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The antifungal activity of cajeput (*Melaleuca cajuputi)*, niaouli (*Melaleuca quinquenervia),* and rosalina *(Melaleuca ericifolia)* essential oils against *Candida albicans*

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ABSTRACT

The recent rise in fungi resistant to common antifungal drugs has led to the need for alternative methods to treat these kinds of infections, whether that be new drugs or natural remedies, the search for a solution is ongoing. Researchers have found tea tree oil to be an effective natural antifungal (*Melaleuca alternifolia*). Few experiments have been conducted on the antifungal properties of other essential oils coming from plants in the same family as tea tree. In this experiment, three oils related to tea tree: cajeput (*Melaleuca cajuputi*), niaouli (*Melaleuca quinquenervia)*, and Rosalina (*Melaleuca ericifolia*), were tested for their antifungal properties at concentrations of: 100%, and dilutions with dimethyl sulfoxide of 75%. 50%, 25%. The fungus chosen for study was *Candida albicans*, a common fungus known to cause irritation upon infection. The oils were tested using a disc diffusion method; potato dextrose agar plates were inoculated with fungus, and oil filled discs were added within 15 minutes. The plates were then incubated at 37 degrees Celsius for 24-48 hours. As a control, the oils were run in a trial against a solution of 1% Clotrimazole, a common antifungal drug. All oils at concentrations of 100%, 75%, and 50% were effective in inhibiting growth, lower concentrations resulted in lower inhibition. At 25% concentration, none of the oils displayed measurable inhibition. Rosalina oil showed the greatest inhibition overall, showing near complete inhibition at 100% concentration.

Keywords: *Antifungal, Candida albicans, Cajeput, Melaleuca genus, Niaouli, Rosalina*

INTRODUCTION

Fungi are classified as eukaryotes that interact with other living organisms in specific relationships known as mutualism, parasitism, or commensalism. Those that cause diseases in their hosts are often classified as pathogens (Sanglard, Coste, Ferrari, 2009). *Candida albicans* is considered the most common species of fungi among the current fungal pathogens, and is classified as parasitic, capable of causing harm to the host and providing no known benefits. The other *Candida* species (*Candida glabrata, Candida parapsilosis, Candida tropicalis*), non-*Candida* species (*Cryptococcus neoformans*) and molds (*Aspergillus fumigatus, Microsporum canis*) are the other common species of pathogenic fungi; infections by which can be fatal (Lass-Florl, 2009). The increase in fungal resistance to antifungal drugs has raised concern over the years and has led to a need for research on new antifungal agents, especially those derived naturally from plants and their oils (Lass-Florl, 2009). In addition, the rise of an immunocompromised population, such as anyone infected by HIV, those born with an immune disorder, or patients undergoing immunosuppressive treatment for a transplant, have also increased the need for research into this topic. (Richardson and Lass-Florl, 2008).

The main virulence factors of the *Candida* genus involve epithelial cell adhesion, hydrolytic enzyme secretions (proteases, phospholipases, hemolysins), biofilm formation, phagocytic cell evasion, and transitions between the yeast and the filamentous hyphal states (Santos, et al., 2018). The choice in initial treatment for a Candida infection is a difficult one due to its many modes of virulence, compounded by the rising development of antifungal drug resistance, makes for a worsening situation in treating these types of infections. The usage of Clotrimazole, a common anti-fungal drug, over the last two decades is believed to be behind a rise in non-albicans species that are less susceptible to the drug (Santos, et al., 2018). This leads to the issue of finding safe, effective, and long-term treatments against fungi without creating dangerous new species or increasing resistance in current ones.

