Investigating the antibacterial and antioxidant properties of slime track and plasmodial extracts from slime mold

Justin Ng Teng Loong, Lu Jin Wei Ethan, Sim Jing Heng

1-19

Abstract

Due to the excessive use of antibiotics, there have been many cases of bacteria that are resistant against commonly-used antibiotics. Research had shown that slime mold possessed antibacterial and antioxidant properties. Slime molds are myxomycetes which have multinucleate trophic stages. In the plasmodium stage of the life cycle, slime molds produce a detectable amount of slime that are left behind as “slime tracks” as the plasmodium moves over a surface of a specific substrate. In this study, *Physarum polycephalum* was used as it is the latest and novel model used for research. Hence, this project aims to investigate antibacterial, antioxidant properties and the effect on UV-treated *Caenorhabditis elegans* of slime track and plasmodial extracts of *Ph. polycephalum* grown on different food wastes as carbon source. This experiment involves conducting various tests on the slime track and plasmodial extracts of *Ph. polycephalum* grown on agar mixed with extracts of honeydew peels, watermelon peels or sugarcane bagasse, and water as the control. Well diffusion and colony count tests were used to determine the antibacterial properties of the extracts. All extracts have been shown to have antibacterial properties against the bacteria tested except against *Pseudomonas putida*, with the extracts displaying the highest antibacterial properties against *Serratia marcescens*. Only plasmodial extracts possessed significant antioxidant properties as shown in the DPPH test. Plasmodial extracts from slime mold grown on agar containing honeydew peel extract showed the highest antibacterial and antioxidant properties. Plasmodial extracts from slime mold grown on honeydew peel extract reduced UV-induced damage on *Caenorhabditis elegans*. As an application, antibacterial agents and sunblocks can be created as protection against UV radiation.

1. Introduction

Antibiotic medications are used to kill bacteria, which can cause illness and disease. However, excessive and unnecessary consumption of antibiotics and lack of research into new antibiotics has led to some bacteria becoming resistant to commonly used antibiotics, and can no longer be controlled or killed by antibiotics (Spellberg & Gilbert, 2014). These antibiotic resistant bacteria are able to survive, or even multiply, in the presence of an antibiotic.

The most serious concern with antibiotic resistance is that some bacteria have become resistant to almost all of the easily available antibiotics. These bacteria are able to cause serious diseases and this is a major public health problem (Ventola, 2015). One example of such bacteria is the multidrug resistant *Mycobacterium tuberculosis* (MDR-TB). In 2012, MDR-TB caused 170, 000 deaths out of 450,000 cases in total, clearly indicating the danger of such antibiotic resistant bacteria (Palomino & Martin, 2014). Thus, there is a need for new antibiotics to inhibit the growth of bacteria that are resistant to currently available antibiotics.

Slime molds are myxomycetes which have multinucleate trophic stages. In the plasmodium stage of the life cycle, slime molds usually produce a detectable amount of slime that are left behind as "slime tracks" as the plasmodium moves over a surface of a specific substrate. *Physarum polycephalum* is one of the latest and novel models used for research in cell biology, biochemistry, genetics, and physical studies (Oettmeier, Brix, & Döbereiner, 2017).

Chemical analyses have suggested that the exopolysaccharides (EPSs) extracted from *Ph. polycephalum* have remarkable antimicrobial properties against the Gram-positive bacterium *Staphylococcus aureus* and the yeast *Candida albicans* (Huynh, Phung Stephenson, & Tran, 2017). The EPSs and slime track also had significant antioxidant activities. The EPSs are important for bacterial cell aggregation, and they also serve as nutrients and protective barrier for the cells. In addition, EPSs from *Ph. polycephalum* showed cytotoxicity activity against the MCF-7 and HepG2 cancer cell lines.

Nguyen, Huynh, Stephenson and Tran (2017) investigated the effects of carbon source and carbon concentration on the growth of *Physarum polycephalum* and evaluated the antimicrobial and anticancer activities of their plasmodial extracts. The rate of growth of *Ph. polycephalum* was highest in 20 g/L glucose as the carbon source, with the highest amount of plasmodia produced. (Nguyen, Huynh, Stephenson, & Tran, 2017).

