Investigating the Antimicrobial and Antioxidant Effects of Vaccinium Corymbosum extract for use in food preservation

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A. Rationale

In recent years, bacterial and yeast resistance to preservatives has been on the rise due to the rampant usage of preservatives (Stratford, 2013). There is too, greater public awareness and scientific studies on the harmful effects of several synthetic preservatives (Siavashpour, Bakhshizadeh, & Rashedinia, 2017; Staff, 2019). Furthermore, nitrates found in preservatives have been shown to posses carcinogenic properties, leading to an increased risk of gastric cancer (Staff, 2019), while sodium benzoate (most widely used in preserving carbonated drinks and other acidic food) impairs memory and motor coordination (Siavashpour, Bakhshizadeh, & Rashedinia, 2017). As can be seen, the need for alternatives possessing antimicrobial compounds is dire, in order to reduce the damage chemical preservatives cause. *Vaccinium Corymbosum* - highbush blueberries - shows great potential as a natural alternative to current synthetic food preservatives.

Anthocyanins were found to be the main component of phenolics in blueberries (Zheng & Wang, 2003). One aspect of food preservation deals with the overall appearance and sensory appeal of food items. In this aspect, antioxidative properties are particularly important. Antioxidants are able to block free radicals from causing lipid oxidation and thereby prevent food items from turning rancid (Shahidi, 2015), thereby prolonging their shelf life. The antioxidant properties of anthocyanins are well established and have been proven in cell culture studies, animal models and human clinical trials (Khoo, Azlan, Tang, & Lim, 2017). Their antioxidant properties arise from their chemical structure consisting of attached hydroxyl groups and phenolic rings (Minatel, Borges, Ferreira, Hector Alonzo Gomez Gomez, & Lima, 2017).

Another important aspect of food preservation is focused on the antimicrobial properties of a potential preservative. In previous studies (Puupponen-Pimiä, et al., 2001; Shen, et al., 2013),

focusing on the phenolic compound in cloudberry, raspberry and strawberry extracts, they proved to be strong inhibitors of the Gram-negative bacteria *Salmonella* and Gram-positive bacteria *Listeria monocytogenes*. Moreover, the malvidins are the dominant anthocyanin present in blueberries, and they possess less hydroxyl groups, making *V. Corymbosum* a possible choice to have good antimicrobial properties. The malvidins are also more hydrophobic and have a greater likelihood of being transported into the cells and tissues, thereby increasing their potential for bioactivity (Silva, et al., 2016). As such, we believe that *V. Corymbosum* is a possible candidate for antibacterial, antifungal and antioxidant active compounds. This study aims to investigate the antimicrobial properties of *V. Corymbosum* extract against Gram-positive bacteria, Gram-negative bacteria and yeasts, as well as its antioxidant properties, through both *in vitro* assays and *in vivo* tests. We see potential in V. Corymbosum to be a natural yet effective alternative in the preservation of food, and we trust that using V. Corymbosum instead of chemical preservatives will be safer for the consumers by reducing the risks that chemical preservatives pose.

B. Research Question / Hypotheses / Engineering Goals

Hypotheses:

We hypothesise that the *V. Corymbosum* extract will exhibit antimicrobial properties, against Gram-negative bacteria and fungi, as well as antioxidant properties, during both *in vitro* assays and *in vivo* tests. We also hypothesise that the antioxidative and antimicrobial properties in *V. Corymbosum* extract will successfully inhibit the growth of harmful microbes during *in vivo* testing.

The objectives of this study are to investigate the antimicrobial properties of *V. Corymbosum* extract, against Gram-negative bacteria and yeasts, as well as its antioxidant properties, through both *in vitro* assays and *in vivo* tests, for potential use in food preservation.

C. Apparatus and Materials:

The table below contains the lists of apparatus and materials used in conducting our experiment.

Apparatus	**Materials**	**Biological Organisms**
Electronic mass balance Blender Centrifuge Microfuge tubes Rotary Evaporator Sterile microfilter UV-Vis Spectrophotometer Cuvettes Micropipette Shaking incubator Tweezers Incubator Sterile plate spreaders Agar plates Autoclave	*V. Corymbosum* 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution 50% Ethanol Deionised water Lysogeny broth (LB) medium Mueller-Hinton agar Potato Dextrose Agar 10% Bleach Fresh Carrots	*Escherichia coli* (*E. coli*, ATCC 25922) *Ralstonia eutropha* (*R. eutropha*, ATCC 17699) *Aspergillus niger* (*A. Niger,* ATCC 6275)

D. Methodology:

Preparation of *V. Corymbosum* Extract:

Fresh *V. Corymbosum* were washed with deionised water, and blended for 30 seconds at room temperature. 10g of the blueberry puree was then mixed with 50ml of 50% ethanol, and agitated for 90 minutes. The mixture was then filtered using a filter paper to remove sediments such as blueberry skin. The filtrate was then centrifuged at 13000 rpm for 10 minutes. The supernatant was decanted and evaporated in a rotary evaporator at 50°C, to remove the ethanol in the mixture. The resulting mixture was filter-sterilised through a sterile microfilter.

