951843358

T-Test of CV Biofilm Concentration (uL/mL) vs. Positive and Negative Control (1.00 group)

Table 4. T-Test statistical analysis of the *C. violaceum*-1.00 group (P-value<0.05). No significant differences.

	CV+SA (1.0 mg/mL)	CV+R (1.0 mg/mL)	CV+ZML (1.0 uL/mL)	CV+ZMB (0.75 uL/mL)
T-Test vs PC	0.2513121213	0.550746958	0.4245652468	0.1665961219
T-Test vs NC	0.4205761923	0.8141900641	0.3131116677	0.06418274241

T-Test of CV026 Biofilm Concentration (uL/mL) vs. PC and NC (0.75 group)

Table 5. T-Test statistical analysis of the CV026-0.75 group (P-value<0.05). ZML and SA have a significant difference to the negative control.

	CV026+SA (0.75mg/mL)	CV026+R (0.75mg/mL)	CV026+ZML (0.75uL/mL)	CV026+ZMB (0.75uL/mL)
T-Test vs PC	0.2087287608	0.03353781303	0.6144205355	0.1155901069
T-Test vs NC	0.02462289772	0.1114163818	0.0197506428	0.05730550439

T-Test of CV026 Biofilm Concentration (uL/mL) vs. PC and NC (1.00 group)

Table 6. T-Test statistical analysis of the CV026-1.00 group (P-value<0.05). ZMB has a significant difference to the positive control.

	SA+CV026 (1.0mg/mL)	R+CV026 (1.0mg/mL)	ZML+CV026 (1.0uL/mL)	ZMB + CV026 (1.0uL/mL)
T-Test vs PC	0.08393238142	0.07398891633	0.4084751247	0.00461992204
T-Test vs NC	0.3045044321	0.2621199525	0.03342964197	0.7430173011

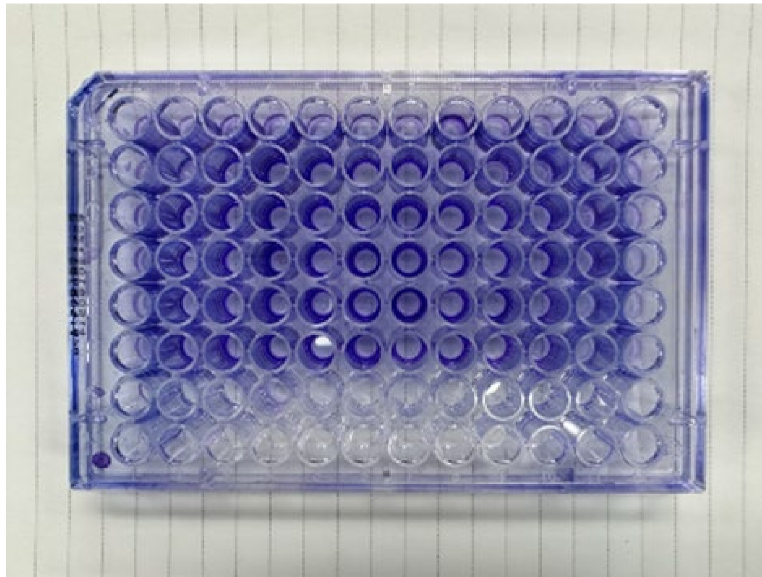


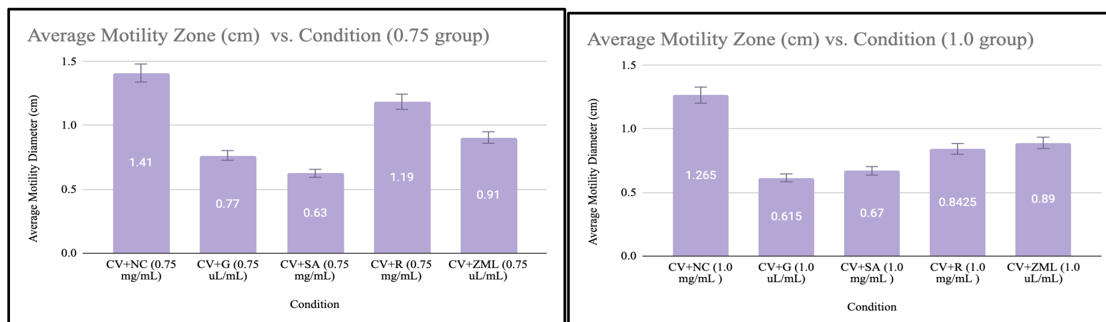
Figure 2. Stained *C. violaceum* biofilms prior to addition of acetic acid for quantification.