In this study, the antibacterial, antioxidant properties and effect against UV-induced damage of the slime track and plasmodial extracts of *Ph. polycephalum* grown on different food wastes are investigated.

2. Objectives and Hypotheses

The objective of this study is to investigate the antibacterial, antioxidant properties and effect on UV-treated *Caenorhabditis elegans* of the slime track and plasmodial extracts *Physarum polycephalum* grown on various food wastes as carbon source.

It was hypothesised that the slime track and plasmodial extracts of *Ph. polycephalum* possess antibacterial activity against Gram-positive and Gram-negative bacteria, antioxidant activity and effect against UV-treated *Caenorhabditis elegans.*

3. Materials and Methods

Preparation of food waste extracts

10 g of food waste was blended in 100 ml of deionised water. The mixture was filtered and the filtrate was centrifuged at 7000 rpm for 10 min. The supernatant was collected.

Dinitrosalicylic acid (DNS) test to determine reducing sugar concentration

The DNS reagent is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid, thus resulting in a colour change, corresponding to the concentration of reducing sugars present. 1.5 ml of DNS reagent was added to 1.5 ml of the food waste extract. The mixtures were then heated in a boiling water bath for 5 min, before 0.5ml of DNS stabiliser was added. The absorbance was read at 530 nm using a spectrophotometer. A higher absorbance corresponds to a higher concentration of reducing sugars present in the samples. The concentration of reducing sugars is read from a glucose standard curve.

Preparation of food waste agar

20 ml of the food waste extract was added to 2 g agar and the volume was made up to 100 ml with deionised water. The mixture was autoclaved at 10 psi for 10 min.

Growth of the slime mold, *Physarum polycephalum*

A piece of agar (about 1 cm x 1 cm) containing active plasmodium of *Ph. polycephalum* growing on a piece of autoclaved oat flake was placed on the surface of a fresh agar plate with food waste extract. 10 pieces of autoclaved, sterile oat flakes was placed next to the agar block. The plate was incubated in the dark at room temperature for 5 days, after which slime tracks and plasmodia were collected. Slime tracks were scraped off the surfaces of the plasmodial cultures and added

to phosphate-buffered saline (PBS). Plasmodial extracts were prepared by homogenising the plasmodium of *Ph. polycephalum* in PBS.

Growth of precultures of microorganisms

Escherichia coli OP50 and the test organisms (*Staphylococcus epidermidis* ATCC12228, *Bacillus cereus* ATCC11778, *Pseudomonas putida* ATCC31800, *Serratia marcescens* Carolina) were inoculated into 10 ml of LB (Luria-Bertani) broth and grown overnight at 30°C in a shaking incubator. The absorbance of the precultures at 600 nm was measured using a UV-vis spectrophotometer and was standardised at 0.8.

Antibacterial test using the well diffusion method

The bacterial culture was spread evenly on Mueller-Hinton agar plate with a sterile swab. 80 µl of slime track or plasmodial extract was added to a well. The positive control was 10% bleach and the negative control was sterile water. The plates were incubated at 30°C overnight and the diameter of the zone of inhibition was measured the next day. Five replicates were conducted for each extract and bacterial species.

Antibacterial test using the colony count method

In the test setup, 0.1 ml of bacterial culture was added to 1.0 ml of slime track or plasmodial extracts and 3.9 ml of LB broth. In the control setup, 1.0 ml of sterile water was added instead of slime mold extract. Five replicates for each setup were prepared. The mixtures were incubated with shaking at 30°C for 2 hours. Serial 10-fold dilutions were done with 0.85% sodium chloride solution to the appropriate dilution factor, and 0.1 ml of the diluted culture was spread on LB agar. Plates were incubated at 30°C overnight. The number of colonies was determined the next day.

