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In Vitro and In Silico Analysis of the Antifungal Activity of Beta-cyclodextrin and Cinnamon Oil Inclusion Complexes against *Fusarium oxysporum f. sp. cubense*-TR4

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ABSTRACT

The Panama Disease-TR4 is a fatal disease of banana plants caused by the fungus *Fusarium oxysporum f. sp. cubense*-TR4 (Foc-TR4). Cinnamon oil has known antifungal properties, while beta-cyclodextrin has been proven to protect essential oils from degradation through encapsulation. The study aims to determine the effectiveness of cinnamon oil, and its beta-cyclodextrin complex against Foc-TR4, through their characterization, in vitro antifungal screening, and in silico docking analysis. The characterization of the complex yielded an encapsulation efficiency of 61.18%, an average particle size of 98.27 μm, while its calorimetry confirmed complex formation. Cinnamon oil, beta-cyclodextrin, and the complex were tested against Foc-TR4 through spread plate method against Ivagard (dialkyl dimethyl ammonium chloride) in triplicate. Among the treatments, the complex exhibited significantly weaker antifungal activity, with only cinnamon oil comparable with Ivagard at $\alpha = 0.05$. Cinnamaldehyde displayed favorable binding energies of -6.1 and -4.9 kcal/mol against the Foc-TR4 proteins, and -3.7 kcal/mol against beta-cyclodextrin. This implies that cinnamaldehyde can spontaneously form stable complexes with the Foc-TR4 proteins or beta-cyclodextrin. These findings suggest the potential of cinnamaldehyde, in cinnamon oil and even in the inclusion complexes, as a viable antifungal treatment against Foc-TR4.

Keywords: *antifungal; characterization; cinnamaldehyde; docking; Foc-TR4*

INTRODUCTION

The Panama Disease-Tropical Race 4 (Panama Disease-TR4), also known as the Fusarium wilt of bananas, is a fast-spreading disease currently targeting both local and international banana production (Karp, 2019). The disease is caused by the infestation of the fungus *Fusarium oxysporum f. sp. cubense*-TR4 (Foc-TR4) that spreads in soil. The disease creeps into the vascular system of banana plants and can only be diagnosed after the plant has already been infected, and possibly has died. The disease is greatly feared in plantations, since it has high occurrences in approximately 91% of the landmass used for banana production (Salvacion et al., 2019). It would be troublesome for the disease to spread as this would cause the death of most, if not all, banana plants grown in that particular area (Karp, 2019), and also render the soil unusable for further banana production, since the fungi can stay within the soil for years.

Foc-TR4 is known to be resistant to commercially available antifungals and fungicides, and so the mitigations undertaken by local and international banana growers are mainly preventative (Ploetz, 2015). These preventative measures include the use of resistant banana variants, vaccine-like treatments, and other phytosanitary practices (Arcalas, 2017; Madrazo-Sta. Romana, 2012; Ploetz, 2015).

Cinnamon oil contains the active compound cinnamaldehyde, which has been found to manifest antimicrobial and antifungal properties, such as in the studies of Hill et al. (2013) and Munhuweyi et al. (2018). Kowalska et al. (2021) reported that the antifungal activity and mechanism of cinnamaldehyde has been assessed against other species of the *Fusarium* genus, such as *Fusarium sambucinum* (causing dry rot disease of potato), *Fusarium proliferatum* (causing dry rot of garlic and bananas), and *Fusarium verticillioides* (causing dry rot of corn). However, cinnamaldehyde has yet to be tested against Foc-TR4, and as such, its mechanism and antifungal potential against it are unknown.

It has been studied that the antifungal mechanism of cinnamaldehyde involves the disruption of the cell wall integrity of fungi, leakage of cytoplasmic contents, and mitochondrial destruction, such as in the case of *Fusarium verticillioides* (Xing et al., 2014). This is congruent with the results of Wei et al. (2020), which identified that cinnamaldehyde attacks the cell membrane of *Fusarium sambucinum*, thus decreasing mitochondrial membrane potential and inhibiting intracellular reactive oxygen species. Similarly, cinnamaldehyde also reduces the synthesis of ergosterol, which is necessary for the growth and reproduction of fungi.

Despite this, the high volatility of cinnamon oil may prevent the full utilization of these properties. For increased protection against degradation and oxidation, microencapsulation of cinnamaldehyde molecules may prove to be effective, such as in the research of Munhuweyi et al. in 2018, where cinnamon oil and beta-cyclodextrin microcapsules were utilized as antifungals for plants infected by the *Botrytis* sp. The protection of the active ingredient cinnamaldehyde in cinnamon oil using beta-cyclodextrin molecules may even increase its effectiveness as an antifungal and enhance its delivery, as encapsulation can protect the active ingredient from different surroundings and conditions, as stated by Hill et al. (2013).

Thus, the project aims to determine the antifungal activity of cinnamon oil and its complex with beta-cyclodextrin against Foc-TR4 in order to determine the viability of these as treatments for the Panama Disease-TR4. Specifically, the project aims to characterize the produced inclusion complexes based on encapsulation efficiency, particle size, and thermal stability; to analyze the in vitro antifungal activity of the inclusion complexes as compared to a commercial antifungal, as well as cinnamon oil and beta-cyclodextrin alone through an antifungal assay; to predict the stability of the inclusion complexes through molecular docking; and, to identify the binding profiles of the inclusion complexes with select Foc-TR4 target proteins through molecular docking.

Through this research, the possibility of the notable antifungal properties of cinnamon oil encapsulated with beta-cyclodextrin to control and/or alleviate the spread of the Panama Disease-TR4 is considered. Furthermore, this study aims to gain insight on the antifungal mechanism of cinnamaldehyde against Foc-TR4 specifically. Lastly, the spread of this disease through the soil-borne pathogen hinders the production of bananas, one of the top export products of the Philippines (Philippine Statistics Authority, 2019). Thus, discovering a cure for the Panama Disease-TR4 can restore and support both local and international banana industries.

