Investigating the effects of carbon chains on the antibacterial and antioxidant properties of catechin and 3-hydroxyflavone

Group 1-21

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Abstract

Recently, there has been growing interest in the research of flavonoids, with many flavonoids discovered to have antimicrobial activities. These flavonoids are commonly found in everyday food as such in green tea. As such, this study aims to contribute to this field of research by finding ways to increase such antimicrobial activities by reacting flavonoids with different acyl chlorides to examine the changes in its antibacterial activity. The procedure involves reacting catechin and 3-hydroxyflavone with different acyl chlorides. The antibacterial activities will then be tested using well diffusion on a gram-positive (Bacillus Subtilis) bacteria and a gram-negative (E.Coli) bacteria, with a positive control of dilute sodium hypochlorite and negative control of deionized water. The investigation will involve the measurement and comparison of the zone of inhibition to determine the effectiveness of the antibacterial properties. The results obtained suggest that there are antibacterial properties present in the two acylated flavonoids, with the acylated flavonoids being more effective against gram-positive bacteria than gram-negative bacteria. The results also found that isomers do not have significant differences on the antibacterial properties, but that acyl chlorides with greater carbon chains will better enhance the antibacterial properties of acylated flavonoids. Acylated catechin is also shown to display greater antibacterial properties as compared to acylated 3-hydroxyflavone. In conclusion, we found that acylated chlorides enhance the antibacterial properties of flavonoids.

Introduction

Catechin and 3-hydroxyflavone are chemical compounds that belong to the chemical group of flavonoids. Flavonoids are a large family that consists of hydroxylated polyphenolic compounds that are ubiquitously distributed in all of the plant cells. (Kumar S., & Pandey A.K, 2013). Flavonoids can be divided into 12 subgroups based on chemical structure, six of which are anthocyanidin, flavan-3-ols, flavonols, flavones, flavanones and isoflavones. Catechin is part of the subgroup flavan-3-ols (refer to Fig 1.1 for chemical structure), while 3-hydroxyflavone is part of the subgroup flavonone, and in fact, is the backbone for all chemical compounds in the flavanonce subgroup (Refer to Fig 1.2 for chemical structure).

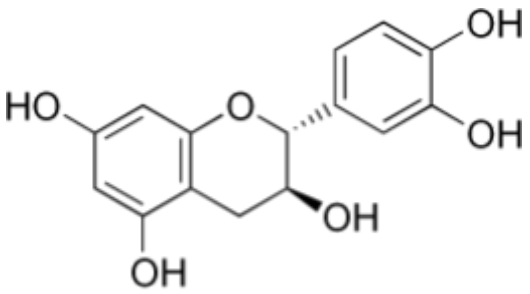


Fig 1.1

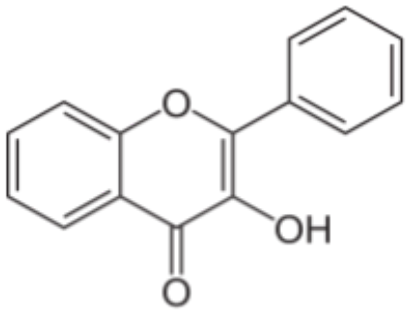


Fig 1.2

Catechin and 3-hydroxyflavone are flavonoids that have been extensively studied and researched for their antibacterial properties. Catechin, which can be easily extracted from green tea leaves, have been found to have antimicrobial properties, combating the bacteria in multiple ways, directly and indirectly. (Reygaert, 2018) One of the major antibacterial properties of green tea catechin is its ability to bind to bacterial cell membranes, disrupting its various functions and increasing the permeability of peptidoglycan cell wall, leading to cell lysis, destroying the bacterial cell. (Sirk, Brown, Sum, & Friedman, 2008). 3-Hydroxyflavone has also been shown to have antibacterial properties. According to research and experiments (Montenegro *et al.*, 2017). One hydroxylated flavone derivative, which is 3-hydroxyflavone, inhibited the growth of all bacterial strains tested. This shows that flavones and its hydroxylated flavone derivatives have moderate antibacterial properties, as large amounts of the chemical were needed to inhibit bacterial growth.

By adding acyl chloride to the flavonoids, the acylation mechanism occurs. An acyl group is an alkyl group attached to a carbon-oxygen double bond. If "R" represents any alkyl group, then an acyl group has the formula RCO-. Acylation means substituting an acyl group into a compound. The most reactive substance containing an acyl group is an acyl chloride (also known as an acid chloride). These have the general formula RCOCl. Thus, we used acyl chloride to be the acylation reagent. When the acylation reaction occurs, the acyl group would be replacing the hydroxyl group in the dihydropan heterocycle of both catechin and 3-hydroxyflavone, forming acylated flavonoids.