Analysis

On average, the CV026 had less biofilm production than the CV. In the CV-0.75 group, only ZMB exhibited significant differences from the positive and negative controls, meaning it has a stronger antibacterial effect than the positive control. In the CV-0.75 group, all of the variables except rutin are suggested to possess antibacterial activity, yet none have significantly more antibacterial activity than the PC in regards to biofilm inhibition; rutin has a significant difference to the PC, suggesting that it has significantly less antibacterial potential than the PC. In the remaining groups, CV026 and CV-1.0, all variables lack a significant difference to the PC and NC, suggesting that there is antibacterial activity, but not as significant as ZMB-0.75 in CV.

Swarming Motility Inhibition Assay

Results and Statistical Analysis



Graph 5. and Graph 6. Average motility diameter in the 0.75 and the 1.0 groups, in which a lower diameter suggests stronger antibacterial activity.

T-Test of Motility Diameter (uL/mL) vs. Positive and Negative Control (0.75 group)

Table 7. T-Test statistical analysis of the 0.75 group (P-value<0.05).

	CV+SA (0.75 mg/mL)	CV+R (0.75 mg/mL)	CV+ZML (0.75 uL/mL)	CV+ZMB (0.75 uL/mL)
T-Test vs PC	0.1054340233	0.005620926166	0.08268495394	0.8045744862
T-Test vs NC	0.002679026308	0.2147669193	0.02550112202	0.01037919441

T-Test of Motility Diameter (uL/mL) vs. Positive and Negative Control (1.00 group)

Table 8. T-Test statistical analysis of the 1.0 group (P-value<0.05).

	CV+SA (1.0 mg/mL)	CV+R (1.0 mg/mL)	CV+ZML (1.0 uL/mL)	CV+ZMB (0.75 uL/mL)
T-Test vs PC	0.7606342452	0.0769043767	0.01602387376	0.1605952959
T-Test vs NC	0.0002455373234	0.008069362856	0.001801935378	0.003291649261

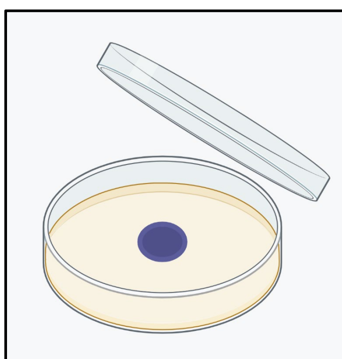


Figure 3. A general model of bacterial motility zone growth

Analysis

In the 0.75 group, all of the variables except R had a significantly different value from the negative control, suggesting that they all possess antibacterial properties; however, no variables had a statistically significant smaller diameter than the PC, so the null hypothesis failed to be rejected. Further, in the 1.0 group all the variables had a statistically significant difference from the negative control, suggesting that they all have anti-bacterial properties, but not to the extent of the PC. When comparing the averages, SA had the smallest diameter, but it was not small enough to be statistically significant from the PC.

SEM Images of Ziziphus mucronata

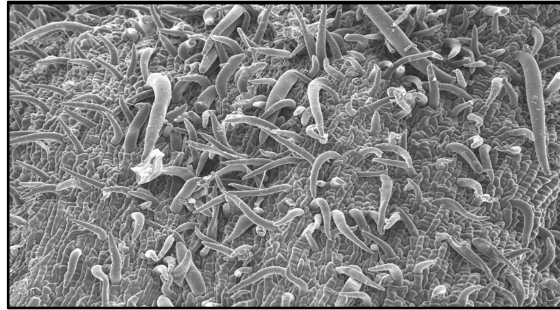


Figure 4. SEM of *Z. mucronata thorn.*

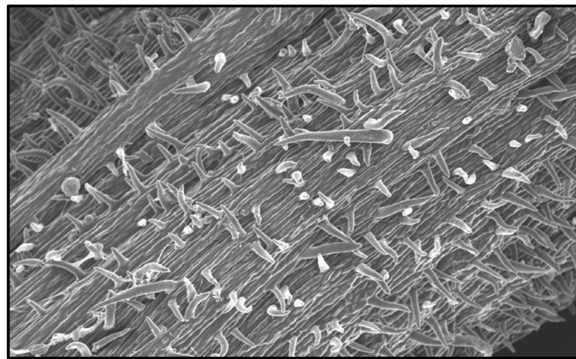


Figure 5. SEM of Z. mucronata bark.