METHODS

Materials and Equipment. Commercially available cinnamon oil was purchased from Chemworld Fragrance Factory in Centris Walk, Quezon City. Beta-cyclodextrin of molecular weight 1134.98 was acquired from HiMedia Laboratories, LLC. A laboratory mortar and pestle were used for the preparation of the inclusion complexes.

Test tubes, sterilized distilled water, and a vortex mixer were used for the preparation of treatments for the antifungal assay. Pure cultures of *Fusarium oxysporum f. sp. cubense*-TR4 were procured from the UTPI-Biotechnology and Research Services laboratory in Bukidnon, and prepared with the use of a cork-borer. Potato dextrose agar and petri dishes were used for replicate plate preparation. A micropipette and a plate spreader were used for treatment dispensing, while a pair of tweezers was used for culture disc application. Lastly, an incubator and a ruler were used for culture growth and measurement.

Preparation of beta-cyclodextrin and cinnamon oil inclusion complexes. A 16 mmol/L concentration mixture of cinnamon oil and beta-cyclodextrin was prepared by dissolving 16 g of beta-cyclodextrin and 1.8630 g of cinnamon oil in 6 mL of absolute ethanol. This mixture was physically kneaded together in a mortar and pestle for 50 minutes, and was then left to dry at room temperature for 24 h (Kamimura et al., 2014).

Characterization of the inclusion complexes based on encapsulation efficiency. For encapsulation efficiency (*EE*), the amount of cinnamaldehyde encapsulated within the inclusion complexes was determined by a spectrophotometry service provided by the Central Instrumentation Facility of DLSU Laguna Campus. The encapsulation efficiency was then calculated using the following equation (Hill et al., 2013):

$$EE = \frac{mass\ of\ complexed\ compound}{initial\ mass\ of\ compound} \times 100\% \quad (1)$$

where the mass of the complexed compound is the amount of cinnamaldehyde extracted from the inclusion complexes, and the initial mass of compound is the amount of input cinnamon oil.

Characterization of the inclusion complexes based on thermal behavior. The thermal behavior of the inclusion complexes was determined by differential scanning calorimetry provided by ADMATEL, located in the DOST Compound in Bicutan, Taguig City.

Characterization of the inclusion complexes based on particle size. The particle size distribution of the inclusion complexes was determined by particle size analysis through SEM imaging provided by ADMATEL, located in the DOST Compound in Bicutan, Taguig City.

Modeling and validation of Foc-TR4 proteins. Two Foc-TR4 proteins, namely chitin synthase and sterol 24-C-methyltransferase, were selected based on their function in Foc-TR4 growth and survival, as well as on the possible targets of cinnamaldehyde for its antifungal activity (Shreaz et al., 2016). The proteins were retrieved from the UniProtKB database with accession numbers Q5YCX1 and A0A559KN81, respectively. The homology models were generated using the SWISS-

MODEL server (Abrigach et al., 2018; Waterhouse et al., 2018; Bienert et al., 2017; Guex et al., 2009; Studer et al., 2020; Bertoni et al., 2017). The homology models were also validated through PROCHECK, ERRAT, and VERIFY3D tools by uploading the PDB file of each homology model to the SAVES (v6.0) structure validation server (Messaoudi et al., 2013).

Docking of Foc-TR4 proteins with cinnamaldehyde. Blind dockings of such proteins with cinnamaldehyde were then conducted using Autodock Vina 1.1.2 (Trott and Olson, 2010). A grid map of 40x40x40 points with a spacing of 0.375 was used, centered at the two respective proteins. The docking results were then visualized using the Protein-Ligand Interaction Profiler server (Adasme et al., 2021).

Docking of beta-cyclodextrin with cinnamaldehyde. Docking analysis of cinnamaldehyde with beta-cyclodextrin was performed using AutoDock Vina 1.1.2. using the default Vina forcefield (Trott and Olson, 2010). A grid map of 40x40x40 points with a spacing of 0.375 was used, centered at beta-cyclodextrin.

Preparation of treatments. A total of five experimental treatments were prepared: cinnamon oil at concentrations of 0.10 and 0.010 mL/mL, beta-cyclodextrin at a concentration of 0.025 g/mL, and beta-cyclodextrin-cinnamon oil inclusion complexes at concentrations of 0.44 and 0.025 g/mL. Ivagard (dialkyl dimethyl ammonium chloride, DDAC) at a concentration of 0.010 mL/mL and sterilized distilled water were used as the positive and negative control treatments, respectively.

Antifungal assay using spread plate method. Twenty-one potato dextrose agar (PDA) plates were prepared. The seven treatments were each delivered at 0.1 mL onto three PDA plate replicates per treatment, and were evenly applied with the use of a plate spreader. Pure Foc-TR4 culture discs were prepared using a cork-borer, and a single culture disc was inoculated on the center of each PDA plate for all replicate plates. The replicate plates were incubated at 25 °C for 14 days, checking the growth and measuring the diameter (cm) of the fungi on the third, seventh, and fourteenth days.

Data Analysis. The colony diameters obtained from the spread plate method per day of reading were compared using the Kruskal-Wallis test and the Dunn test at $\alpha = 0.05$. This was done for all days of reading to observe the significance of the differences between the means.