In this project, we were originally going to investigate the antibacterial and antioxidant properties of acylated flavonoid samples. However, due to unforeseen circumstances and time constraints, we were unable to conduct the antioxidant tests on the samples. Thus, this report will be investigating the effects of carbon chains on the antibacterial properties of the chosen flavonoids. More specifically, the number of carbon chains in an alkyl substituent. Then, we will be using the product in order to determine if there is an enhancement in the antibacterial properties of the flavonoids.

Objectives and Hypotheses

The objective of this study is to investigate the effect of different carbon chains and structure of the acyl chloride on the antibacterial properties of acylated flavonoids against Gram-positive and Gram-negative bacteria, for being a potential substitute for antibiotics

We hypothesise that:

1. Antibacterial properties of flavonoids can be enhanced by synthesis of acyl chloride
2. Acyl chloride with more carbon branches can better enhance the antibacterial properties of flavonoids
3. Isomers of acyl chlorides will have different effects on the antibacterial properties of flavonoids
4. Acylated catechin and 3-hydroxyflavone would have greater antibacterial effects on Gram positive bacteria than Gram negative bacteria.
5. Acylated catechin samples would have greater antibacterial properties than acylated 3-hydroxyflavone

Apparatus and Materials

The apparatus we require will be Electronic Mass Balance, Fume Hood, Micropipettes, Round Bottom Flask, Shaking incubator, Bacterial Culture 1 (BSL 1), Silica Gel Chromatography Paper, Tweezers, Incubator/Dry Oven, Sterile plate spreaders and Agar plates.

As for the chemicals, this study will need the flavonoids, which are catechin(powdered) and 3-hydroxyflavone(powdered).

For the acyl chlorides, Acetyl Chloride (CH_3COCl), Propionyl Chloride (C_3H_5ClO), Butyryl Chloride (C_4H_7ClO), Isobutyryl Chloride (C_4H_7ClO), which is a isomer of Butyryl Chloride, and Isovaleryl Chloride (C_5H_9ClO) were used.

Other chemicals include Pyridine, Dimethyl sulfoxide(DMSO), Hexane, Acetone, Dilute sodium hypochlorite(10% Bleach Solution), Sterile Water, Lysogeny Broth Medium and Mueller-Hinton Agar.

Lastly, for the biological organisms, gram-negative *Escherichia coli* (E. coli, ATCC 25922) and gram-positive *Bacillus subtilis (*ATCC 19659) were chosen.

Methodology

Preparation of Acylated Flavonoids

Reacting With Catechin

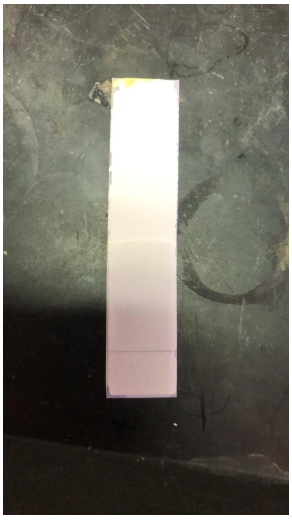
5ml of pyridine was added to 0.001 mol of catechin. 0.001 mol of each acyl chloride is added to 0.001 mol of catechin. The mixture was stirred at 30 degrees celsius for 12 hours using a magnetic stirrer. Product obtained and purified using thin layer chromatography

Reacting With 3-Hydroxyflavone

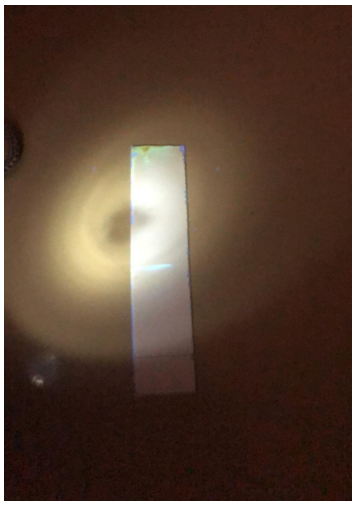
0.001 mol of each acyl chloride was added to 0.001 mol of 3-hydroxyflavone. The suspension rested undisturbed for 3 days until a yellow-green mixture formed. Product obtained and purified using thin layer chromatography. The reaction mixture was left evaporated in a fume hood until a white powder was observed. 2g of the white solid was then dissolved in 2ml of DMSO.