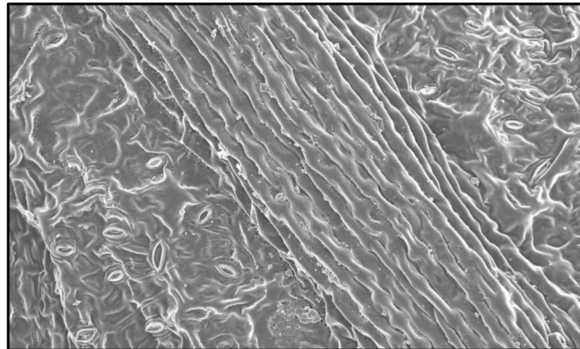


Figure 6. SEM of *Z. mucronata* leaf underside.

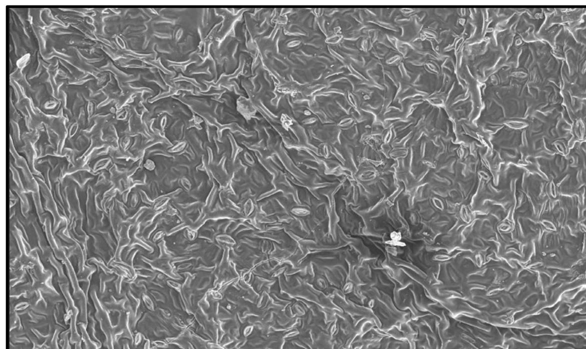


Figure 7. SEM of *Z. mucronata* leaf upperside.

Conclusion

Neither the null nor alternative hypotheses were consistently supported or unsupported as the results were not consistent throughout each assay. This could be explained through the use of various pathways of *quorum* sensing tested and the different effects the variables may have on each. However, when comparing all of the results, it is suggested that all of the phytochemicals tested have antimicrobial properties at both concentrations as they would average below the negative control throughout the majority of the trials. The most notable of these variables is the *Ziziphus mucronata* leaf extract and the *Ziziphus mucronata* bark extract. These two variables would consistently average the lowest for *quorum* sensing indicators compared to the other variables and occasionally less than the positive control gentamicin. In the violacein inhibition assay, the 0.75 uL/mL concentration of the *Z.mucronata* leaf extract was statistically different from the positive control; further, in the biofilm inhibition assay, the same is true for the 0.75 uL/mL concentration of the *Z.mucronata* bark extract. This suggests that the scarcely studied *Z.mucronata* could be a notable candidate for further "*quorum* quenching" research and development. After the *Z.mucronata* extracts*,* syringic acid was the strongest of the variables, but it did not have any statistically significant results.

Applications

Often, people will have to undergo multiple antibiotic treatments if their bacterial infection begins to develop resistance, and there is only a limited amount of options. Therefore, the development and research of "*quorum* quenchers" is of growing importance. They allow the containment and elimination of bacterial infections while decreasing the chances of antibiotic resistant mutations developing. As suggested by the data, the phytochemicals tested in this experiment show antimicrobial properties and could be a contender for further development. The development and research of these phytochemicals in the medical field could add to the production of future antibiotics.

Limitations and Error Analysis

One of the principal difficulties associated with this project was obtaining materials within time constraints. Most notably, the dried *Ziziphus mucronata* extract suffered numerous shipping delays, which caused most of the experiment to be placed on hold for multiple weeks. Further, initial data collection was unable to begin until weeks after planned due to difficulty in growing the *C. violaceum,* and later the same issue arose causing an

additional week delay. Additionally, as the bacteria had to be periodically replated, there is a chance of it developing mutations at some point during the experimentation process. In the future, there can be improvements made to overall planning and obtaining materials.

Random errors that could have potentially occurred through the course of this project could have been mutations developing in the bacteria within the experiment and contamination of LB stock concentrations used throughout the experiment. Another error that occurred, but was then resolved, was the appropriate temperature in which to incubate the *C. violaceum*: prior papers recommended 37°C, but the bacteria only grows once the incubator was at 30°C. Systematic errors could have occurred through malfunctions with the equipment used and human handling: interference with machine scans, inaccurate micropipetting, and incorrectly measuring solution contents.

Future Research

In the future, there can be further testing of the phytochemicals discussed in this project to develop a more complete understanding of their antibacterial properties. Possible ways to do this are testing other assays or testing other bacteria; some potential assays are qPCR for different genes, SEM biofilm analysis, and human tissue assays. Mixtures between the phytochemicals and mixtures between the phytochemicals and antibiotics could also be created then tested at different concentrations. Additionally, other phytochemicals not used in this project can be studied to further develop the field of "*quorum* quenching."

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