CHAPTER 6

DISCUSSION

This experiment was done to prove that carrot (*Daucus carota*) extract has antifungal effects on *Candida albicans* in vitro. To obtain the Minimal Inhibitory Concentration (MIC) and the Minimal Fungicidal Concentration (MFC), the tube dilution method is used. *Candida albicans* 1×10^3 is mixed with different extract concentrations (100%, 90%, 80%, 70%, 60% and 50%) and incubated for 24 hours. After examining the turbidity, the broths are then inoculated on Sabouraud's Dextrose Agar (SDA) plate and incubated again for 24 hours.

In many clinical situations, the tube dilution method of determining antimicrobial susceptibilities is generally recognized as providing more accurate and useful information as compared with results obtained by disc method (Chitwood, 1969). Therefore, the tube dilution method is preferred in this research. After 24 hours incubation, the broth is then inoculated in plates with Sabouraud's Dextrose Agar. This agar is specifically used for the cultivation of commensal fungi and yeasts. The high dextrose concentration and acidic pH of the formula permits selectivity of fungi because an acidic pH inhibits many species of bacteria.

The Minimal Inhibitory Concentration is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MIC is considered the 'gold standard' for determining the susceptibility of organisms to antimicrobials.

Minimal Fungicidal Concentration (MFC) is the lowest concentration of a substance that is capable to kill all fungal colonies under a defined set of conditions. The MFC value is important therapeutically as it determines the drug dosage to be given to treat a candidal infection.

In this research, after 24-hours incubation, the turbidities of the broths are examined to determine the MIC value. Because of the turbid state of the original extract, the MIC value cannot be determined. Same as the MIC, the MFC value in my experiment cannot be determined also. At 100% extract concentration which is the full 1ml extract solution, the amount of fungi colonies is still abundant, but there is a slight decrease compared to its control where it kills some of the fungi colonies.

The results are then analyzed using statistical tests. Since the results are numerical, a one-way ANOVA test is used. Before that, a normality test is done to determine whether a data set is well-modeled by a normal distribution or not. The test used is the One-Sample Kolmogorov-Smirnov Test because of the nominal independent variable and the numerical dependent variable. Data distribution was normal with $p = 0.047$ ($p>0.05$). So, the data came from a normal distributed population, was accepted**.** The conclusion of normality test was the data of bacterial colony came from population which had a normal distribution.

Test of homogeneity of variances is done to show that the population variances are equal. A Levene's test is used to assess the equality of variances in different samples. If the resulting p-value of Levene's test is less than 0.05, the obtained differences in sample variances are unlikely to have occurred based on random sampling. Thus, the hypothesis of equal variances is

rejected and it is concluded that there is a difference between the variances in the population. Base on homogeneity test done in this research, the data variance of bacterial colony was homogenous ($p = 0.143$; $p > 0.05$). Because the data distribution was normal and the data variance was homogenous, it was eligible to perform one way ANOVA to investigate whether different treatment dose have different effects on colony numbers.

In the one-way ANOVA test done to see the effect of treatment on the number of colony, it was shown the p value 0.000 ($p < 0.05$) , it means that Ho: $\mu1 = \mu2 = \mu3 = \mu4 = \mu5$ is rejected or treatments have effect on the number of colony. Different dose of extract would give a different number of colony. $\mu1$ is the number of colonies in dose 1, $\mu2$ in dose 2; $\mu3$ in dose 3 and so on.

To determine the optimal dose, a multiple comparison (Pos Hoc Tukey) test is done. Based on this test, there is a significant difference in the number of colonies in all treatment groups when compared to negative control ($p < 0.05$), or there is significant decrease in the colony numbers in all treatment group. There is no significant difference among the treatment group ($p>0.05$), which means that dose 25% till dose 50% have similar effects on the colony number.

Correlation test is used when both variables are at least at interval level and data is parametric. This test is done to see if there is correlation between the two variables. The significance of the correlation is determined by the Sig. value, and it should be < 0.05. In this research, there is significant correlation between the number of colony and treatment ($p = 0.000$). Pearson correlation coefficient (r value) is the strength of correlation. A weak correlation is at $r < 0.500$, moderate correlation is at 0.500-0.59, a strong correlation is 0.600-0.799 and a very strong

correlation is r > 0.799. The *r-value* between treatment and colony number was -0.876, which means that there is a strong correlation between treatment and colony number.