DPPH antioxidant test

In the test set-ups, 0.1 ml of slime track or plasmodial extract sample was mixed with 1.9 ml of methanol and 1.0 ml of DPPH solution. In the control setup, 0.1 ml of PBS replaced the sample. For the respective blanks, 1.0 ml DPPH solution was replaced with 1.0 ml methanol. Five replicates of each test and control set-ups were prepared. The mixtures were left in darkness for 20 min. The absorbance readings were taken. The radical scavenging activity was calculated:
[(absorbance of control – absorbance of test) / absorbance of control] x 100%

Effect on survival of UV-treated *Caenorhabditis elegans*

The composition of NGM was as follows: 0.9 g NaCl, 7.5 g agar, 0.75 g bacto peptone in 300 ml water. After autoclaving, 0.3 ml cholesterol (5 mg/ml), 0.3 ml $MgSO_4$ (1 M), 0.3 ml $CaCl_2$ (1 M), 7.5 ml potassium phosphate buffer pH 6.0 (1M) were added. 0.05 ml of *E. coli* OP50 was added to the centre of a fresh NGM plate and grown overnight at 37°C. A block of agar containing *C. elegans* N2 was placed on the plate and incubated at 20°C for 2 days. *C. elegans* and *E. coli* were collected in M9 buffer and filtered through a sterile 8 µm membrane filter. *C. elegans* were resuspended in M9 buffer, and added to test tubes with 10% slime track/plasmodial extract. Drops of the solution were shone with UV for 2 minutes. For the control set-up, PBS replaced the sample. The percentage survival of worms was then determined. Worms were considered dead if they did not move and were straight.

4. Results and Discussion

Dinitrosalicylic acid (DNS) test to determine reducing sugar concentration

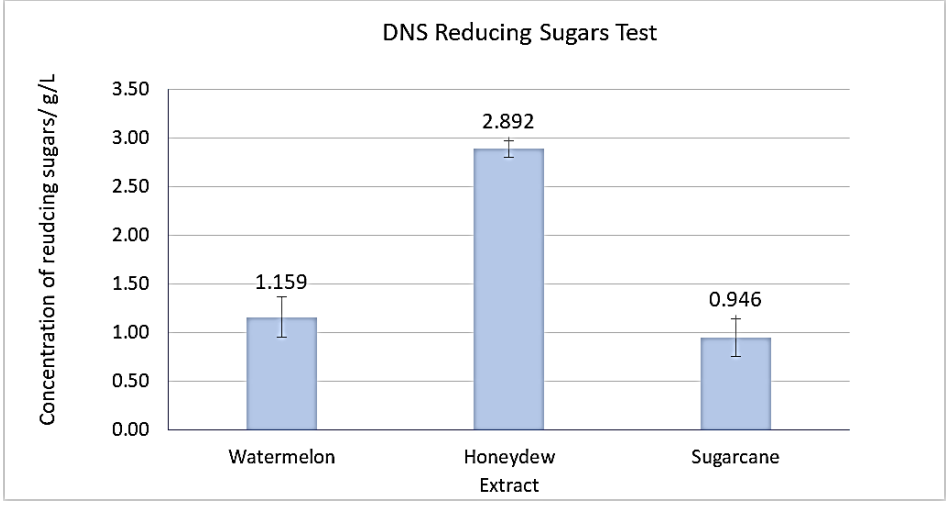


Fig. 1: Graph showing the reducing sugar concentration of the various extracts

Honeydew extract had a significantly higher amount of reducing sugars compared to extracts from watermelon and sugarcane. It was observed that the growth rate of slime mold was higher in the presence of food waste extracts than water due to the sugars present in the extracts.