Thin layer chromatography

The reaction products were analyzed using thin layer chromatography by varying the polarity of the solvent ranging from volume ratio of acetone : hexane of 20%:80% to 70%: 30%



Silica Gel under UV Light (70% Acetone 30% Hexane)



Silica Gel under UV Light (60% Acetone, 40% Hexane)

There were no observable spots in the chromatogram viewed under UV light and iodine vapour. This could be due to insufficient samples being loaded onto the silica gel. We didn't repeat the experiment due to time constraints. Hence, we didn't purify the products from the reactions

Growth of Pre-cultures of Bacteria

Escherichia coli (E. coli, ATCC 25922), Bacillus Subtilis (ATCC 19659) were used as the bacteria and fungal test organisms. Each test organism was inoculated with 10ml Lysogeny Broth (LB) medium and grown overnight at 26 degrees celsius in a shaking incubator.

Agar Well Diffusion Test

The test bacteria Bacillus Subtilis and E.coli were spread evenly on Mueller-Hinton (MH) agar plates with a sterile cotton swab. For the acylated 3-hydroxyflavone sample, 8 agar plates were then divided into 3 sections, for the sample, negative control (Sterile Water) and positive control (DMSO). Circular wells of diameter 1cm were made in the center of each section, and thereafter filled with 0.8 ml of acylated 3-hydroxyflavone for the test section, sterile water for the negative control section and 10% bleach for the positive control section. The agar plates were then sealed with parafilm and incubated at 30°C for 4 days. For the acylated catechin samples, 10 agar plates were then divided into 4 sections, for the sample, negative control (Sterile Water) and positive control (10% Bleach), and pyridine. We had to include pyridine as the catechin was dissolved in pyridine before the acyl chloride was added, so it had to be there to act as another control.

Results and Evaluation

Characterisation

Due to time constraints, we are not able to perform any characterization tests such as FTIR or H-NMR. Hence, we can only assume that our intended products were formed in the acylation reaction.

Tests Against Gram-Negative Bacteria (E.coli)

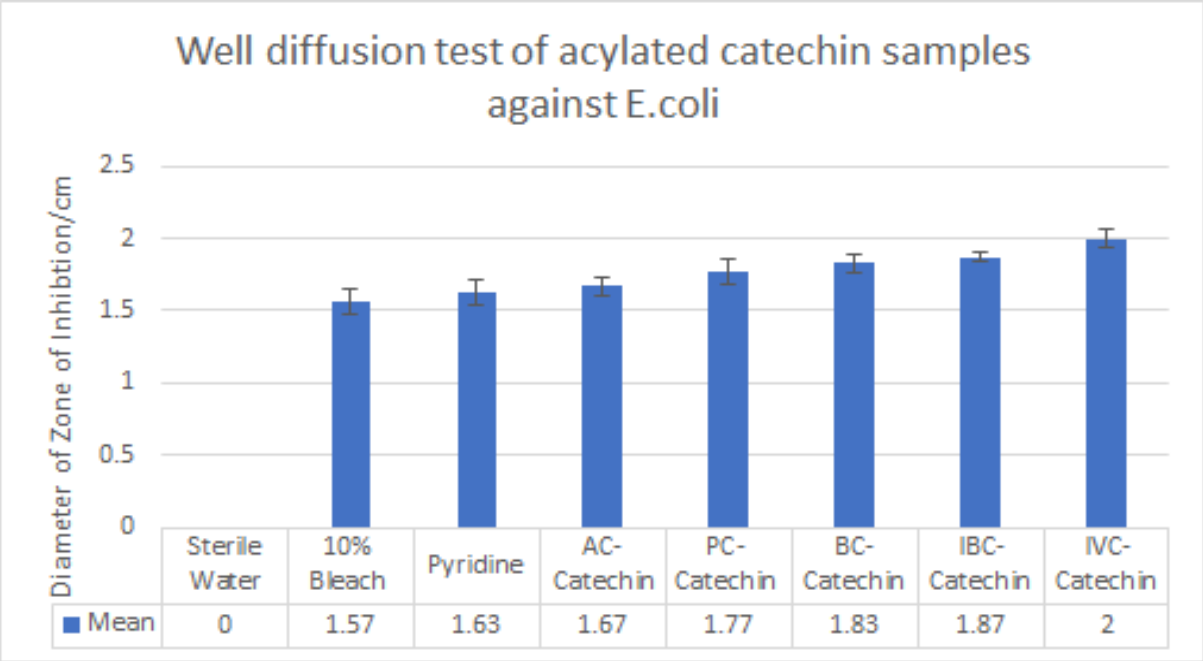


Fig 2.1:
Graph showing average diameters of Zone of Inhibition (cm) generated by acylated catechin samples against E.coli

(Take note that AC refers to Acetyl Chloride, PC refers to Propionyl Chloride, BC refers to Butyryl Chloride, IBC refers to Isobutyryl Chloride, while IVC refers to Isovaleryl Chloride.)

