Investigating the antibacterial properties of *Citrus limon* peel extract and its effectiveness in preventing bacterial wilt caused by *Ralstonia solanacearum*

Group 1-48

Abstract

This study investigates the antibacterial properties of *Citrus limon* (*C. limon*) extract extracted with acetone and methanol separately against the plant pathogen *Ralstonia solanacearum* (*R. solanacearum*). The extent of the *C. limon* peel extracts' antibacterial property was determined through the well diffusion test, minimum inhibitory concentration (MIC) test, as well as a test on the inhibition of bacterial wilt in infected *Solanum melongena* (*S. melongena*) leaves. Both acetone and methanol extracts were found to have antibacterial properties in the well diffusion tests, and the extracts have an average MIC of 25% and 18.75% respectively. The results of the test on inhibition of bacterial wilt in infected *Solanum melongena* (*S. melongena*) leaves was inconclusive but provided some information on the feasibility of using the extract on a large scale in farms to prevent bacterial wilt, and how to conduct the test better in future. This project also opened up the possibility of using a cheap and usually discarded material like *C. limon* peels to help prevent bacterial wilt caused by *R. solanacearum* in farms, hence helping farmers and the economy lose less of their profit to bacterial wilt.

1. Introduction

R. solanacearum is the causative agent of bacterial wilt. The bacterium infects the roots of host plants, rapidly colonizes the vascular system and releases large amounts of exopolysaccharide that prevent water flow within xylem vessels, causing wilting symptoms and subsequent plant death (Genin & Denny, 2012). The pathogen is distributed worldwide, affects over 200 species and induces a destructive economic impact. Direct yield losses by *R. solanacearum* vary from 0 to 91% in the tomato, 33 to 90% in the potato, 10 to 30% in tobacco, 80 to 100% in the banana, and up to 20% in the groundnut (Elphinstone, 2005).

Currently, *R. solanacearum* control methods include bactericides and pesticides. However, these may remain in the environment for many years, and become a contaminant in soil and/or groundwater, and become poisonous to humans. Another method of *R. solanacearum* control is the use of biological control agents (Yuliar, Nion, & Toyota, 2015). Biological control agents of *R. solanacearum* include, but are not limited to, *Bacillus amyloliquefaciens*, *Ralstonia pickettii*

and *Pseudomonas mallei* which are able to control bacterial wilt diseases caused by *R. solanacearum* by production of indole acetic acid and siderophores, competition, and production of inhibitory compounds and siderophores respectively (Yuliar et al., 2015). However, suppression of diseases by biological control agents has only been observed in a narrow range of host plants or restricted to a single pathogen or disease. The degree of suppression is also sometimes too low to be commercially acceptable (Yuliar et al., 2015).

Hence, using plant extracts to prevent bacterial wilt by *R. solanacearum* is preferred. Rawson, Ho, and Li, (2014), found that *C. limon* peels contained many common flavonoid compounds such hesperidin, naringin, neohesperidin, narirutin, eriocitrin, didymin and rutin among others, and that these compounds had antibacterial properties. Hence, we hypothesised that the flavonoid compounds in *C. limon* peels had antibacterial properties against *R. solanacearum*, and would be able to prevent bacterial wilt caused by *R. solanacearum*.

2. Objectives and Hypotheses

Objectives

1. Investigate the antibacterial properties of both *C. limon* peel extracts against *R. solanacearum*
2. Determine the MIC of both *C. limon* peel extracts against *R. solanacearum*
3. Investigate the effectiveness of both *C. limon* peel extracts in preventing bacterial wilt

Hypotheses

1. Both *C. limon* peel extracts will prevent the growth of *R. solanacearum*, and *C. limon* peel extract extracted from methanol will be more effective in preventing the growth of *R. solanacearum* than the C. limon peel extract extracted from acetone.
2. The MIC of both *C. limon* peel extracts will be able to prevent the growth of *R. solanacearum* in infected *S. melongena* leaves

3. Outline of Methods

Preparation of *C. limon* peel extract

C. limon peels were cut into small pieces and left to sun dry for 3 days. The dried peels were blended into fine powder using a blender. Soxhlet extraction was then performed with acetone and methanol separately. The extracts were then concentrated using a rotary flash evaporator.

Preparation of *R. solanacearum*

R. solanacearum was inoculated into sucrose-peptone (SP) broth inside a centrifuge tube using an inoculating loop. The centrifuge tubes were left to incubate in the shaking incubator at 34°C overnight.