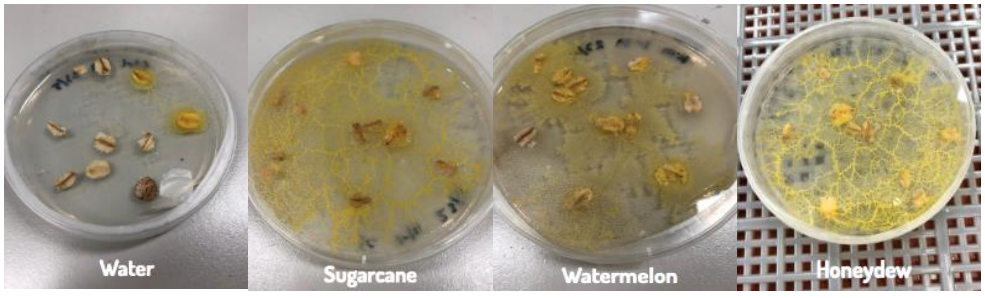


Fig. 2a, 2b, 2c, 2d (left to right): Photos showing the growth of slime mold on different media.

Antibacterial test using the well diffusion method

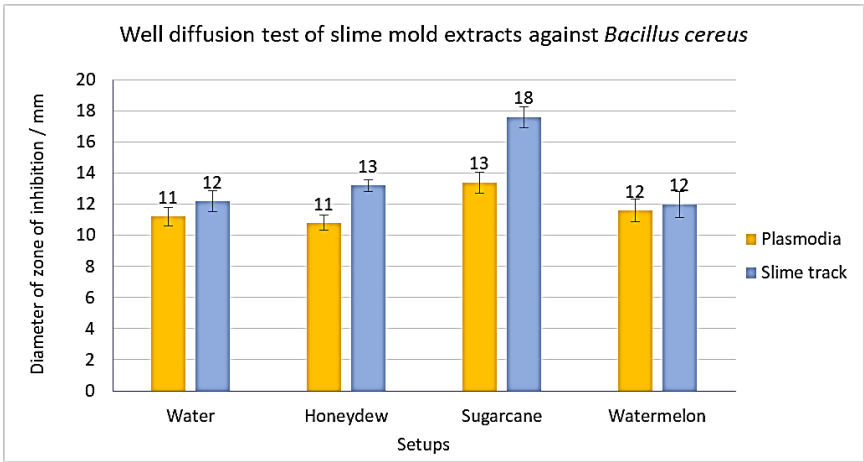


Fig. 3:
Graph showing the well diffusion test of slime mold extracts against *Bacillus cereus*

Samples were less effective against *B. cereus* than bleach. Extracts of slime mold grown on sugarcane possessed the highest antibacterial property against *B. cereus*. Extracts of slime tracks grown on honeydew and sugarcane had more significant antibacterial properties than plasmodia. (Mann-Whitney p-values: 0.014 (Honeydew) & 0.011 (Sugarcane))

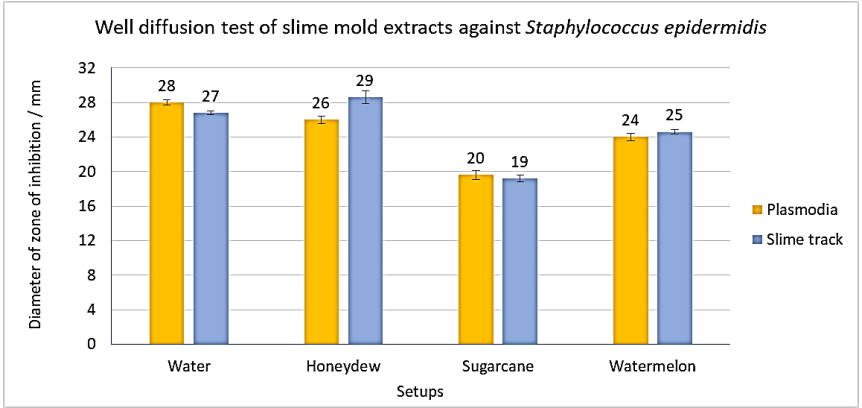


Fig. 4:
Graph showing the well diffusion test of slime mold extracts against *Staphylococcus epidermidis*

Samples were more effective against *S. epidermidis* than bleach. As seen from the Kruskal-Wallis p-value of 0.001, which is less than 0.05, slime mold extracts grown on water and honeydew possessed the highest antibacterial properties against *S. epidermidis.*