DPPH Antioxidant Assay:

2,2-diphenyl-1-picrylhydrazyl (DPPH) is a free radical which produces a purple solution when dissolved in methanol. When it is reduced by antioxidants, a change of colouration from purple to yellow can be observed.

In the test setup, 0.1 ml of *V.corymbosum* extract was mixed with 1.9 ml of methanol and 1.0 ml of DPPH solution. In the control setup, the *V.corymbosum* extract was replaced with 0.1ml of deionised water. For the respective blanks, the 1.0 ml DPPH solution was replaced with 1.0 ml methanol. Three replicates were prepared for each test and control setup. The mixtures were left in the darkness for 15 minutes. The 3ml mixtures were then poured into 4ml cuvettes. Using a UV-Vis Spectrophotometer, the absorbance of each mixture was measured at 517nm. The final absorbance reading and the radical scavenging activity (%) was recorded as ($\frac{Abs\ of\ control - Abs\ of\ sample}{Abs\ of\ control}$) x 100, with all values representing the mean of readings obtained.

Growth of Pre-cultures of Yeast and Bacteria

Escherichia coli (*E. coli*, ATCC 25922), *Ralstonia eutropha (R. eutropha*, ATCC 17699) and *Aspergillus niger* (*A. niger,* ATCC 6275) 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 *R.eutropha* and *E.coli* were spread evenly on Mueller-Hinton (MH) agar plates with a sterile cotton swab. The agar plate was then divided into 3 sections, for the test (*V.corymbosum* Extract), negative control (Sterile Water) and positive control (10% Bleach). Circular wells of diameter 1cm were made in the center of each section, and thereafter filled with 0.1ml of *V.corymbosum* extract 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 24 hours.

Determining the Minimum Bactericidal Concentration (MBC)

8 serial 2-fold dilutions (ranging from 1/2 to 1/256) were conducted using LB medium to get different concentrations of *V.corymbosum* extract. 50μℓ of the respective bacterial cultures were then added into each microfuge tube. The microcentrifuge tubes were then incubated at 30°C for 24 hours in an incubator. After incubation, 100μℓ of the diluted culture from each microcentrifuge tube was spread on a MH agar plate. The agar plates were then sealed tightly with parafilm and incubated at 30°C for 24 hours in an incubator to allow the bacteria, if any, to grow. Thereafter, the MH agar plates were observed to see if bacterial growth was present. The lowest concentration of the *V.corymbosum* extract with no bacterial growth on the Mueller Hinton agar plate was recorded as the minimum bactericidal concentration. The procedures were carried out in quintuplicates.

Fungi Growth Test (In-vitro)

A.niger was inoculated in 20 mℓ of potato dextrose broth and incubated at 30°C for 24 hours with shaking in an orbital shaker. 50μℓ of the *A. niger* overnight culture was then pipetted into the center of a potato dextrose agar plate, and incubated at 30°C for 72 hours. Thereafter, 100% potato dextrose agar (PDA) plates were used in the control setup, while 90% potato dextrose and 10% *V.corymbosum* extract agar plates were used in the test setup. 1cm by 1cm squares were cut out of the PDA plates containing the grown fungi, and placed at centre of each agar plate. The agar plates were then sealed tightly with parafilm and incubated in an incubator at 30°C for 120 hours. After incubation, the diameter of fungal growth was measured with a meter rule. Seven replicates were conducted.

Carrot Fungi Growth Test (In-vivo)

Fresh Australian carrots were sliced to slices of diameter 3cm, and thickness of 0.5cm. Each carrot slice was then placed on a petri dish. The carrot slices were soaked in 70% ethanol for 15 minutes entirely. Thereafter, for the test setup, 500μℓ *V.corymbosum* extract was pipetted into the center of the carrot slice. For the negative control, 500μℓ of sterile water was pipetted, while in the positive control, 500μℓ of 10% bleach was pipetted. The agar plates were then sealed

tightly with parafilm and incubated in an incubator at 30°C for 120 hours. After incubation, the diameter of fungal growth was measured. The procedures were carried out in quintuplicates.