Well diffusion test

A sterile cotton swab was immersed in the *R. solanacearum* and swabbed evenly onto 6 agar plates. Sterile pipette tips and sterile forceps were used to cut and remove 4 holes in each agar plate. 70 microlitres of *C. limon* peel extract extracted with acetone was pipetted into one of the holes. This was repeated using *C. limon* peel extract extracted with methanol, bleach as positive control and sterile water as negative control and in different holes. This was repeated for the 5 other agar plates. The agar plates were sealed with parafilm and left to incubate at 30°C. The zone of inhibition of each substance was measured using a ruler after 24 hours and recorded.

Minimum Inhibitory Concentration (MIC) Test

9 microfuge tubes were labelled from 1 to 9. 0.5ml of sterile SP broth was placed in each tube. 0.5 ml of *C. limon* peel extract extracted with methanol was added to tube 1 and was mixed by gently shaking. Serial two-fold dilutions of the plant extract was performed from tube # to tube # by transferring 0.5 ml of the contents of tube 1 to tube 2 using a pipette and gently mixing the contents of tube 2 well. This was repeated until tube 7 with new pipette tips for each transfer. After the contents of tube 7 were mixed well, 0.5ml of the contents in tube 7 was discarded. 0.1 ml of *R. solanacearum* was added to each tube 1 to 7. 0.1 ml of *R. solanacearum* was added to tubes 8 as control. 0.1 ml of sterile water was added to tube 9 as control. The tubes were placed in an incubator at 30°C for 24 hours. The procedure was repeated for the *C. limon* peel extract extracted with acetone. After 24 hours, the contents of tubes 1 to 7 were swabbed onto agar plates for the *C. limon* peel extract extracted with methanol. This was repeated for the tubes containing *C. limon* peel extract extracted with acetone. The agar plates were placed in an

incubator at 30°C. After 24 hours, the plates were checked for presence of bacteria and recorded.

In Vivo Test

6 GA7 containers of plant culture medium were prepared. 6 pieces of *S. melongena* leaves were sterilized in ethanol for 5 minutes, then rinsed with sterile water and left to dry. The MIC of *C. limon* peel extract extracted from acetone was prepared by dissolving the extracts with dimethyl sulfate accordingly in centrifuge tubes. *R. solanacearum* was mixed with the MIC of *C. limon* peel extract extracted from acetone in a centrifuge tube in a 1:1 ratio. The mixture was swabbed onto both sides of a sterilised leaf using a cotton swab. This was repeated with *C. limon* peel extract extracted from methanol. Another leaf was swabbed with *R. solanacearum*. The remaining leaves were swabbed with both *C. limon* peel extracts separately, and sterile water as control. Each leaf was placed in a separate container and left in the plant culture room. The leaves were checked for bacterial wilt every 2 days for a week and recorded.

4. Results and Discussion

Overall

4 batches of *C. limon* peel extracts were prepared. The well diffusion test was conducted 5 times, MIC test was conducted 4 times, and the in vivo test was conducted 2 times.

Well Diffusion Test

Across all batches of experiments, bleach had the largest average zone of inhibition of 2.25 cm, followed by the extract extracted with acetone with an average zone of inhibition of 1.52 cm, then the extract extracted with methanol which had an average zone of inhibition of 1.51 cm. Sterile water showed no zone of inhibition across all batches. This is represented in Fig 1. The t-test between the methanol extract and the acetone extract had a t-value of 0.0714, which was insignificant as the t-value had to be more than 0.1 to be considered significant. The t-test between the methanol extract and bleach had a t-value of 9.765, which was significant. The t-test between the acetone extract and bleach had a t-value of 8.8970, which was significant. Hence, both extracts had similar antibacterial properties to each other and weaker antibacterial properties as compared to bleach. Fig 2 shows pictures of finished well diffusion tests.