From this graph, we can understand that the acylated catechin samples exhibited antibacterial properties against the Gram-negative E.coli bacteria, and that the diameter of the zone of inhibition of each acylated catechin sample is greater than that of the 10% bleach and pyridine controls. After conducting Kruskal-Wallis Tests between bleach, pyridine and each sample, we can conclude that AC-Catechin and PC-Catechin do not have significant differences when

compared to the controls, but BC, IBC and IVC-Catechin samples do have significant differences when compared to bleach and pyridine, with p-values at 0.02712, 0.01674 and 0.00842 respectively.

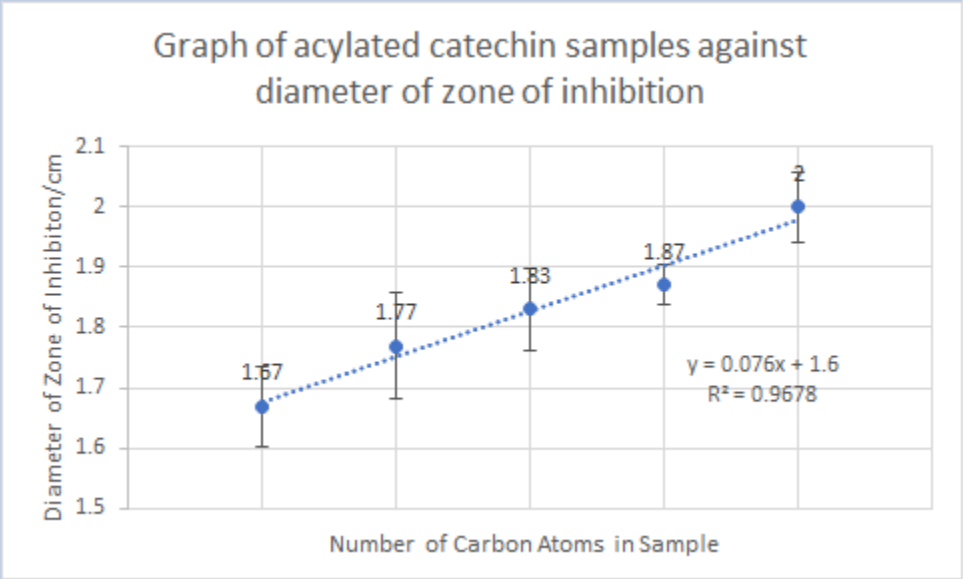


Fig 2.2
Graph showing the increase in the diameter of the zone of inhibition as the carbon chains in acylated catechin samples increase.

(Take note that in this graph, each dot from left to right, represents AC, PC, BC, IBC, and IVC respectively , in an ascending order in terms of the number of carbon chains)

From Fig 1.2, we can conclude that the antibacterial properties of the acylated catechin samples increase as the number of carbon chains in the sample increases. Acyl chlorides with a different number of carbon molecules starting from acetyl chloride to isovaleryl chloride show not only a wide range of molecular weight but also a variable degree of polarity. (Koushik *et al.*, 2017) The decreasing polarity of each acylated flavonoid due to their increasing carbon chain results in an increasing positive charge of each acylated catechin. Since acylated catechin samples have a greater positive charge as the length of the carbon chain increases, acylated catechin samples that have a longer carbon chain length are more attracted to the negatively charged peptidoglycan bacterial cell wall of both Gram-positive and Gram-negative bacteria, resulting in greater antibacterial activity. Due to time constraints, we could not repeat the experiment to have a larger sample size.