E. Results & Discussion:

DPPH Antioxidant Assay

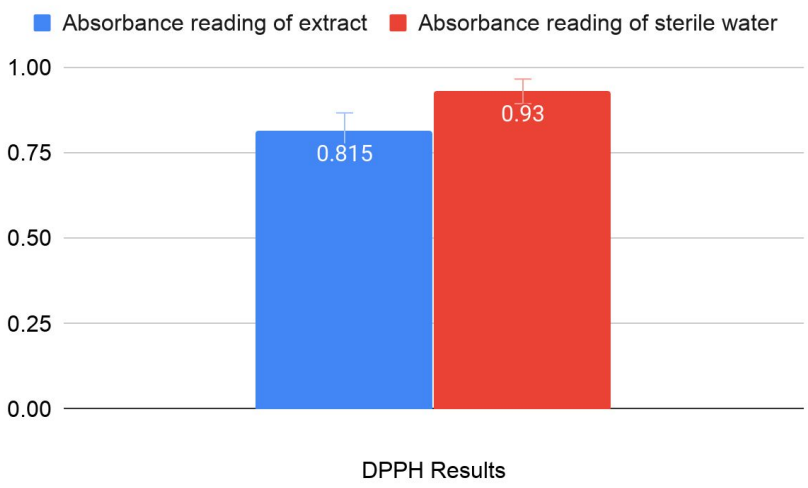

Fig 1: Graph showing the average absorbance readings generated by *V. Corymbosum* extract against sterile water (control)

Final radical scavenging activity of *V.corymbosum* extract (%):

$$\left(\frac{0.930 - 0.815}{0.930}\right) \times 100 = 12.37\%$$

As seen from Fig 1, the *V.corymbosum* extract and the control have similar absorbances at 517 nm, showing only slight reduction of the free radical, DPPH, and thus the *V.corymbosum* extract is not shown to have significant antioxidant activity, with low radical scavenging activity of 12.37%.

Agar Well Diffusion Test

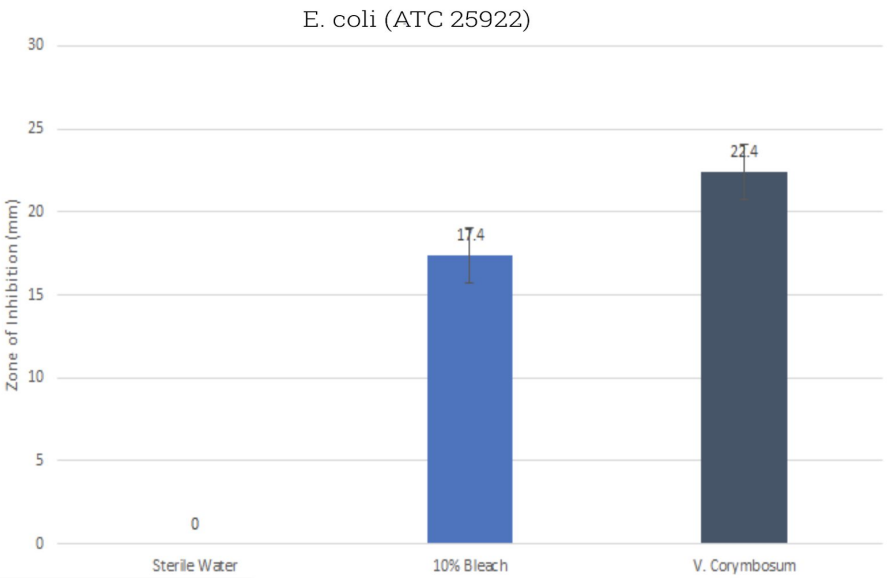

Fig 2.1: Graph showing average diameters of Zone of Inhibition (mm) generated by *V. Corymbosum* extract against *E. coli*