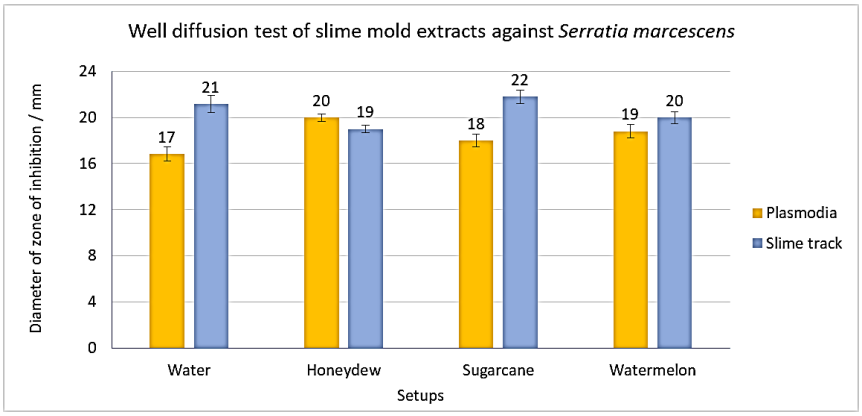
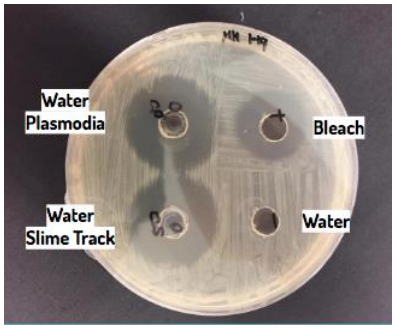


Fig. 5:
Graph showing the well diffusion test of slime mold extracts against *Serratia marcescens*

Besides having greater antibacterial properties than bleach, slime track extracts from water and sugarcane mediums possessed significant antibacterial properties against *S. marcescens* than its plasmodial extracts, as seen from the Mann-Whitney p-values of 0.010 (Sugarcane) and 0.015 (Water) which were less than 0.05.

Against *Pseudomonas putida,* slime mold extracts possessed no antibacterial properties.



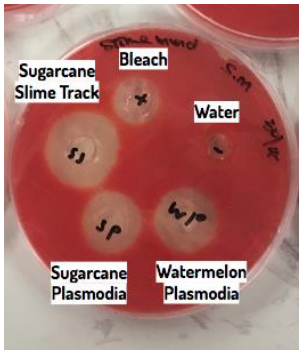


Fig. 6a (left) & 6b (right):
Photos showing the antibacterial well diffusion test of slime mold extracts against *Staphylococcus epidermidis* and *Serratia marcescens*

Antibacterial test using the colony count method

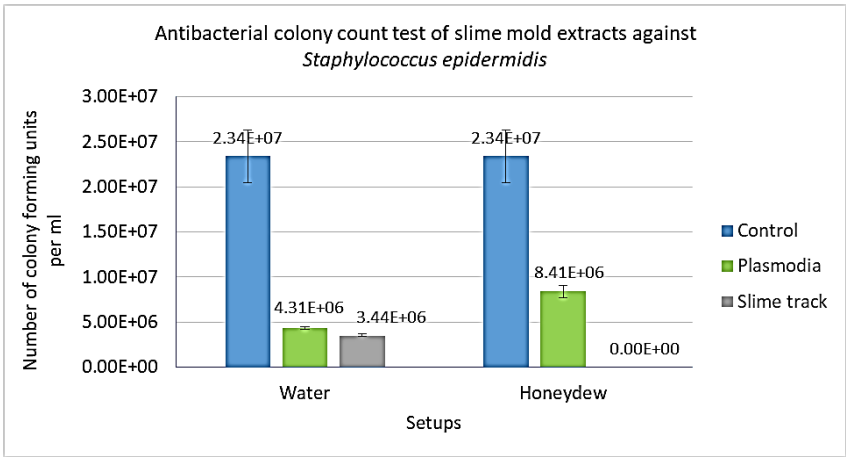


Fig. 7:
Graph showing the colony count test of slime mold extracts against *Staphylococcus epidermidis*

Slime mold extracts grown on water possessed higher antibacterial properties than those grown on honeydew, as shown by the Kruskal-Wallis p-value of 0.000 which is less than 0.05.