RESULTS AND DISCUSSION

Characterization of the inclusion complexes based on encapsulation efficiency. Beta-cyclodextrin formed complexes with cinnamaldehyde with an encapsulation efficiency of 61.18%. The inclusion complexes exhibited a considerably high degree of encapsulation considering the use of cinnamon oil instead of pure cinnamaldehyde as the guest compound in the inclusion complexes, as well as considering the utilization of the kneading method for the production of the inclusion complexes. A full copy of the spectrophotometry results and the encapsulation efficiency calculations can be found in Appendix A.

In a study by Hill et al. (2013), beta-cyclodextrin exhibited an encapsulation efficiency of 41.72% with cinnamon bark extract, while it exhibited an encapsulation efficiency of 84.70% with pure cinnamaldehyde. Similar to cinnamon bark extract, cinnamon oil contains components other than cinnamaldehyde such as eugenol and linalool, with cinnamon oil comprising 85% cinnamaldehyde (Ooi et al., 2006). The presence of these substances may have led to a lower encapsulation efficiency for the produced inclusion complexes than it would have otherwise exhibited if it were produced using pure cinnamaldehyde.

The inclusion complexes may have also exhibited a lower encapsulation efficiency due to the kneading method that was employed for their formation. In a study by Tao et al. (2014), the kneading method led to a lower encapsulation efficiency compared to the freeze-drying method, primarily due to the volatility of the essential oil. The kneading method, which includes a mixing and drying step at room temperature, may induce some vaporization of the cinnamon oil, leading to a lower encapsulation efficiency.

Characterization of the inclusion complexes based on thermal behavior. The resulting thermogram of the produced inclusion complexes has been compared with the thermograms of cinnamaldehyde, beta-cyclodextrin, and the inclusion complexes produced by Hill et al. (2013), as can be seen in Figure 1.

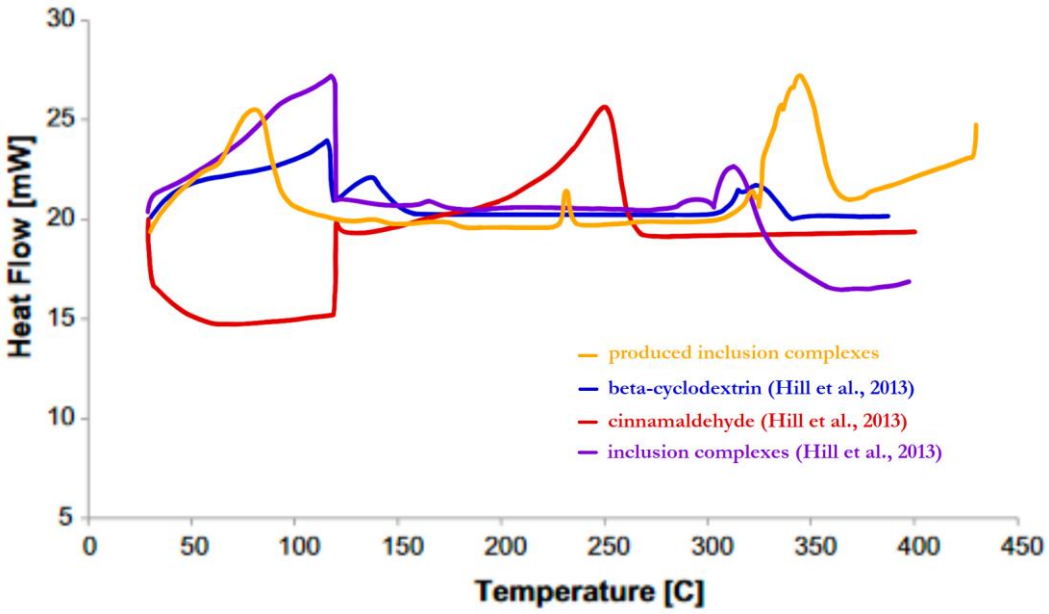

Figure 1. Differential scanning calorimetry thermograms of beta-cyclodextrin (blue), cinnamaldehyde (red), and inclusion complexes (violet) by Hill et al. (2013), and the produced inclusion complexes (yellow).

The most significant endothermic peaks produced by the inclusion complexes can be observed at temperatures of 80.69 °C, 231.45 °C, and 344.65 °C. A full copy of the differential scanning calorimetry results can be found in Appendix B.

The thermogram of the produced inclusion complexes show similarities to the thermograms by Hill et al. in 2013, especially that of their own beta-cyclodextrin-cinnamaldehyde complexes. The endothermic peak displayed by the inclusion complexes before 100°C corresponds to the evaporation of water, while the peak after 300°C corresponds to the thermal decomposition of beta-cyclodextrin (Tao et al., 2014). The reduction of the endothermic peak displayed by cinnamaldehyde at 230°C to 260°C—which can be attributed to its hydrolysis or oxidation (Tao et al., 2014)—in the produced inclusion complexes thermogram should indicate that beta-cyclodextrin was able to successfully encapsulate and protect most of the cinnamaldehyde from degradation.

Characterization of the inclusion complexes based on particle size. The produced inclusion complexes exhibited diameter sizes between 68.92 µm and approximately 350.00 µm, with an average size of 98.27 µm. A full copy of the optical microscopy and particle size analysis results can be found in Appendix C. Microcapsule size ranges from 200 nm to 5000 µm (da Silva et al., 2014), which means that the produced inclusion complexes are confirmed to be microcapsules.

The kneading method employed to create the microcapsules may have contributed to a relatively small particle diameter, due to the high stress used on the complexes (Tao et al., 2014). The inclusion complexes, due to the beta-cyclodextrin coating, may also have had a tendency to agglomerate and assemble in water (Kamimura et al., 2014). However, since the kneading method makes use of less water than other encapsulation methods, this may have led to less agglomeration of the beta-cyclodextrin, which consequently may have also contributed to a smaller particle size and size diversity.