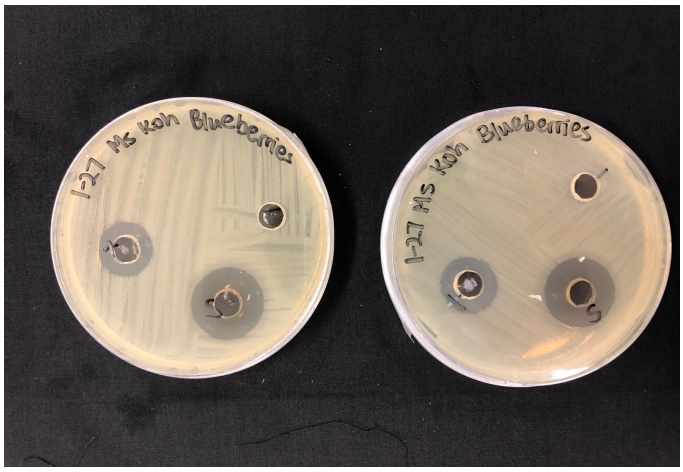

Fig 2.2: Agar plates shown after conducting well diffusion test of E. coli. S represents V. Corymbosum extract, + represents 10% bleach, - represents sterile water.

(note that the agar plates as shown are the best results in the quintiplicates we did)

As seen from the graph in Figure 2.1 and the agar plates in Figure 2.2, the zone of inhibition of the *V. Corymbosum* extract was 22.3% greater than the zone of Inhibition of the 10% bleach. Since the error bars did not overlap, there was an observable difference between positive control bleach and the extract. We can conclude that the V. *Corymbosum* extract exhibited a more

promising effect in inhibiting growth of *E. coli* than 10% bleach solution. This proves our hypothesis that *V. Corymbosum* would exhibit antimicrobial properties against *E. coli.*

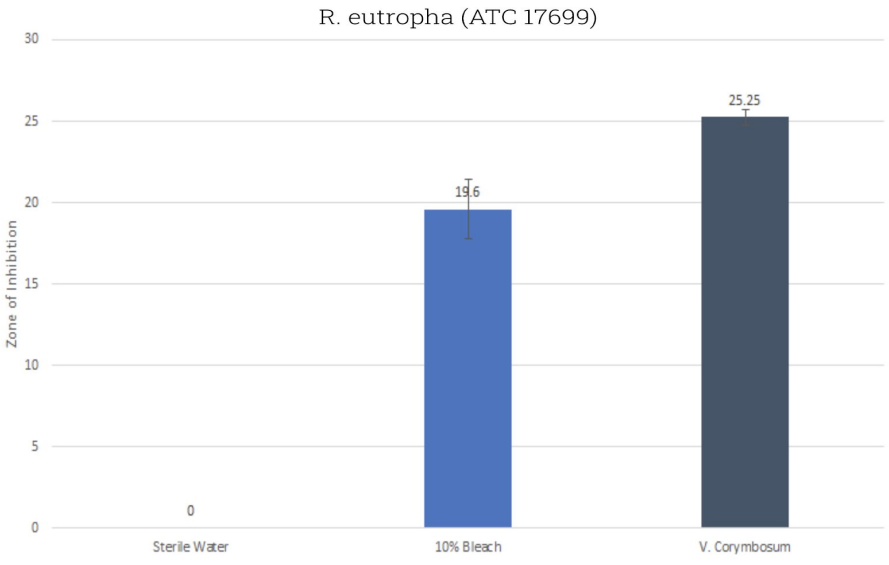

Fig 3.1: Graph showing average diameters of Zone of Inhibition (mm) generated by *V. Corymbosum* extract against *R.Eutropha*

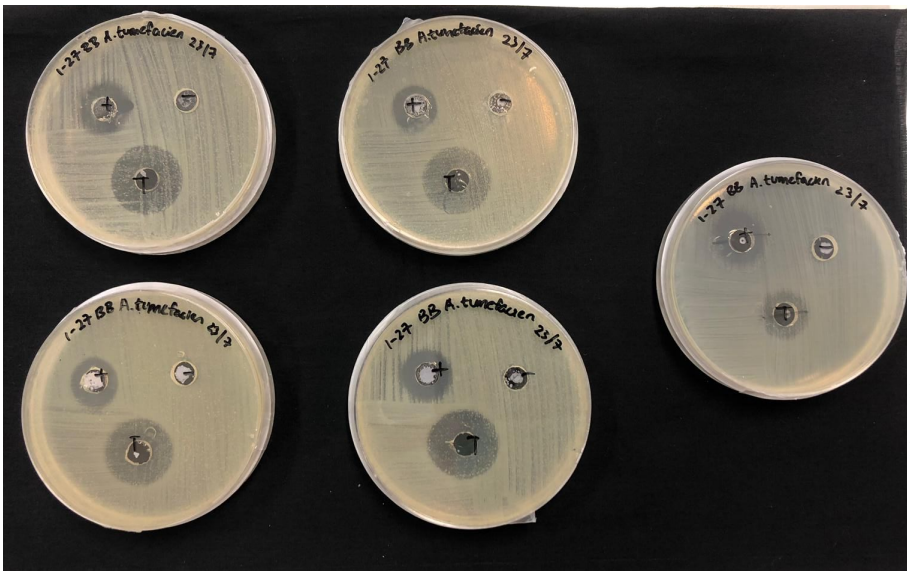

Fig 3.2: Agar plates shown after conducting well diffusion test of R. eutropha. T represents V. Corymbosum extract, + represents 10% bleach, - represents sterile water.