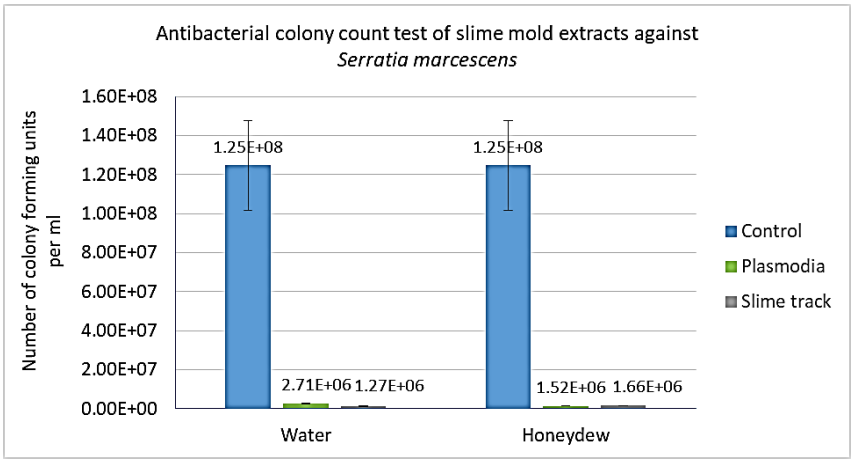


Fig. 8:
Graph showing the colony count test of slime mold extracts against *Serratia marcescens*

Against *Serratia marcescens,* the Mann-Whitney p-values are 0.012, which is less than 0.05. Thus, slime mold extracts possessed significant antibacterial properties.

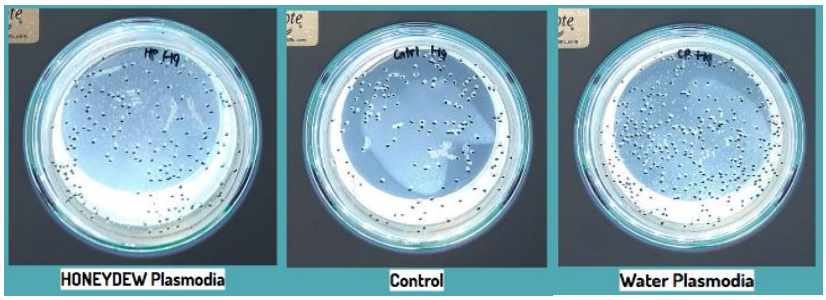


Fig. 9a, 9b, 9c (left to right):
Photos showing the colony count test of plasmodial extracts against *Serratia marcescens*

DPPH antioxidant test

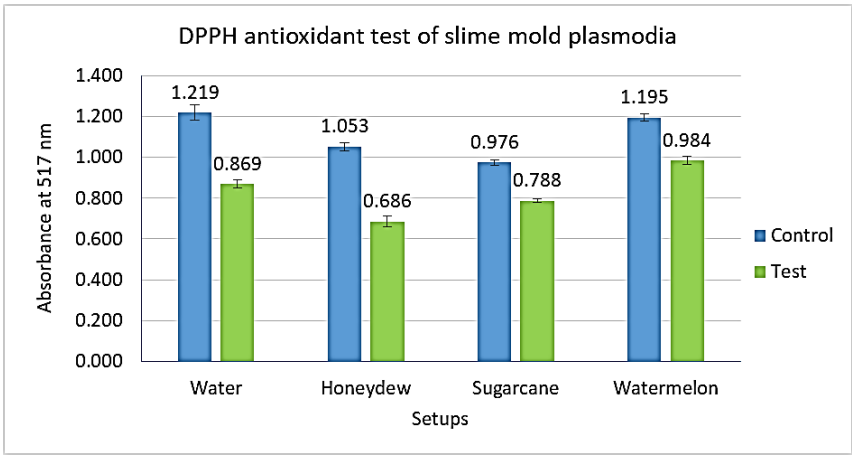


Fig. 10:
Graph showing the DPPH antioxidant test of plasmodial extracts

As the Mann-Whitney p-values are both 0.012, which is less than 0.05, plasmodia grown on water and honeydew possessed significant antioxidant effect, with potential to protect from UV-induced damage. (Kruskal-Wallis p-value: 0.000)