Modeling and validation of Foc-TR4 proteins with cinnamaldehyde. Validation tests for the homology models using PROCHECK, VERIFY3D, and ERRAT tools yielded intermediary results. A full copy of the statistics and plots generated by the three validation tools can be found in Appendices D, E, and F. The Ramachandran plots obtained from PROCHECK for both homology models can be found in Figure 2.

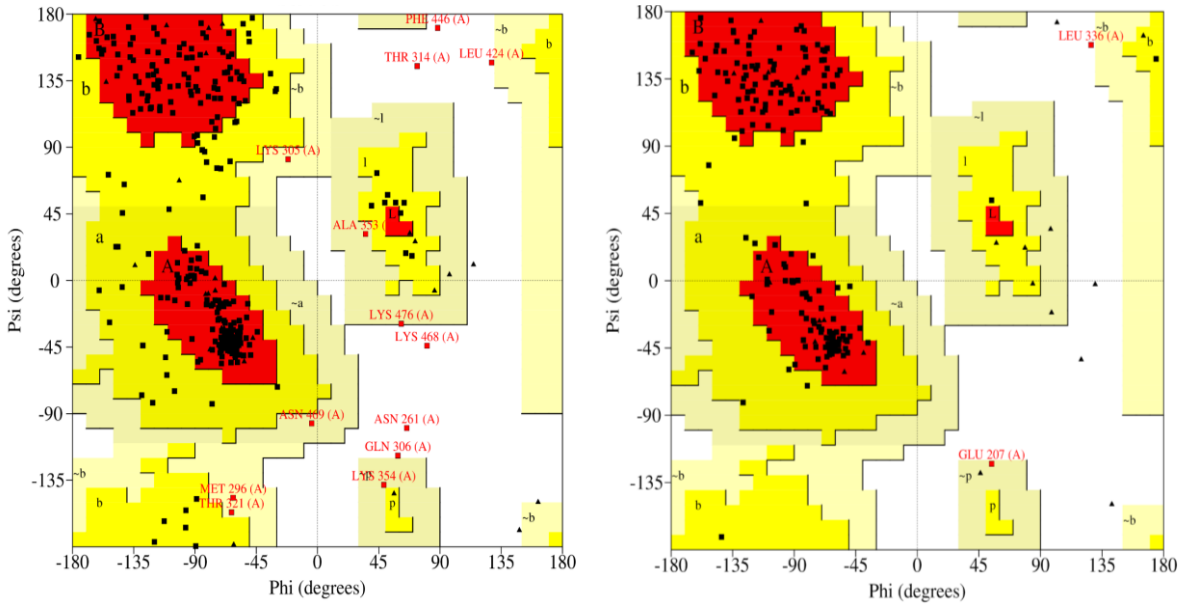

Figure 2. PROCHECK's Ramachandran plots for the two homology models ([left-right]: chitin synthase, sterol 24-C-methyltransferase).

The Ramachandran plot for the chitin synthase model revealed that among 292 non-glycine and non-proline residues, 77.1% (225) of the residues are in most favored regions (shown in red) while 18.5% (54) are in additional allowed regions (shown in yellow). Meanwhile, the Ramachandran plot for the sterol 24-C-methyltransferase model declared that among 182 non-glycine and non-proline residues, 89.6% (163) of residues are in most favored regions (shown in red) while 9.3% (17) are in additional allowed regions. These results confirm that the homology models produced are of good quality, as most or all residues are in allowed regions (Messaoudi et al., 2013).

Moreover, the homology models for chitin synthase and sterol 24-C-methyltransferase garnered overall quality factors of 76.8987 and 85.8696 from ERRAT. A quality factor above 50 indicates a model of good quality (Lagares et al., 2020), which suggests that both homology models produced are of good quality. Lastly, results from the 3D-1D averaged score plot generated by VERIFY3D revealed that 57.70% of residues in the chitin synthase model and 58.41% of residues in the sterol 24-C-methyltransferase model achieved 3D-1D scores >= 0.1. These results are considered low, as VERIFY3D requires at least 80% of the amino acids to obtain average scores above 0.1, and ideally above 0.2 to consider these as models of good quality (Jain et al., 2014). The lower scores obtained from VERIFY3D despite the acceptable results from PROCHECK and ERRAT suggest that

loop modeling, refinement, and energy minimization can be pursued in order to increase the validity of the models (Haddad et al., 2020).

Docking of Foc-TR4 proteins with cinnamaldehyde. According to Shreaz et al. (2016), cinnamaldehyde has been studied to express antifungal activity through disrupting cell wall and chitin synthesis and through inhibiting ergosterol biosynthesis. This is supported by several studies, wherein cinnamaldehyde has been found to express antifungal activity against other *Fusarium* species through disrupting cell wall integrity (Xing et al., 2014), as well as reducing the synthesis of ergosterol (Wei et al., 2020).

Chitin synthase has been actively studied as a target for antifungal agents (Lima et al., 2019), as the function of chitin synthase is important in the maintenance of the fungal cell wall, which provides the cell structural integrity and protection from environmental stressors. Furthermore, several studies such as those conducted by Azam et al. (2014) and Nes et al. (2009) have identified sterol 24-C-methyltransferase as a potential drug target due to its role in ergosterol biosynthesis, which is necessary in maintaining the permeability and fluidity of fungal cell membranes. As such, these proteins were chosen for docking as these may be efficiently targeted to limit the spread of Foc-TR4 due to their functions in fungal growth and survival, as well as in accordance with cinnamaldehyde's antifungal mechanism.