As seen from the graph in Figure 3.1 and the agar plates in Figure 3.2, the zone of inhibition of the *V. Corymbosum* extract was 22.4% greater than the zone of Inhibition of the 10% bleach.

Since the error bars did not overlap, there was an observable difference between positive control bleach and the extract. *V. Corymbosum* extract. The plant extract exhibited promising effect in inhibiting growth of *R. Eutropha* than 10% bleach solution. This proves our hypothesis that *V. Corymbosum* would exhibit antimicrobial properties

Minimum Bactericidal Concentration (MIC)

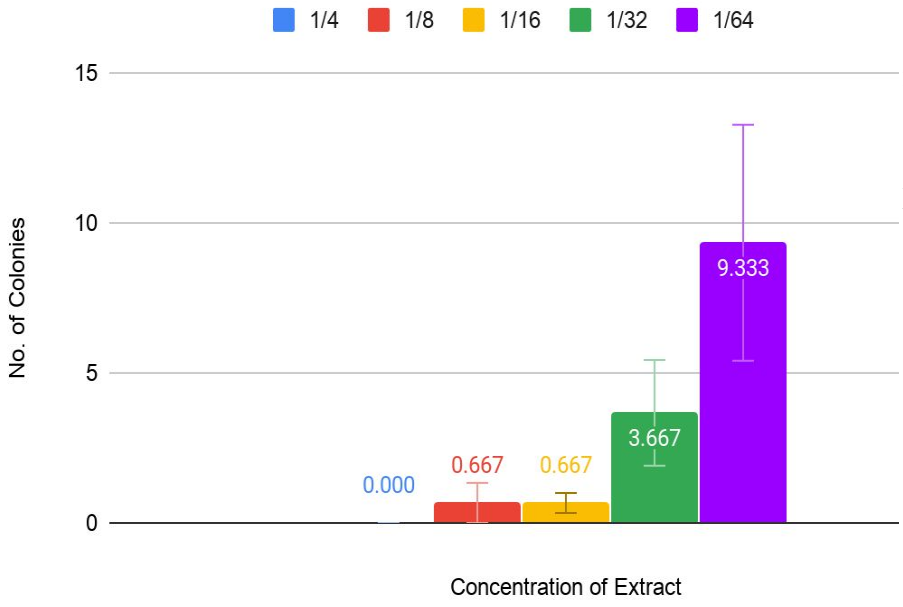

Fig 4: Graph showing number of *E.coli* colonies formed on agar plate with each concentration of *V.Corymbosum* extract **(1/128 concentration had an uncountable number of colonies and is thus not included in the graph)**

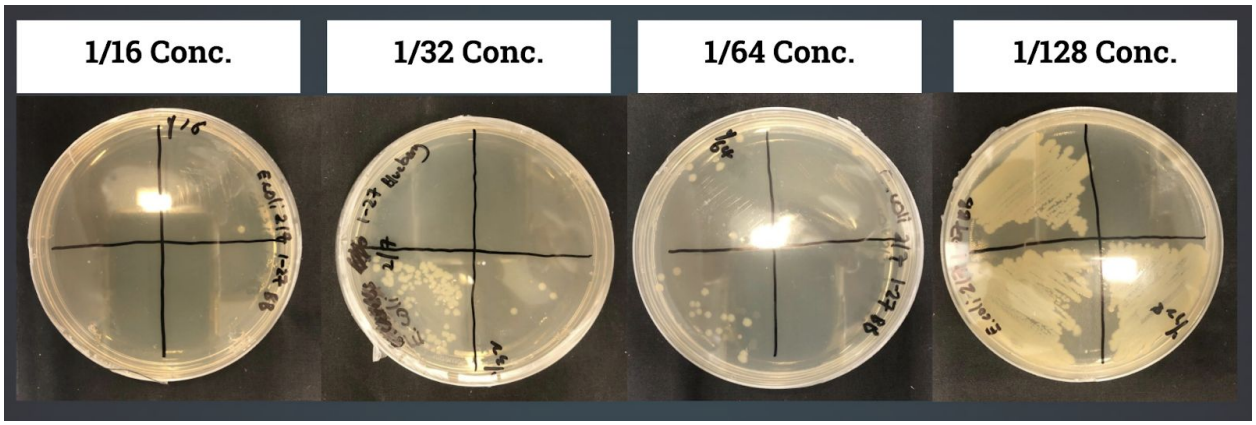

Fig 5: Picture showing the agar plates obtained after spreading solutions containing different concentrations of extract and a fixed concentration of LB broth containing E. coli.