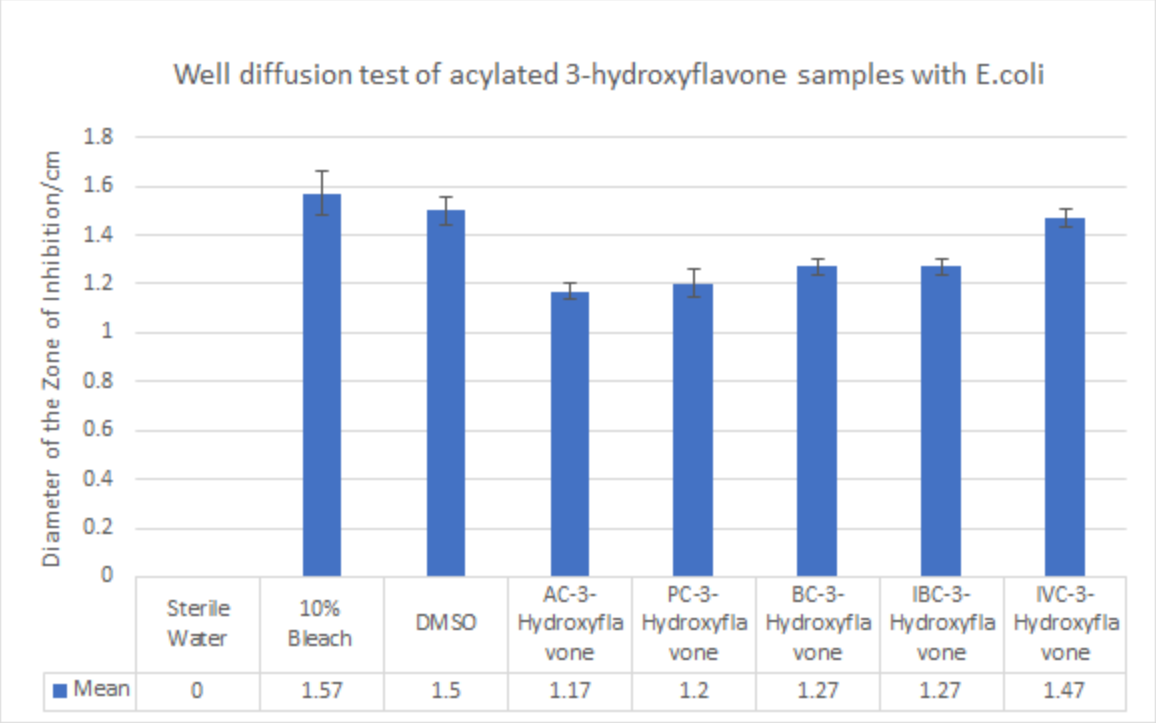


Fig 2.3:
Graph showing average diameters of Zone of Inhibition (cm) generated by acylated 3-hydroxyflavone samples against E.coli

From this graph, we can understand that the acylated 3-hydroxyflavone samples exhibited antibacterial properties against the Gram-negative E.coli bacteria, but the diameter of the zone of inhibition of each acylated catechin sample is smaller than that of the 10% bleach control. After conducting a series of Kruskal Wallis Tests, we can conclude that the samples are significantly less effective or possess insignificant differences in terms of antibacterial properties when compared to bleach and DMSO. AC, PC, BC, IBC and IVC have p-values of 0.01208, 0.01208, 0.0164, 0.0164 and 0.4009 respectively, which shows that AC, PC, BC and IBC are significantly less effective, while IVC does not have any significant difference when compared to bleach and DMSO.

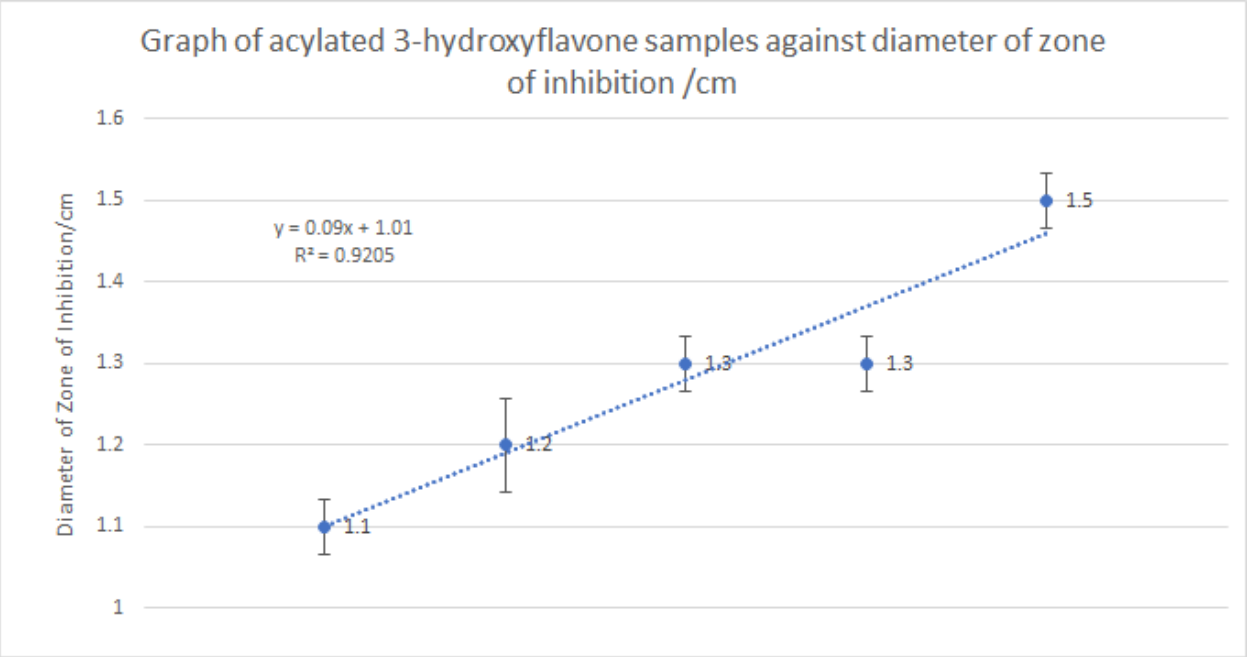


Fig 2.4
Graph showing the increase in the diameter of the zone of inhibition as the carbon chains in acylated 3-hydroxyflavone samples increase.