The binding profiles of cinnamaldehyde with each of the two proteins can be seen in Figures 3 and 4, respectively. The blind docking of cinnamaldehyde with the Foc-TR4 proteins yielded nine docking profiles for each protein, and the representative docking profile per protein was chosen to be the one yielding the most negative energy, being -6.1 kcal/mol for chitin synthase and -4.9 kcal/mol for sterol 24-C-methyltransferase. The negative binding energies cinnamaldehyde displayed towards both proteins indicate that the interactions between them release energy, and that the complexes between cinnamaldehyde and the proteins are thermodynamically stable (Du et al., 2016). This communicates that cinnamaldehyde can readily and spontaneously interact with the Foc-TR4 proteins. These values are also similar to binding energies of cinnamaldehyde when docked against proteins of other pathogens, such as *Escherichia coli* (-5.65 kcal/mol) and non-typhoidal *Salmonella* spp. (-4.65 kcal/mol) from the study of Abishad et al. (2021), as well as UCF1 and YPW1 proteins of *Candida albicans* (-5.45 kcal/mol and -4.42 kcal/mol, respectively) from the study of Khadke et al. (2022).

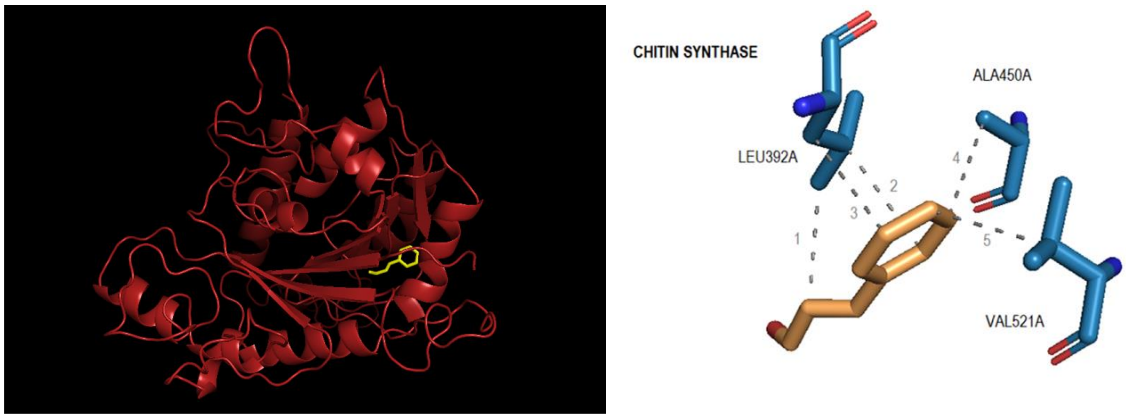

Figure 3. Binding profile [left] and non-bonded interactions [right] of cinnamaldehyde (yellow) and chitin synthase (red).

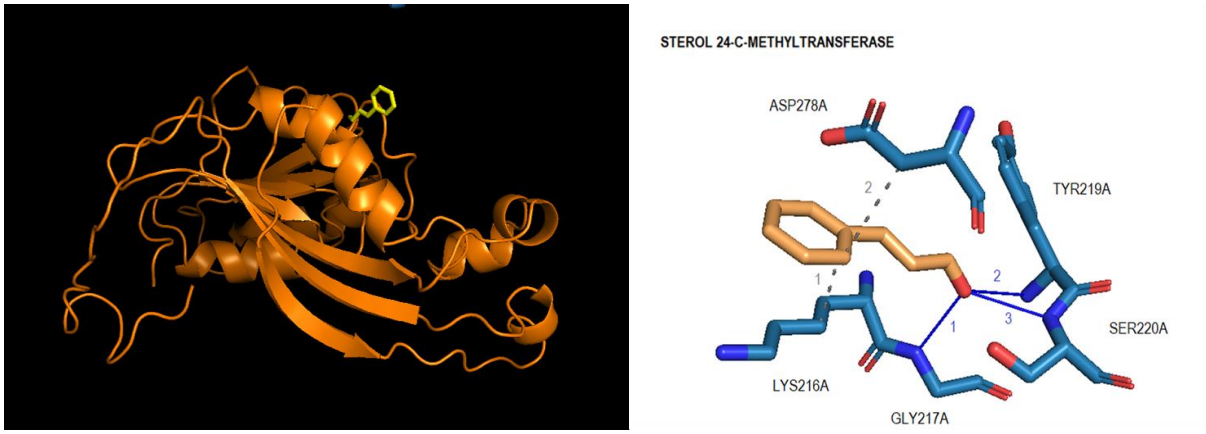

Figure 4. Binding profile [left] and non-bonded interactions [right] of cinnamaldehyde (yellow) and sterol 24-C-methyltransferase (orange).

Cinnamaldehyde was found to exhibit hydrophobic interactions (shown in gray dotted lines) with both proteins. It can also be seen that cinnamaldehyde formed hydrogen bonds (shown in blue solid lines) with sterol 24-C-methyltransferase in particular. A full copy detailing the bond interactions of cinnamaldehyde with the amino acid components of the two proteins can be found in Appendices G and H, respectively.

Cinnamaldehyde had hydrophobic interactions with Leu382, Ala450, and Val521 residues of chitin synthase, and Lys216 and Asp278 residues of sterol 24-C-methyltransferase. Cinnamaldehyde also had hydrogen bond interactions with Gly217, Tyr219, and Ser220 residues of sterol 24-C-methyltransferase. Additionally, findings from Cheng et al. (2008) indicate that the presence of an aldehyde group, conjugated double bond, and both aromatic and aliphatic hydrocarbon chains all contribute to cinnamaldehyde possessing strong antifungal activity.

Docking of beta-cyclodextrin with cinnamaldehyde. The docking of cinnamaldehyde with beta-cyclodextrin yielded a binding energy of -3.7 kcal/mol, and its binding profile can be found in Figure 5.