As seen from Fig 4 and Fig 5, the agar plates had sporadic colonies at 1/64 concentration but a huge confluence at 1/128 concentration. Hence, we can conclude that the minimum bactericidal concentration of the extract is between 1/64 to 1/28 concentration.

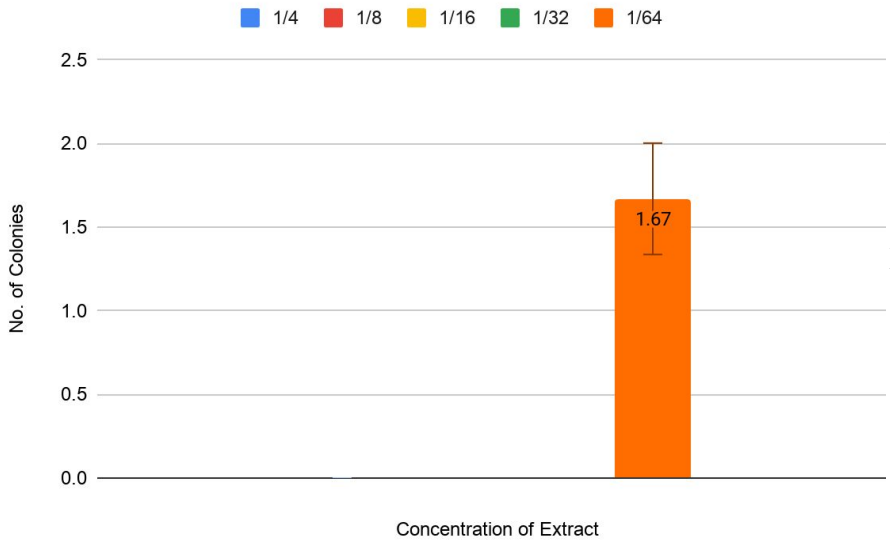

Fig 6: Graph showing number of *R.eutropha* colonies formed on agar plate with each concentration of *V.corymbosum* extract

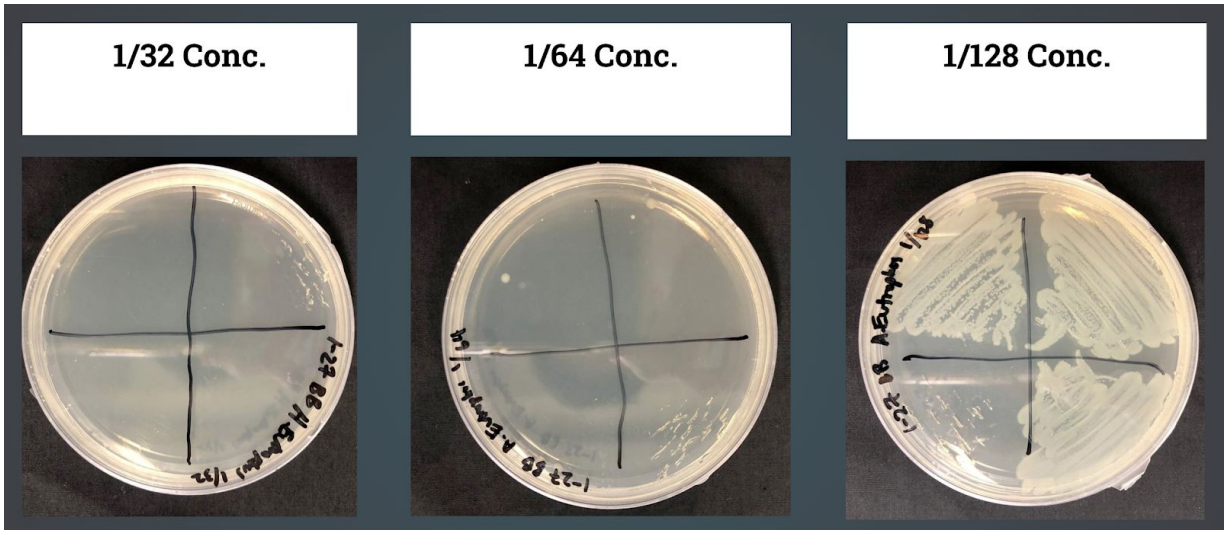

Fig 7: Picture showing agar plates obtained after spreading solutions containing different concentrations of extract, and a fixed concentration of LB broth containing R. eutropha.