(Take note that in this graph, each dot from left to right, represents AC, PC, BC, IBC, and IVC respectively , in an ascending order in terms of the number of carbon chains)

From Fig 1.4, we can conclude that the antibacterial properties of the acylated 3-hydroxyflavone samples increase as the number of carbon chains in the sample increases in a somewhat linear fashion. We can also conclude that there are no significant differences in the antibacterial properties of isomers, as BC and IBC have the equal diameters of zone of inhibition. Based on the Kruskal-Wallis tests, the results indicate that there are no significant differences between isomers too, as the p-values of both BC and IBC are equal as well.

Tests Against Gram-Positive Bacteria (Bacillus Subtilis)

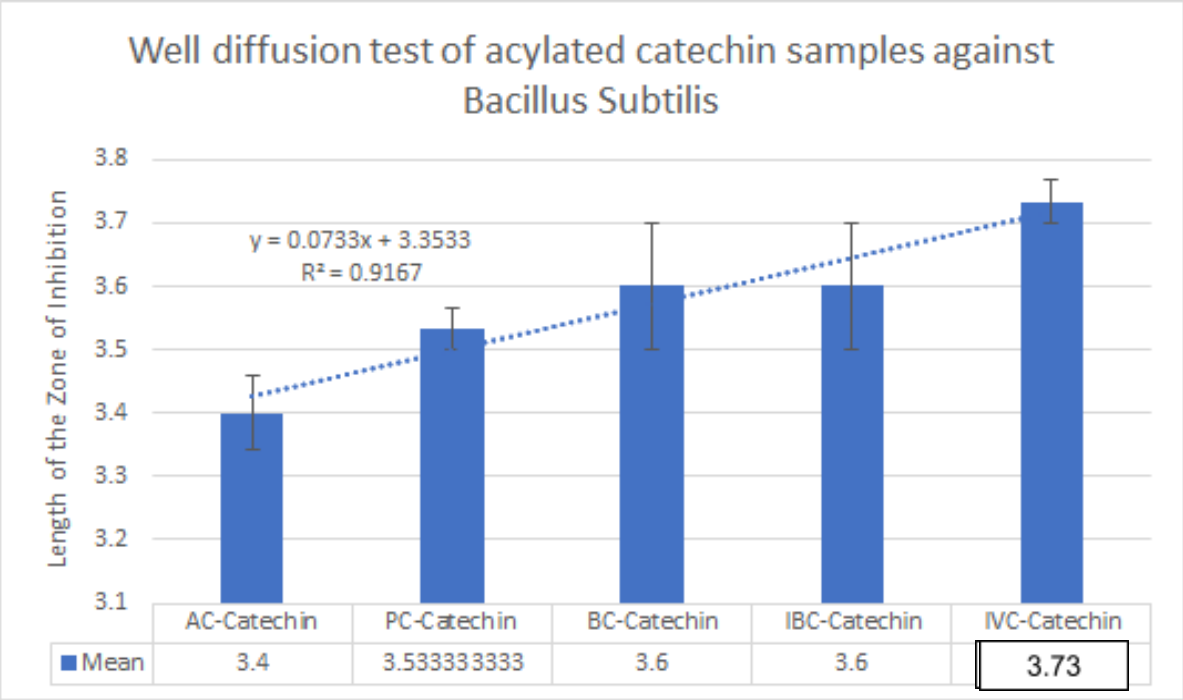


Fig 3.1:
Graph showing average length of Zone of Inhibition (cm) generated by acylated catechin samples against Bacillus Subtilis

From this graph, we can understand that the acylated catechin samples exhibited antibacterial properties against the Gram-positive E.coli bacteria, and that the length of the zone of inhibition of each acylated catechin sample is much greater than that of the 10% bleach control (refer to Fig 2.2 for data), thus showing that the samples are more effective than bleach against Bacillus Subtilis. This is expected as catechin is able to break down the bacterial cell membrane, leading to cell lysis. We can also conclude that the antibacterial properties of the acylated 3-hydroxyflavone samples increase as the number of carbon chains in the sample increases in a linear fashion. We can also conclude that there are no significant differences in the antibacterial properties of isomers, as BC and IBC have the equal average lengths of the zone of inhibition.