Tea tree oil shows promise as a topical antifungal agent, with some clinical research indicating its capability as a treatment for dandruff and oral thrush (Hammer, Carson, Riley, 2004). Tea tree oil is obtained through steam distillation of the leaves from *Melaleuca alternifolia* (tea tree), a native Australian plant, containing around 100 components, mostly being monoterpenes, sesquiterpenes and other similar alcohols. This plant has been used medicinally for almost a century and studies of *in vitro* work have shown that tea tree oil components can cause leakage of intracellular compounds and even inhibit respiration in bacteria, explaining some its well-known antibacterial properties (Hammer, Carson, Riley, 2004). The usage of tea tree oil as a topical treatment against fungi has been shown as effective,

but the mechanism by which tea tree oil acts as an antifungal is still under research despite it commonly appearing as a home remedy for oral thrush or yeast infections. However, some recent studies have found tea tree oil capable of destroying the fungal cell wall and changing the composition of membrane fatty acids, leading to a release of intracellular materials in *B. cinerea* (Shao, Cheng, Wang, Yu & Mungai, 2013).

The Myrtaceae family, of which Melaleuca alternifolia is a part of, can be commonly found in Brazil and other South American countries (Hammer, Carson, Riley, 2004). Narrowing this down further, the genus *Melaleuca*, of which this experiment is focused on, is a fast-growing species of plant that is indigenous to: Australia, New Zealand, Papua, New Guinea, Solomon Islands, Indonesia, Malaysia, Myanmar, Cambodia, Thailand, and Vietnam. This genus of plants, of which tea tree is a part of, can appear as either tall shrubs or small trees, with a height up to 7 m. This genus often displays distinct features such as a bushy crown, papery bark, and leaves with pronounced glands enriched with aromatic oils (Sharifi-Rad, et al., 2017). This experiment focuses on the oils of three plants from the Melaleuca genus: *Melaleuca cajuputi* (cajuput or white samet), *Melaleuca quinquenervia* (broad-leaved paperbark, paper bark tea tree, punk tree or niaouli) and *Melaleuca ericifolia* (swamp paperbark or rosalina). The oils distilled from the leaves, being cajuput oil, niaouli oil and rosalina oil, have not been as extensively studied as their counterpart, tea tree oil, for their antifungal properties, but remain commonly utilized medicinal oils (Keereedach, Hrimpeng, Boonbumrung, 2020). All three oils come from the same family and genus as tea tree oil, which as stated before is proven to be an effective antifungal, and so this represents an opportunity to expand research into this genus for effective treatments against fungi species such as Candida.

The objective of this study is to investigate the *in vitro* effect of cajuput, niaouli, and rosalina oil on the growth inhibition and drug action potential against *Candida* fungi. This kind of research is important as fungal diseases are severe and can cause up to a 60% mortality rate for patients diagnosed with these kinds of parasitic fungal infections (Staniszewska, 2020). The overall goal of this study is to uncover new potential solutions to prevent development of antifungal resistance or less susceptible species, and potentially lower these mortality rates.

MATERIALS AND METHODS

Candida albicans strains were obtained through the Carolina Biological Company Supply and grown on a potato dextrose agar, also provided by this company. Testing was done using the disk diffusion method in which agar plates and fungi were treated with four sterile paper disks inoculated with a specific oil. The cajeput, niaouli, and rosalina oils were obtained from Mountain Rose Herbs in 10 mL bottles.

The first tests were initially run at a 100% concentration, and then at 75%, 50%, and 25% dilutions of each oil using 99.9% dimethyl sulfoxide, provided by Sigma-Aldrich. The level of inhibition present in each dish was figured by studying the zone of inhibition after each treatment in which the diameter was measured in millimeters for each of the four individual discs on the plate. The potato dextrose agar, cotton tipped swabs, and tweezers were sterilized for 30 minutes in a Brinkman 3870 autoclave to ensure the agar was thoroughly melted and that all tools were sterile. The first five plates made were inoculated with the fungi using the cotton tipped swab in a sweeping motion to achieve full coverage of the plate. Then the 6 mm discs were each filled with 60 microliters of one of the three oils, undiluted for a 100% concentration, and placed on the designated plates (four discs per plate). This was done within 15 minutes of the fungus being added to the agar plates. Each of the 4 discs on a plate were measured individually, providing four trials for each oil at each concentration. Two of the plates inoculated with the fugus were set aside to grow in order to provide fresh samples for the next rounds of testing. The plates were then incubated at 37 degrees Celsius for 24-48 hours in a Fisher Scientific isotemp incubator. The zone of inhibition was checked by taking a measurement of the diameter of each circular zone formed in millimeters. Rosalina oil at 100% concentration had to have the zone of inhibition checked to the nearest edges of fungal growth, as the zones merged together, preventing distinct circles from appearing.