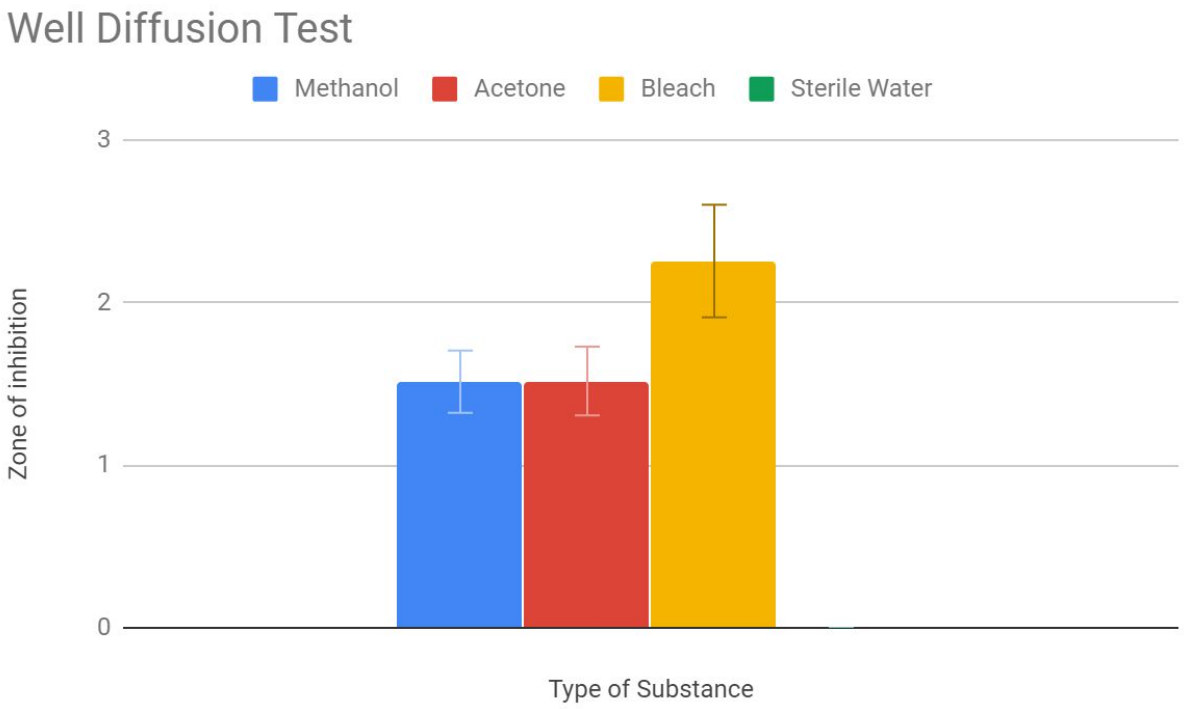

Fig 1: Graph of average zone of inhibition across all 5 batches of well diffusion test

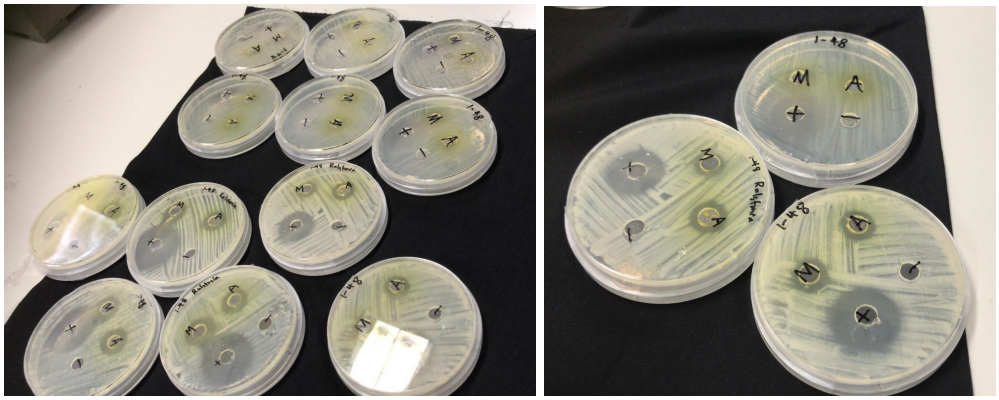

Fig 2: Pictures of finished well diffusion test

MIC Test

The MIC of *C. limon* peel extract extracted from acetone is 12.5% (average) while the MIC of *C. limon* peel extract extracted with methanol is 18.75% (average). This is shown with Fig 3. Hence, it can be seen that the acetone extract has slightly better antibacterial properties, which is consistent with our results from the well diffusion test. Fig 4 shows pictures of the microfuge tubes and the agar plates used in the MIC Test.