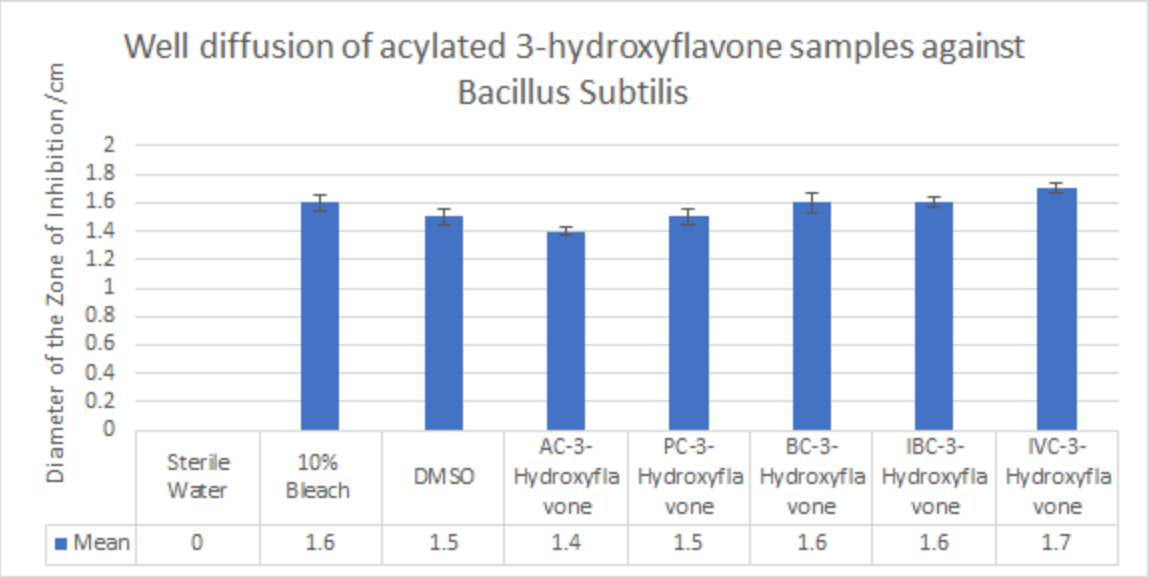


Fig 3.2:
Graph showing average diameters of Zone of Inhibition (cm) generated by acylated 3-hydroxyflavone samples against Bacillus Subtilis

From the graph, we can see that the antibacterial properties of some acylated 3-hydroxyflavone samples are only slightly more effective than the control substances, which is the 10% bleach solution and the DMSO (Dimethylsulfoxide). Some samples, such as the AC-3 Hydroxyflavone and the PC-3 Hydroxyflavone are less effective in combating Gram-positive bacteria too. After conducting a series of Kruskal-Wallis Tests, we know that most of the samples do not have a significant difference in terms of antibacterial properties when compared to the control substances, with the exception of IVC-3 Hydroxyflavone. AC, PC, BC, IBC and IVC have p-values of 0.39952, 0.94885, 0.14086, 0.06409 and 0.013 respectively As only IVC has a p-value of less than 0.05, we can conclude that it has a significant difference when compared to bleach and DMSO, while AC, PC, BC and IBC, which shows that AC, PC, BC and IBC do not have any significant difference while only IVC does.do not. Thus, we can conclude that acylated 3-hydroxyflavones are ineffective antibacterial agents.