This exact process was repeated three more times for each of the different concentrations of the oils. These concentrations were diluted with the DMSO using C1V1 = C2V2 in order to determine the amount of DMSO needed to make such dilutions.

The final test done used Clotrimazole as a control, as it is a common treatment for this type of fungi. Because Clotrimazole is only used at 1% in over-the-counter medications, 0.1006 grams of pure Clotrimazole was added to 10 milliliters of DMSO to make a 1% solution for testing. This solution was thoroughly mixed and then added in 60 microliters to four discs to be tested the same way the oils had been and was compared to the previous tests with the oils. Once all the rounds of testing were completed, a two-way ANOVA test was done to analyze the relationship between the concentrations and oil types.

RESULTS

The results of this study, using a two-way ANOVA test, found that there was an overall significant effect of oil type, concentration, and a significant interaction

between oil type and concentration (see Table 1).

Table 1. Two-Way ANOVA Test in analysis of the relationship between concentrations and oil types.

	Sum of Squares	df	Mean Square	F	p
Oil	323	2	161.44	29.5	<.001
Concentration	3452	2	1762.08	315.4	<.001
Oil ✻ Concentration	281	4	70.22	12.8	<.001
Residuals	148	27	5.47		

The F-value for the oils alone was 29.5 with a p-value of less than 0.001. The F-value for concentration is extremely high at 315.4 and the p-value again sits at less than 0.001. Finally, the F-value for oil and concentration was 12.8 and the p-value was less than 0.001. The two-way ANOVA test also provided the data for the mean of the inhibition zones for each oil at the different concentrations (see Figure 1).

The data displayed on Figure 1 suggest that rosalina oil (*Melaleuca ericifolia*) had the greatest effect on fungal growth at concentrations of 100% and 50%, but that cajeput oil (Melaleuca cajuputi) showed the greatest inhibition at a concentration of 75%. The data also suggests that niaouli oil (*Melaleuca quinquenervia*) was the least effective of the three.

The visual results of this experiment, which cannot be fully depicted by the numerical date are shown in Figures 2 and 3. Figure 2 displays the plate in which rosalina oil was at 100% concentration; in the photo it can be see that this plate had near total inhibition of growth, and in fact took 48 hours to show any growth at all. It can also be seen that rosalina at 100% shows no distinct zones of inhibition, which are clearly seen in figure 3, containing plates treated with niaouli and cajeput oil, in which significantly more fungal growth is visible. The experiment was run for a 25% concentration for each oil, but no significant zone of inhibition could be found for this percentage, and so the lowest concentration to display inhibition was 50%.

DISCUSSION

Due to the fact that no distinct zones of inhibition could be seen for rosalina at 100%, measurements had to be done by measuring to the nearest edge of fungal growth on each of the four discs, giving the radius of each one, which was then multiplied by two to give a diameter. This was the only case in which measurements had to be taken this way, as concentrations of 75% and 50% for rosalina yielded distinct circles in which the diameter of the inhibition zones were clearly seen. The bars of standard deviation on Figure 1