	Acetone extract	Methanol extract
1st batch	12.5%	25%
2nd batch	12.5%	25%
3rd batch	12.5%	12.5%
4th batch	12.5%	12.5%

Fig 3: Table of MIC of all 4 batches of extracts

Fig 4: Pictures of the microfuge tubes and the agar plates used in the MIC Test

In Vivo Test

Due to time constraints, the In Vivo test was only conducted twice. The 1st test had favourable outcomes while the 2nd test did not have favourable outcomes. In the 1st In Vivo test, after 7 days, the leaves swabbed with *R. solanacearum*, sterile water,and the acetone extract was observed to have bacterial wilt. This can be shown in Fig 5. In the 1st In Vivo test, apart from the leaves swabbed with sterile water and the acetone extract, the results of all other leaves were consistent with our hypothesis and results from the earlier tests. The most likely reason for the leaves swabbed with sterile water and acetone extract to have bacterial wilt was due to contamination. Another possible reason was that the leaves were not perfectly identical in size and this may have affected the time it took for the leaf to get bacterial wilt.

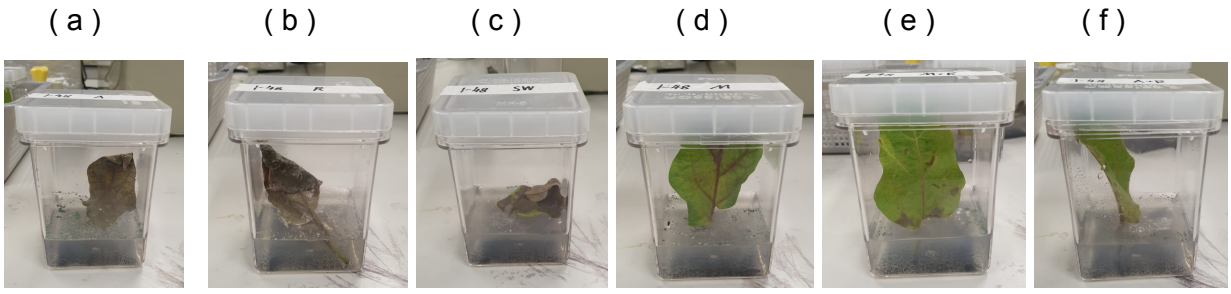

Fig 5: 1st In Vivo test after 7 days, (a) shows leaf swabbed with acetone extract,
(b) shows leaf swabbed with *R. solanacearum*,
(c) shows leaf swabbed with sterile water,
(d) shows leaf swabbed with methanol extract,
(e) shows leaf swabbed with methanol extract plus *R. solanacearum,*
(f) shows leaf swabbed with acetone extract plus *R. solanacearum*

The 2nd test showed unfavourable results, likely due to contamination, as it was not performed in a fully sterile environment.

As can be seen from the 1st In Vivo test, the *C. limon* peel extracts are able to prevent bacterial wilt caused by *R. solanacearum* to a certain extent in *S. melongena* leaves.

5. Conclusions and recommendations for future work

In conclusion, from the well diffusion test and the MIC Test, it can be seen that the *C. limon* peel extract extracted with acetone was slightly more effective at inhibiting the growth of *R. solanacearum* as compared to *C. limon* peel extract extracted with methanol, but both were not significantly different. However, both extracts were significantly weaker than Bleach at inhibiting the growth of *R. solanacearum.* The results of the in vivo tests were not conclusive as there were many complications that happened, such as contamination, and the leaves wilting before we could see the results. However, from the 2 previous tests and the uncontaminated leaves in the 1st In Vivo Test, it can be seen that the 2 extracts have antibacterial properties against *R. solanacearum* and are able to prevent bacterial wilt to some extent. Hence, our extract can potentially be produced in large scale in the future to help prevent bacterial wilt in plants during farming. This can help farmers and the economy as they will not lose as much of their profit to bacterial wilt. Another benefit of this extract is that unlike the many studies done on other parts of the *C. limon* plant such as the juice of the *C. limon* fruit, *C. limon* peels are usually discarded

and using them for this extract will not cost as much as using other parts of the *C. limon* plant such as the fruit.

For future work, the experiments can be repeated for reproducibility. Due to time constraints, the In Vivo test was performed only twice and due to many complications, the results were unreliable. Tighter measures can be used to minimise the complications, such as parafilming the GA7 containers to prevent contamination, and the use of large, identically sized leaves from the same plant so that the leaves are able to survive and will only get the bacterial wilt at the same time. Another possible way to perform the In Vivo test is to use the entire plant instead of leaves and infecting the plant through the soil, as this is how *R. solanacearum* infects plants during farming. It is also possible to perform the In Vivo on different plants which are also affected by *R. solanacearum*. It is also possible to perform the experiments with other solvents, such as ethanol, or with other citrus fruits, such as *Citrus X sinensis*, and compare the results with this study's result.

References

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