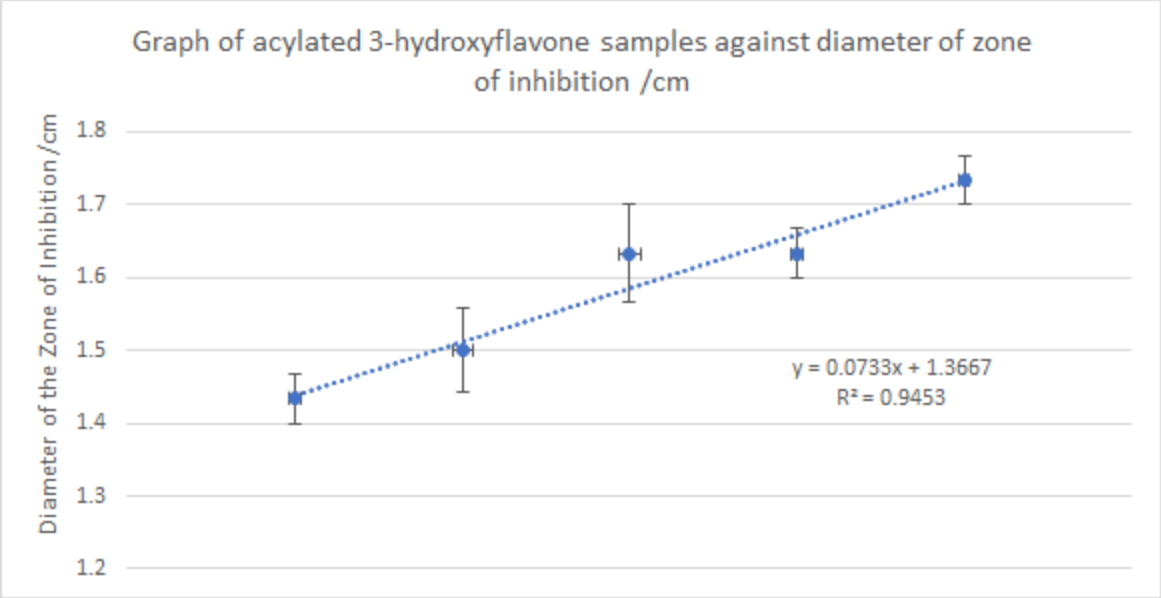


Fig 3.3
Graph showing the increase in the diameter of the zone of inhibition as the carbon chains in acylated 3-hydroxyflavone samples increase.

(Take note that in this graph, each dot from left to right, represents AC, PC, BC, IBC, and IVC respectively , in an ascending order in terms of the number of carbon chains)

From Fig 3.3, we can conclude that the antibacterial properties of the acylated 3-hydroxyflavone samples increase as the number of carbon chains in the sample increases in a linear fashion. We can also conclude that there are no significant differences in the antibacterial properties of isomers, as BC and IBC have again, equal average diameters of zone of inhibition.

Conclusion

From the results we conclude that the antibacterial activities of Catechin and 3-Hydroxyflavone have been enhanced by the reaction with acyl chlorides. The zone of inhibition of acylated flavonoids is greater than bleach and pyridine, suggesting it possesses antibacterial properties. This conclusion supports our hypothesis that antibacterial properties of flavonoids can be enhanced by synthesis of acyl chloride.

The study's results from our study also indicates that the acylated flavonoids are more effective against gram-positive bacteria than gram-negative bacteria. The zone of inhibition on Bacillus Subtilis bacteria is significantly larger than that in E.coli bacteria, indicating a stronger effect on gram-positive bacteria than gram-negative bacteria, for both catechin and 3-hydroxyflavone. This supports our hypothesis that acylated catechin and 3-hydroxyflavone would have greater antibacterial effects on Gram positive bacteria than Gram negative bacteria. This is because the lipopolysaccharide (LPS) on the outer membrane of gram negative bacteria makes them more resistant to binding by flavonoids, thus making gram-negative bacteria harder to destroy.

As for the difference in antibacterial properties between acyl chlorides of different carbon chains, we have obtained positive results. The zone of inhibition increases as the number of carbon chains increases, indicating that antibacterial properties of acylated flavonoids with more carbon chains are stronger. This supports our hypothesis that acyl chloride with more carbon branches can better enhance the antibacterial properties of flavonoids.

Our results also show that acylated catechin displays greater antibacterial properties than acylated 3-hydroxyflavone. From our results, we can see that the zone of inhibition for acylated catechin samples is significantly greater as compared to the acylated 3-hydroxyflavone sample. This supports our hypothesis that acylated catechin samples would have greater antibacterial properties than acylated 3-hydroxyflavone.

However, there is little difference in antibacterial properties between synthesis of isomers of acyl chlorides. From the results, we can see that IBC and BC have almost the same antibacterial properties. Hence, there is no significant difference in antibacterial properties between synthesis of isomers of acyl chlorides, contradicting our hypothesis that isomers of acyl chlorides will have different effects on the antibacterial properties of flavonoids.

With the positive results from our antibacterial tests, we can conclude that acylated flavonoids can be used as an alternative antibiotics.

Future Work

Due to limited time and unforeseen circumstances, we were unable to carry out the FTIR test and the antioxidant test. We intend to continue with both tests in the future for further research and to make a better conclusion. Our results also suggest possible properties of acylated flavonoids that may trigger induced cell cycle arrest. We will carry out more future experiments to conclude this possible property of acylated flavonoids.

Challenges

Performing the experiments for our project was quite a challenging task for us. The required chemicals took a long time to arrive and when it did, the lab was closed due to tightened restrictions from COVID-19. Furthermore, our initial plans to obtain acyl chlorides from the reaction between carboxylic acids and phosphorus pentachloride was cut short as the company that the lab orders phosphorus pentachloride from stopped selling them. We also struggled to purify the product due to time constraints. Lastly, we met unexpected results from the well diffusion test, as we did not expect the results to be extremely effective. However, we did not have the time to repeat the experiment with a bigger sample size due to time constraints as well.

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