Based on the statistics, it is shown that the carrot extract can inhibit the growth of *candida albicans* in general. Based on observation, the carrot extract did a good job in inhibiting the fungi colonies but not as expected it will be. This is because, at 50% extract which is pure extract only it only kills 57.25 colonies out of 170.50 colonies according to the mean value. This value also shows that the carrot extract is effective in inhibiting the colonies but in small amount. This may be due to its active ingredient which is the falcarinol, it is possible that the active ingredient which the carrot contains is in very small amount or there is other factors from the carrot extract itself inhibiting the falcarinol from working at 100%.

Other method, namely agar overlay technique has been used to check the Minimum Inhibitory Concentration (MIC) of the carrot extract because the initial test for the MIC cannot be determined. This method is used to the determine the diameter of inhibition zone on the agar plate. This technique allows you to produce a homogeneous lawn of fungi within a thin layer of agar across the surface of a plate. Fungi are added to a soft top agar (0.75% agar, as opposed to the usual 1.5% for agar plates) which has been melted at 100°C and cooled to 45°C. This is warm enough so the agar remains liquid, but cool enough so that the fungi are not killed (for a period of time). The melted agar/bacterial suspension is mixed and poured evenly across the top of an agar plate and allowed to solidify.

The fungi distributed through the top agar will grow to produce a homogeneously turbid lawn. If the freshly seeded lawn is exposed to various antifungal agents and then incubated at 37°C, any inhibition of fungi growth will cause a reduction in the turbidity of the lawn near the agent: the greater the antifungal action, the wider the zone of inhibition. Thus, the antifungal strength of the agent may be judged by the width of the zone of inhibition around it. (Frankhauser, 2001).

The result obtain from this experiment is the same as the begining of the experiment, which is no changes in the diameter of the inhibition zone from the control which is all 7 millimeter. This result shows that quantity wise, the colonies is not affected to the naked eyes, but if tested thoroughly and in detail maybe there is substantial decrease of amount of the fungi colonies.

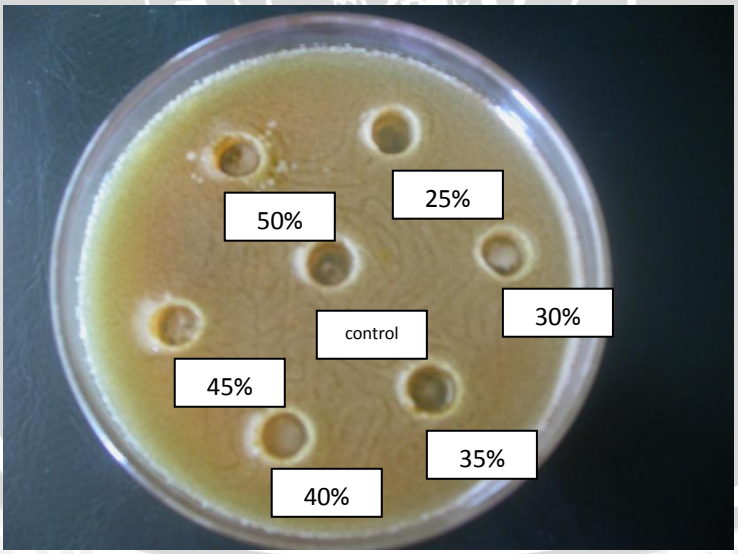

Figure 9 Result of agar overlay technique

The major bioactive constituents of *Dactus Carota* are group of polyacetylenes comprising of of falcarinol, falcarindiol, and falcarindiol 3-acetate.

Carrot also contains carrotene, lycopene, sugar, fatty oil, essential oil, caffeic acid, chlorogenic acid, gallic acid, vitamins B1, B2 and C, and umbelliferone. (Purup, 2009;Larsen,2009; Christensen,2009). Polyacetylenes (also known as polyynes) are defined as either hydrocarbons with more than one triple bonds or the oxidized form of polyenes. Falcarinol, with a molecular formula of C7H24O and having two triple bonds, can therefore be concluded to be an oxidized form of polyene (Reference.MD, 2009). Polyynes interact with fungal membrane sterols to form aqueous pores or channels through the membrane, leading to altered permeability, leakage of vital cytoplasmic components, and death of the organism. They also bind to membrane structure or characteristics to damage transport systems for small molecules, causing a loss of intracellular loss of potassium from fungal cells. In addition to that, polyynes afflict oxidative damage to fungal cells, leading to their death (Reference.MD, 2009).