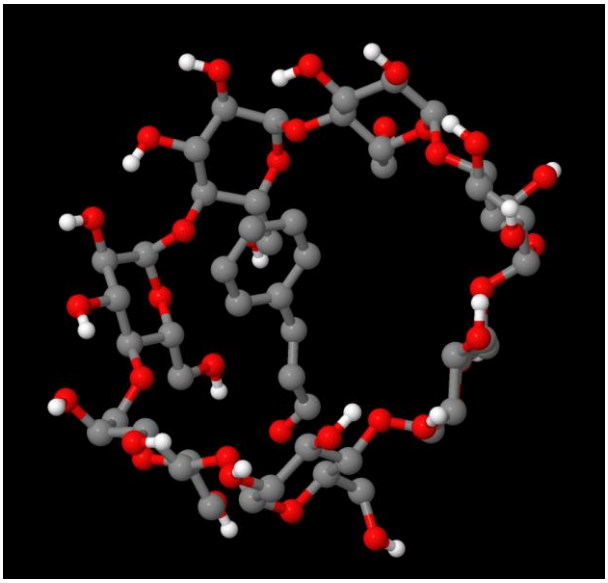

Figure 5. Binding profile of cinnamaldehyde with beta-cyclodextrin.

The binding energy of -3.7 kcal/mol indicates that the complex formation between cinnamaldehyde and beta-cyclodextrin is thermodynamically stable, given that its ΔG value is negative (Du et al., 2016). This communicates that the formation of the complex is favorable and reproducible (Herrera et al., 2019). This value is also similar to the binding energy Herrera et al. (2019) obtained for their own cinnamaldehyde and beta-cyclodextrin docking at −3.03 kcal/mol. Additionally, this also corroborates the calculated encapsulation efficiency of the inclusion complexes at 61.18%, as the output of both measures support the likelihood of the formation of complexes between cinnamaldehyde and beta-cyclodextrin.

As seen in the binding profile, it is primarily the nonpolar side of cinnamaldehyde that is enclosed within the beta-cyclodextrin ring. This suggests that cinnamaldehyde and beta-cyclodextrin possess hydrophobic interactions with each other, which can be supported by the fact that the inner pocket of beta-cyclodextrin is hydrophobic (Ponce Cevallos et al., 2010). Additionally, their interaction may also be driven by dispersion forces and hydrogen bonding (Tao et al., 2014).

Antifungal assay using spread plate method. The mean colony diameters (and their respective standard deviations) for each treatment recorded on all three days of bioassay reading can be found in Table 1, while photos of the replicate plates for each treatment can be found in Figure 6. A full copy of the raw data from the third, seventh, and fourteenth days of bioassay reading can be found in Appendices I1, J1, and K1, respectively.

When subjected to the Kruskal-Wallis and Dunn tests for mean differences, significant differences in antifungal activities of the treatments were observed on the third day of bioassay reading. Cinnamon oil at 0.10 mL/mL expectedly displayed a significantly higher antifungal activity than the negative control ($p = 0.0113$) and beta-cyclodextrin at 0.025 g/mL ($p = 0.0480$), as cinnamon oil is the active compound in its inclusion complex with beta-cyclodextrin. The positive control also displayed a significant difference with every other treatment except for cinnamon oil at 0.10 mL/mL ($p = 1.0000$); these two treatments displayed similar antifungal activities, having been effective in controlling the growth of Foc-TR4 due to all replicates of both treatments showing no growth beyond the culture discs.

The inclusion complexes (0.025 and 0.44 g/mL), on the other hand, provided intermediary results, possibly due to having a lower concentration of cinnamaldehyde compared to other treatments ($p = 0.5789$ and $p = 0.3671$, respectively). Cinnamon oil (0.010 mL/mL) exhibited significantly lower antifungal activity compared to its 0.10 mL/mL counterpart, and exhibited similar antifungal strength to the inclusion complexes (0.025 g/mL). This could imply that if the concentration of the inclusion complex treatment was increased, thus increasing the cinnamon oil content, the treatment could possibly have expressed a more significant antifungal activity. A full copy of the third day statistical test results can be found in Appendix I2.

From the Kruskal-Wallis and Dunn tests for the seventh day of bioassay reading, cinnamon oil at 0.10 mL/mL had still shown significantly more antifungal activity than the negative control ($p = 0.0005$) and beta-cyclodextrin at 0.025 g/mL ($p = 0.0437$). Similar to the third day of reading, the positive control also displayed a significant difference with the other treatments except for cinnamon oil at 0.10 mL/mL ($p = 0.5517$). However, it can be observed that cinnamon oil (0.10 mL/mL) was still able to inhibit the growth of Foc-TR4 completely, while the positive control began waning in strength over the fungi, which can be seen from the slight growth of Foc-TR4 on its replicate plates. It is plausible that the positive control had already diffused into the plate, lowering its concentration, which would explain the start of fungal growth on the seventh day after the treatment was applied.

Table 1. Colony diameter of Foc-TR4 set-ups with different treatments based on the spread plate method on the third, seventh, and fourteenth days of bioassay reading.