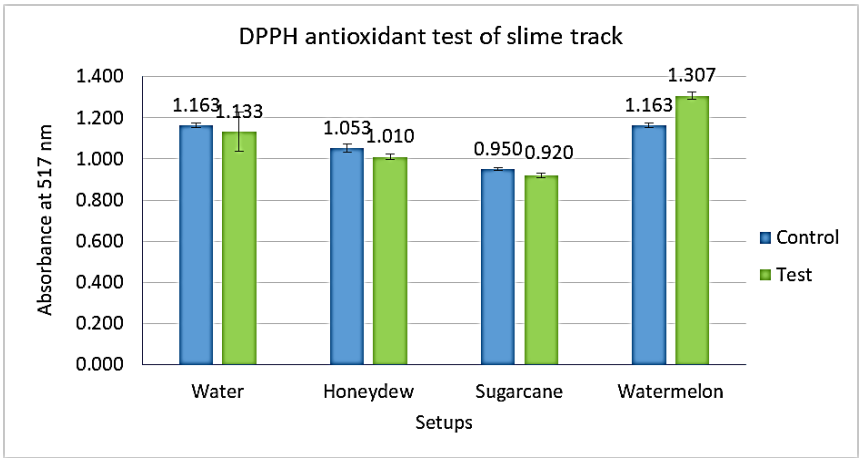


Fig. 11: Graph showing the DPPH antioxidant test of slime track extracts

Slime tracks grown on watermelon possessed no antioxidant properties. The Mann-Whitney p-values for the other 3 extracts more than 0.05, suggesting that all slime track extracts possessed insignificant antioxidant properties. (Kruskal-Wallis p-value: 0.000)

MEDIUM	Slime Track	Plasmodia
Water	2.58	28.71
Honeydew	4.08	34.85
Sugarcane	3.16	19.26
Watermelon	0.00	17.66

Table 1: Table showing the radical scavenging activity of slime mold extracts

Slime mold extracts grown on honeydew had the most significant antioxidant properties, followed by the extracts grown on water, sugarcane, and finally watermelon. Plasmodial extracts possessed more significant antioxidant properties than slime track extracts.

Effect on UV survival of *Caenorhabditis elegans*

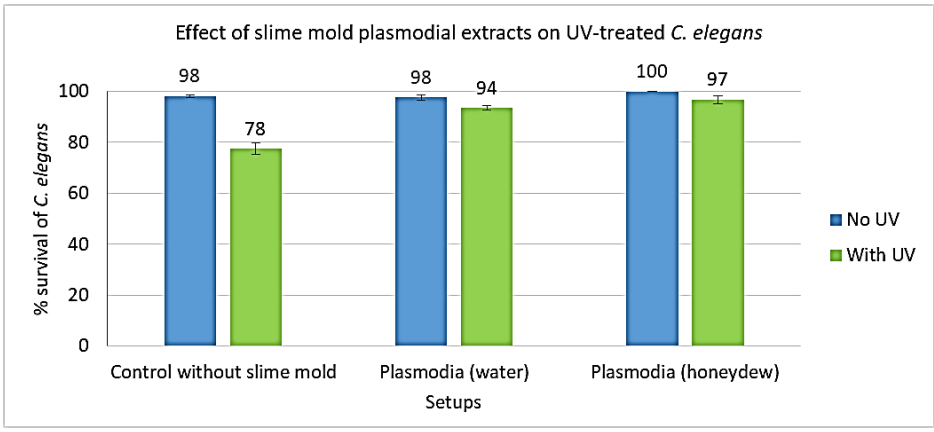


Fig. 12: Graph showing the effect of water and honeydew plasmodial extracts on UV-treated *C. elegans*

As the Kruskal-Wallis p-value was less than 0.05, plasmodial extracts grown on water and honeydew were able to protect *C. elegans* from UV-induced damage.