As can be seen from Fig 6 and Fig 7, the extract exhibited significant bactericidal properties for concentrations below 1/128 concentrations, with little to no colonies forming. Thus we can

conclude that the minimum bactericidal concentration of the extract against R. eutropha lies between 1/64 to 1/128 concentration.

Fungi Growth Test (In-vitro)

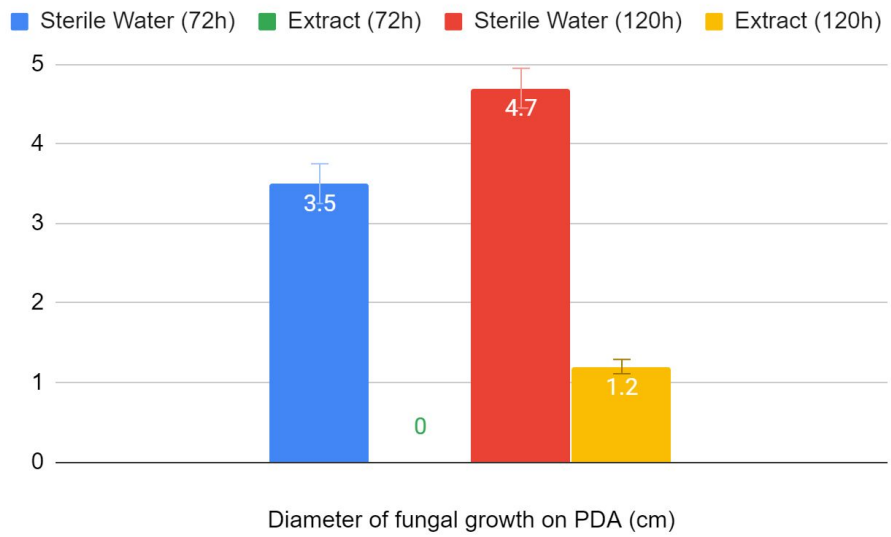

Fig 8: Graph showing the average diameter of fungal growth on potato dextrose agar (cm) for *V.corymbosum* extract, against sterile water (control), after 72h and 120h of incubation

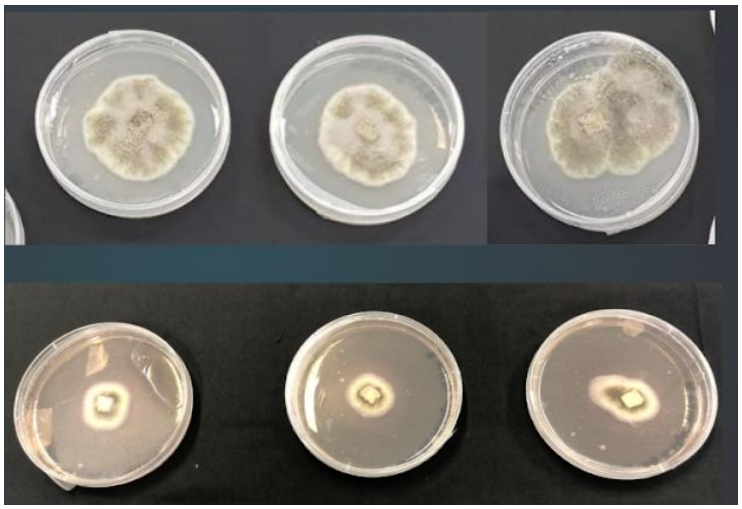

Fig 9: Pictures showing the fungal growth of *A.niger* on potato dextrose agar plates with sterile water (above), and with *V.corymbosum* extract (below), after 120h

As seen from Fig 8, there is no observable diameter of fungal growth in the test set up with *V.corymbosum* extract, while a significant amount of fungal growth is observed in the control set

up. At 120h, the diameter of fungal growth continued to be relatively larger in the control set up, while the test set up with *V.corymbosum* extract started to show a small amount of fungal growth. From the data collected, the *V.corymbosum* extract exhibited significant antifungal properties against A.niger in-vitro, and is effective in inhibiting its growth.