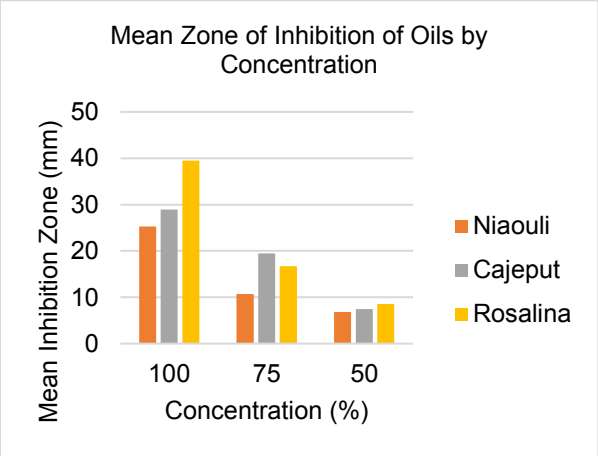

Figure 1. Mean Zones of Inhibition with Lines of Standard Deviation

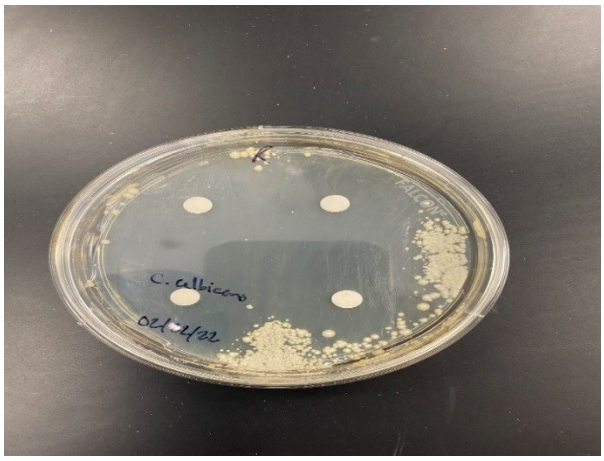

Figure 2. Rosalina Oil Plate at 100% concentration.

Figure 3. Cajeput and Niaouli plates with Distinct Zones of Inhibition.

suggest that rosalina oil had the largest deviations away from the mean value, which may be explained by the difference in measurement taking done for the 100% concentrations.

The results of the two-way ANOVA test gave large F-values, which tell us that the variation among the group means was more than expected. Because the mean values of the experiment vary so highly it can be inferred that the oils work differently at different concentrations. This is important because this means that these oils could be effective at concentrations other than 100%, as applying an essential oil undiluted could lead to a poor reaction such as hives or chemical burns.

The control of the experiment used Clotrimazole at a 1% concentration, which is the standard for the antifungal drug, the inhibition zones displayed were similar to that of the results of the oils at 50% concentrations. Clotrimazole functions as an antifungal primarily through damaging a fungus' cytoplasmic membrane, leading ultimately to the inhibition of ergosterol synthesis and the loss in ability to make a functional membrane (Khatter and Khan, 2021). This result suggests that these oils could potentially function similar to an antifungal drug, and that new research into their commercial usage in the medical industry would be beneficial.

Tea tree oil is a mixture of terpene hydrocarbons and tertiary alcohols, but the main compounds responsible for its antimicrobial activity are terpinen-4-ol and 1,8-cineole (Shao, Cheng, Wang, Yu & Mungai, 2013). Cajeput oil actually contains a higher concentration of 1,8 Cineole (Eucalyptol), limonene and alpha-Terpineol, but tea tree oil still contains more Terpinen-4-ol and gamma-Terpinene (Foreverest Resources, 2022). Niaouli oil is composed of: 1,8-cineole, alpha-terpineol, alpha-pinene, beta-pinene, limonene, a-phellandrene, nerolidol, linalool, piperitone and gamma terpineol (Ayurvedic Oils, 2022). Finally, rosalina oil is composed of mostly 1,8-cineole, linalool, and low levels of terpen-4-ol (Napp Global Essential, 2022). Knowing the makeup of these three oils and that of tea tree, it can be assumed the method by which tea tree acts as an antifungal is highly similar in the other three oils if not exactly the same, this can lead to future research into this mechanism to narrow it down further.

Based off the results of this experiment, rosalina oil was the best for inhibiting growth of the fungus, and cajeput oils is a close second. However, the results also suggest that concentrations of the oils lower than 50% may not be very effective in treating an infection, as higher concentrations may not be safe to use on patients. Overall, the findings of this experiment, while miniscule in scale, open more doorways to research on essential oils as natural remedies for fungal infections, and may offer a future solution to the rising anti-fungal resistant drug crisis.

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