5. Conclusion and Recommendations for future work

Four main conclusions can be derived from this study. Firstly, slime mold grown on various food wastes had shown antibacterial properties. Secondly, slime tracks possessed greater antibacterial properties than plasmodial extracts. Thirdly, slime mold grown on water and honeydew mediums had shown significant antibacterial and antioxidant properties. Lastly, plasmodial extracts have shown greater antioxidant properties than slime tracks.

Little research has been done into slime mold, with only one research conducting antibacterial tests on *Staphylococcus aureus* and *Candida albicans* (Hyunh, Phung Stephenson, & Tran., 2017). However, our research conducted tests on a wide range of Gram-positive and Gram-negative bacteria. Hyunh, Phung Stephenson, & Tran. (2017) have suggested that exopolysaccharides (from plasmodium) possessed remarkable antibacterial and antioxidant properties while slime tracks had shown antioxidant activities. On the contrary, our research has shown that plasmodial extracts possessed significant antioxidant properties while slime track extracts possessed greater antibacterial properties. The limitation of our project was the difficulty in standardising the amount of antibacterial and antioxidant substances in the slime mold extracts.

Slime mold is not known to be pathogenic. Plasmodial extracts were effective in reducing UV-induced damage. Thus, purified plasmodial extracts grown from water and honeydew can be used to manufacture sunblocks. In addition, slime track extracts have shown to possess antibacterial properties. Hence, antibacterial agents can be created. For instance, skin-care products can be made from slime track extracts to treat acne, which is caused by *Staphylococcus epidermidis*. According to the NEA, 7.70 million tonnes of solid waste was generated in 2018, of which only 17% of food wastes were recycled. Hence, using food wastes as carbon source not only recycles them, but promotes growth of slime mold.

For further work, two different extracts can be mixed together to check whether there are synergistic effects against bacteria and UV-induced damage. We also aim to isolate the active compound within the extracts that exhibits the tested properties.

References

Huynh, T.T.M., Phung, T.V., Stephenson, S.L., & Tran, H.T.M. (2017). Biological activities and chemical compositions of slime tracks and crude exopolysaccharides isolated from plasmodia of *Physarum polycephalum* and *Physarella oblonga*. BMC Biotechnology, 17:76. doi:10.1186/s12896-017-0398-6

Nguyen, N.T.N., Huynh, T.T.M., Stephenson, S.L., & Tran, H.T.M. (2017). Study of the biological activities of Physarum polycephalum and *Physarella oblonga* plasmodial extracts. Mycosphere, 8(7), 968-976. doi: 10.5943/mycosphere/8/7/12

Oettmeier, C., Brix, K., & Döbereiner, H.-G. (2017). *Physarum polycephalum* — a new take on a classic model system. Journal of Physics D: Applied Physics, 50(41). doi:10.1088/1361-6463/aa8699

Palomino, J.C., & Martin, A. (2014). Drug resistance mechanisms in *Mycobacterium tuberculosis*. Antibiotics (Basel), 3(3), 317-340. doi: 10.3390/antibiotics3030317

Spellberg, B., & Gilbert, D.N. (2014). The future of antibiotics and resistance: a tribute to a career of leadership by John Bartlett. Clinical Infectious Diseases, 59 (suppl 2), S71-S75. doi: 10.1093/cid/ciu392

Ventola, C.L. (2015). The antibiotic resistance crisis. Part 1: causes and threats. Pharmacy and Therapeutics, 40(4), 277-283. Retrieved February 14, 2019, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4378521/

Waste Statistics and Overall Recycling. Retrieved July 3, 2019, from https://www.nea.gov.sg/our-services/waste-management/waste-statistics-and-overall-recycling