Fungi Growth Test (In-vivo):

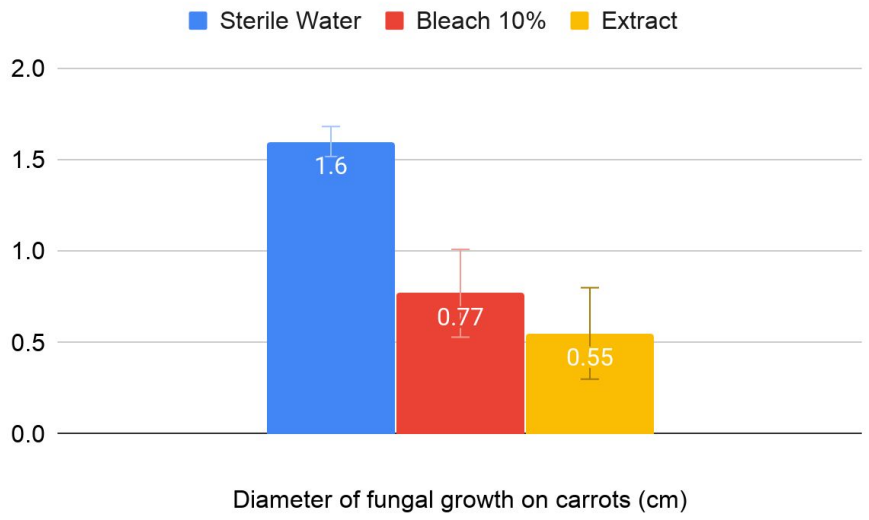

Fig 10: Graph showing the average diameter of fungal growth on sliced carrots (cm) for *V.corymbosum* extract, against sterile water (negative control) and 10% bleach (positive control)

Fig 11: Pictures showing fungal growth of *A.niger* on sliced carrots after 72h, with sterile water (left), *V.corymbosum* extract (middle) and 10% bleach (right)

As seen in Fig 10, the diameter of fungal growth on carrots in the set up with sterile water, is significantly larger than that of the fungal growth observed on 10% bleach and the *V.*

corymbosum extract, which have similar diameters of fungal growth. From the data collected, it can be concluded that the *V. corymbosum* extract exhibited significant antifungal properties against *A.niger* in-vivo on carrots, with an effectiveness comparable to that of 10% bleach.

Conclusion and Recommendations for Future Research

From the results obtained above, we can conclude that the *V.corymbosum* extract possesses a low level of antioxidant properties, and thus will not be effective in inhibiting oxidation. This finding was not only against our hypothesis, but also contradictory to the research conducted by Burdulis, D (2009), which concluded that *V.corymbosum* had strong antioxidant properties. Further research can be conducted, to find out if the discrepancy may have been attributed to the antioxidant properties being lost during the process of evaporating the ethanol in the *V.corymbosum* extract.

The zone of inhibition for *V.corymbosum* was 22.3% and 22.4% larger than 10% bleach, the positive control, for both E.coli and R.eutropha respectively. This proves our hypothesis that *V.corymbosum* would exhibit antimicrobial properties. *V.corymbosum* can be seen as an effective alternative to 10% bleach to inhibit growth of *E.coli* and *R.eutropha*. These results were further reinforced by our MBC results, with *V.corymbosum* extract having a final MBC of between 1/64 and 1/128 for both *E.coli* and *R.eutropha.*

In addition, *V.corymbosum* extract was also able to successfully inhibit the growth of the *A.niger* fungi*,* both in-vitro and in-vivo, with a similar effectiveness to 10% bleach. This proves our hypothesis that the *V.corymbosum* extract would exhibit antifungal properties against the *A.niger* fungi. This finding also suggests that the *V.corymbosum* extract would be a useful fungicide, protecting crops and food from being infected and contaminated by black mold.

As an extension of this study, additional tests can be carried out on a wider range of bacteria and fungi such as *Agrobacterium tumefaciens* and *Erwinia carotovora*, which are pathogenic organisms with a higher biosafety level, and have been commonly associated with crown gall disease and soft rot in crops respectively.

Currently, no research has concluded on the presence of any harmful substances in *V. corymbosum*. In contrast, earlier parts of our report have cited numerous research showing the potential side effects of consuming synthetic preservatives and fungicides.

With the positive results from our antimicrobial tests, *V. corymbosum* extract can thus be regarded as an natural alternative to synthetic fungicides and food preservatives.

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