Based on the results of the study, the hypothesis is proven. It can be concluded that the carrot extract significantly inhibits *Candida albicans* growth. Furthermore, there was a negative correlation between the extract concentrations and its inhibitory effect on *Candida albicans* colony formed. The higher the carrot extract concentration, the further the reduction in *Candida albicans* colony growth seen. In this study the carrot extract can inhibit the fungi, but not 100%, due to factor of the less amount of the major bioactive ingredient which is the falcarinol.

In recent years, very few studies have been carried out on carrot as a form of antifungal hence the clinical application of this study needs to be further studied. Future studies should be extended to an investigation on the appropriate amount and concentration of the *Candida albicans* used and using narrower concentration ranges to obtain more accurate results leading to eventual

realization of the carrot extract as an alternative treatment for candidiasis. In this respect, the determination of the MIC will be one of the crucial factors to see the suitability of carrot extract as a potential antifungal therapy.

As plant extracts are frequently turbid or precipitates when mixed with microbial growth media, the use of turbidity to determine the MIC will be difficult. This was the reason why the MIC for carrot extract on *Candida albicans* in this study could not be elucidated. Other methods to determine the MIC should be done in future studies such as the p-iodonitrotetrazolium violet (INT) reaction. The reaction is based on the transfer of electrons from NADH, a product of threonine dehydrogenase (TDH) catalyzed reaction to the tetrazolium dye (INT). TDH from fungi catalyzes the NAD-dependentoxidation of threonine to form 2-amino 3-ketobutyrate and NADH. During the active growth of fungi, an electron is transferred from NADH (which is colourless) to p-iodonitrotetrazolium violet resulting in a formazon dye, which is purple in colour. Therefore, the clear zones in the chromatogram indicate areas of inhibition (zones where no active growth of fungi has taken place). This method eliminates the dependence on turbidity to determine the MIC and will be a better option for testing the MIC for carrot extract. A low MIC for carrot extract will make it a potential candidate to treat candidal infections (Masoko, 2007).

The use of plant product to treat fungal infection and in particular, *Candida albicans* has been made possible by the success story of the oregano oil. The oregano plant was initially used as spices in cooking but their ability to exert therapeutic effects was well recognized since the ancient Greek (Leung et al. 1996). Based on this fact, many studies were conducted to establish their potential for therapeutic use. Various studies have proven its antifungal activity.

The active compound found in the oregano leaves which exert this effects were phenolic derivatives such as carvachol and thymol. In 2001, Manohar et al. has shown its antifungal activity against *Candida albicans* in vitro and in vivo. The MIC for oregano was found to be at 0.125 mg/ml while its MFC was 0.25 mg/ml in their study. These findings were significantas they confirmed the possibility of using oregano as a therapeutic agent. Today, oregano oil is already being commercialised for the treatment of *Candida* infection. However, with the cost of 15 ml of the oil stands around USD 25 it might not be so cost effective especially when these infections mostly affect the immunocompromised group in the developing world.

Carrot is economically available worldwide, therefore should be researched on to determine its antifungal effect in candidiasis patients. It can likely be a home-remedy medicine that is affordable and obtainable in Indonesia or other part of the world easily for it can be grown in all seasons/weathers and does not require a huge financial investment to cultivate the plant. Ideally, the active compounds of the carrot should be tested against a more established substance such as the phenolic derivatives of the ivy leaf plants. The composition of the substance, the pharmacodynamics and the pharmacokinetics should be studied to see whether it can offer a safe and effective treatment option. This study has clearly shown that carrot extract can inhibit the growth of *Candida albicans* and a high concentration of the extract is needed to produce a better effect. However, we need more studies to support these findings. It is with hope that the findings of this study will provide and impetus for future research on the antifungal effect of carrot extract on the diseases brought on by pathological fungus.

Due to the fact that the MFC value of the carrot extract towards *Candida albicans* cannot be determined but it strongly contributes to the conclusion that carrot extract is proven to have antifungal effects towards *Candida albicans,* therefore, the previous hypothesis is accepted.