Treatment	Mean Colony Diameter (cm)[1]		
	Third Day	Seventh Day	Fourteenth Day
Negative Control	2.67 (0.24)	6.63 (0.69)	9.00 (0.00)
Positive Control	0.70[2] (0.00)	0.93 (0.05)	1.30 (0.14)
Cinnamon Oil (0.10 mL/mL)	0.70[2] (0.00)	0.70[2] (0.00)	0.70[2] (0.00)
Cinnamon Oil (0.010 mL/mL)	2.63 (0.13)	3.90 (0.22)	6.57 (1.14)
Beta-cyclodextrin (0.025 g/mL)	2.42 (0.31)	5.47 (1.01)	6.80 (1.69)
Inclusion Complexes (0.44 g/mL)	2.47 (0.05)	3.83 (0.05)	6.77 (0.40)
Inclusion Complexes (0.025 g/mL)	2.50 (0.00)	5.50 (0.71)	7.17 (0.24)

[1] three replicates per treatment
[2] culture disc size is 0.70 cm

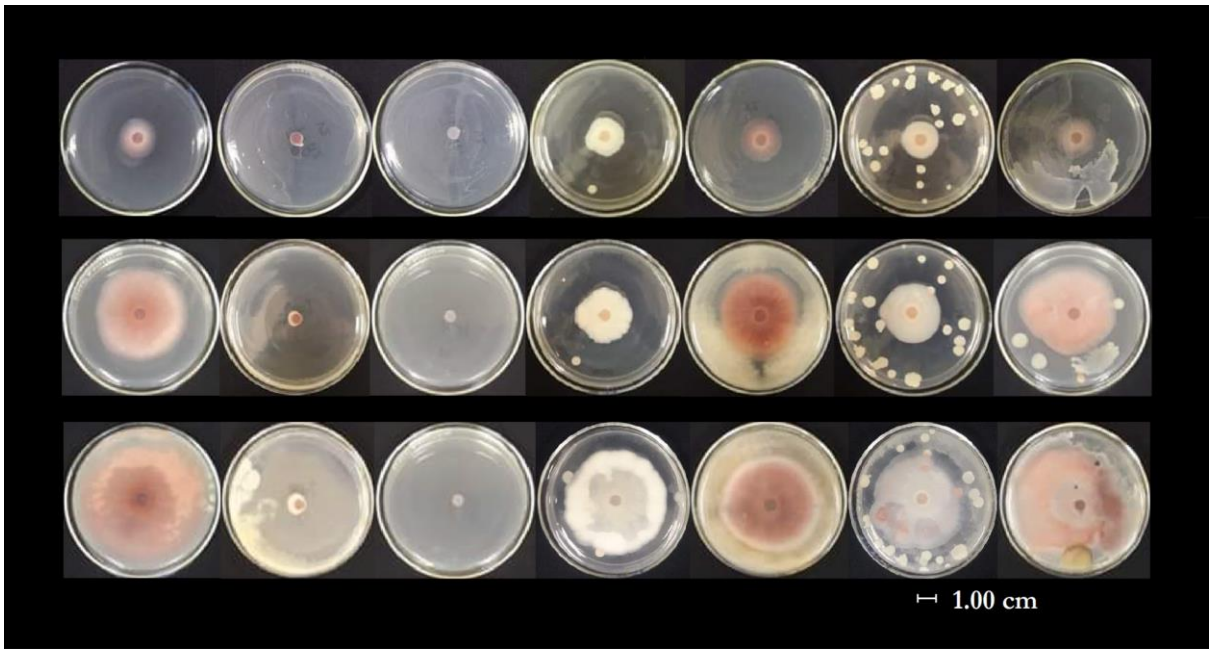

Figure 6. Growth of Foc-TR4 ([top-bottom]: third day, seventh day, fourteenth day) under different treatments ([left-right]: negative control, positive control, cinnamon oil (0.10 mL/mL), cinnamon oil (0.010 mL/mL), beta-cyclodextrin (0.025 g/mL), inclusion complexes (0.44 g/mL), inclusion complexes (0.025 g/mL)).

Compared to the third day of reading, the inclusion complexes at 0.44 g/mL showed a higher degree of antifungal activity despite not registering as having significant differences from the negative control ($p = 0.1456$). The inclusion complex treatment at 0.44 g/mL also displayed a higher antifungal activity than its 0.025 g/mL counterpart. Similar to the third day of reading, cinnamon oil (0.010 mL/mL) also proved to be less effective compared to its 0.10 mL/mL counterpart treatment ($p = 0.0372$) as well as to the positive control ($p = 0.1367$). The negative control, beta-cyclodextrin, and inclusion complex treatments (0.025 and 0.44 g/mL) all showed continuous growth of Foc-TR4, with the negative control exhibiting the most fungi growth on all its replicate plates. A full copy of the seventh day statistical test results can be found in Appendix J2.

From the Kruskal-Wallis and Dunn tests for the fourteenth day of bioassay reading, cinnamon oil at 0.10 mL/mL had again shown significantly more antifungal activity than the negative control ($p = 0.0005$) and beta-cyclodextrin ($p = 0.0430$). The positive control also displayed significant

differences with the other treatments except for cinnamon oil at 0.10 mL/mL ($p = 0.5505$). However, cinnamon oil (0.10 mL/mL) continued to inhibit the growth of Foc-TR4 completely, while the positive control continued to lose its antifungal strength as shown by increased colony growth in all plates.

Similar to the seventh day reading, cinnamon oil at 0.010 mL/mL and the inclusion complexes at 0.44 g/mL both showed a higher degree of antifungal activity despite not registering as having significant differences from the negative control ($p = 0.1636$ and $p = 0.1445$, respectively). Cinnamon oil (0.010 mL/mL) was still less effective than its 0.10 mL/mL counterpart treatment ($p = 0.0202$) and the positive control ($p = 0.1356$). On the last day of bioassay reading, it was observed that both inclusion complex treatments displayed similar results in antifungal strength despite their concentration difference. This was not the case for the seventh day of reading, where the higher concentration treatment produced smaller fungi colony diameters. The negative control still exhibited the most fungi growth among all treatments. A full copy of the fourteenth day statistical test results can be found in Appendix K2.

The growth of the Foc-TR4 colonies under each of the treatments in the span of fourteen days is graphed and summarized in Figure 7. The negative control was not able to inhibit fungal growth throughout the entire bioassay period. Cinnamon oil (0.10 mL/mL) significantly inhibited the growth of Foc-TR4 all throughout the bioassay period. Although some fungi growth was observed on the seventh day onwards for the positive control, the antifungal activities of cinnamon oil (0.10 mL/mL) and the positive control have not been found to be significantly different for all days of bioassay reading. Cinnamon oil (0.010 mL/mL), the inclusion complexes (0.025 and 0.44 g/mL), and beta-cyclodextrin all inhibited fungi growth to some extent, but were not observed to be significantly different to the negative control for all bioassay readings.

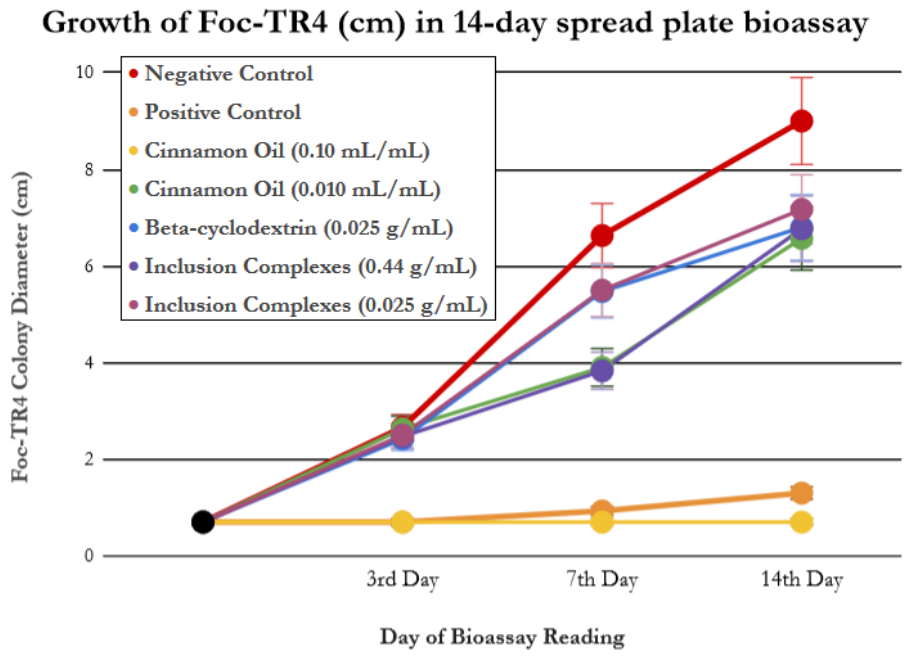

Figure 7. Growth of Foc-TR4 (cm) in 14-day spread plate bioassay.

Overall, beta-cyclodextrin was not able to significantly inhibit the growth of Foc-TR4, which is believed to be so since beta-cyclodextrin alone may stimulate fungal growth. Beta-cyclodextrin, given that it is a sugar, may act as a carbon source for the fungi, allowing it to thrive (Munhuweyi et al., 2018). Cinnamon oil at 0.10 mL/mL was able to consistently inhibit the growth of Foc-TR4,

while cinnamon oil at 0.010 mL/mL was only able to do so to some extent. This implies that the strength of antifungal activity is directly proportional to the amount of cinnamon oil, and in turn cinnamaldehyde, within the treatment. Also, given that cinnamon oil is expected to be momentarily effective as an antifungal due to its high volatility, it is possible that the antifungal properties of cinnamon oil were preserved in the in vitro set-ups, as the controlled environment did not allow for its vaporization. This may not be the case for in vivo set-ups.

As for the inclusion complex treatments (0.025 and 0.44 g/mL), both treatments were only able to inhibit Foc-TR4 growth to some extent. Although the encapsulation of cinnamon oil is believed to be able to protect cinnamon oil from oxidation or degradation (Munhuweyi et al., 2018), as well as enhance its delivery and effectiveness (Hill et al., 2013), the intermediary results provided by the experimental inclusion complex treatments may be due to low set treatment concentrations. It is highly likely that setting higher concentrations of the inclusion complex treatment can yield higher antifungal activity. This may be supported by the fact that the higher-concentrated inclusion complex treatment had a higher antifungal activity compared to the lower-concentrated treatment, despite neither displaying strong antifungal activity.

CONCLUSIONS

The project successfully determined the antifungal activity of cinnamon oil and beta-cyclodextrin inclusion complexes against Foc-TR4 through the characterization of the said complexes, the molecular docking of cinnamaldehyde with beta-cyclodextrin and select Foc-TR4 proteins, and the analysis of in vitro antifungal activity of the complexes. For the characterization of the inclusion complexes, differential scanning calorimetry analysis confirmed the proper formation of the complexes, while the spectrophotometry and the particle size analysis characterized them as having an encapsulation efficiency of 61.18% and an average size of 98.27 μm. For in silico studies, modeling and validation of the two proteins chitin synthase and sterol 24-C-methyltransferase produced moderately favorable results, and the docking of said proteins with cinnamaldehyde yielded binding energies of -6.1 and -4.9 kcal/mol, respectively. Cinnamaldehyde also exhibited a binding energy of -3.7 kcal/mol when docked against beta-cyclodextrin. These imply that cinnamaldehyde readily interacts and can form complexes with both the proteins and beta-cyclodextrin. In particular, cinnamaldehyde interacted with beta-cyclodextrin and the two proteins through hydrophobic interactions, as well as through hydrogen bonding with sterol 24-C-methyltransferase. For in vitro studies, cinnamon oil (0.10 mL/mL) proved effective in inhibiting Foc-TR4 growth ($p = 0.0005$), while the inclusion complexes (0.025 and 0.44 g/mL) proved somewhat effective in inhibiting Foc-TR4 growth ($p = 0.1445$ and $p = 0.2457$, respectively).

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