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Testing natural substances as antifungal agents on *Saccharomyces cerevisiae* using the zone of inhibition

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Abstract

Three natural essential oils, apple cider vinegar, and 1% Butenafine hydrochloride antifungal cream were tested for their ability to inhibit the growth of *Saccharomyces cerevisiae* (baker's yeast). The essential oils included lemongrass, coconut, and tea tree oil. Petri dishes, previously streaked with yeast and incubated, were separately exposed to each essential oil, the apple cider vinegar, and the antifungal cream, and the zone of inhibition diameter was measured. At their full concentrations, the lemongrass oil and the apple cider vinegar were found to be as effective as the 1% butenafine hydrochloride cream in inhibiting the growth of *Saccharomyces cerevisiae*, whereas the coconut oil and the tea tree oil were significantly less effective as antifungal agents. These natural substances, such as lemongrass oil and the apple cider vinegar, can be formulated into medicinal antifungal agents with the advantage that they may cause considerably fewer side effects than commercially available drugs.

Key words

Yeast, Essential oils, Lemongrass, Fungal infections, Coconut oil, Apple cider vinegar, Antifungal, Yeast infections, Tea tree oil, Butenafine hydrochloride

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Introduction

Yeasts are single-celled eukaryotic organisms that are considered to be part of the Fungi kingdom. Yeasts also live inside and outside living organisms, such as various animals and humans. However, yeasts have been known to overgrow and cause infections in humans on various body parts. A commonly known infection called Candidiasis, which is caused by a species of Candida yeast, causes vaginal infections in women, oral thrush, and deep organ candidiasis. These conditions are highly uncomfortable and painful, and can sometimes be serious if the infection targets other organs, such as the heart. Yeast infections are often treated with various types of antifungal creams, depending on the type and severity of the infection. These medications tend to have some unwanted side effects, such as inflammation, nausea, dizziness, and liver dysfunction. These side effects can occur when using the medications because fungal cells are similar to human cells, making it difficult for a medical treatment to target only the fungal cells. This is where alternative natural substances, such as essential oils or legacy antifungal therapies, can fill an unmet clinical need.

Materials and Methods

Materials

The materials used were: Nona® white agar agar powder, Uvelka® white rolled oats, Poland Spring® distilled bottled water, Mainstays™ slimline digital scale (d= ± 0.01 g), Nature's Alchemy® lemongrass oil, *Life Plus Style Gourmet* coconut oil, Gold Emblem Abound™ apple cider vinegar, Nature's Alchemy® tea tree oil, CVSHealth™ 1% Butenafine hydrochloride Antifungal cream, and Fleischmann's® active dry baker's yeast.

Preparation of the oatmeal agar

5.0 grams of oatmeal was placed into a mortar and finely ground using a pestle. The ground oatmeal was combined with 0.5 L of distilled water in a pot and the mixture was heated to boiling. After boiling for 20 minutes, the mixture was poured through a cheesecloth into another bowl and transferred back to the pot. 4.0 g of Agar was added and the contents of the pot were heated until the agar dissolved. The contents were then allowed to cool to a temperature between 50 and 55^{o} C. The oatmeal agar solution was evenly distributed into sterile Petri dishes, filling each dish to the graduated mark. The dishes were left to cool undisturbed at room temperature before placing the lids over them.

Preparation of a 10% yeast solution

2.5 grams of dry baker's yeast and 25 mL of distilled water at 40^{o} C were added to a bowl. The mixture was added to the beaker of warm water, and the solution was stirred until the yeast was thoroughly incorporated into the water.

Testing the natural antifungal agents

All experiments were performed in a work area that was wiped down with 70% isopropyl alcohol. Gloves were worn throughout the procedure. Using a sterilized loop, the yeast solution was streaked in a zig-zagged direction across ⅓ of the petri dish. This procedure was repeated until the yeast contacted all the agar in the petri dish. A total of 27 petri dishes were contacted with the yeast solution. In five petri

dishes, one antifungal agent was placed at full concentration (2 drops for liquids or a small portion for cream or thick oil) on the surface in the center of each petri dish. The name of the antifungal agent and the trial number were written on the cover using a marker, and the dishes with the cream or coconut oil were lightly shaken to ensure the substances were in proper contact with the agar. This procedure was repeated for the other antifungal agents. A Petri dish served as a positive control where the yeast was allowed to grow without any antifungal agents. Another Petri dish served as a negative control where no yeast or antifungal agent was present in order to provide evidence that no rogue bacteria or fungi could grow using the procedure. The Petri dishes were placed in an incubator at 30 °C for 3 days. After 3 days, the Petri dishes were removed from the incubator, and the diameter (mm) of the area where the yeast did not grow (zone of inhibition) was measured. The data was tabulated, and the antifungal agents were categorized according to their zone of inhibition diameters: resistant (less than 13mm, weak antifungal activity), intermediate (14-17mm, intermediate antifungal activity), and susceptible (18 or more mm, strong antifungal activity). The entire process, starting from making the oatmeal agar, was repeated two more times for a total of three experimental sets.

Results and Discussion

Table 1. Zone Diamaters For 5 Trials For Set 2, Mean, and Standard Deviation (SD)

Set 2	Lemongrass Oil	Coconut Oil	Apple Cider Vinegar	1% Butenafine hydrochloride cream	Tea Tree Oil
Trial 1	30. mm	N/A	29 mm	30. mm	20. mm
Trial 2	31 mm	9.0 mm	23 mm	N/A	14 mm
Trial 3	25 mm	12 mm	30. mm	27 mm	N/A
Trial 4	32 mm	20. mm	28 mm	29 mm	22 mm
Trial 5	N/A	19 mm	24 mm	32 mm	27 mm
Mean	30. mm	15 mm	27 mm	30. mm	21 mm
SD	3.1	5.4	3.1	2.1	5.4

Three sets of trials were performed, with 5 trials in each set. The data from the first set was not taken into consideration for calculating the results because; when compared with sets two and three, it seemed to signify errors in performing the experiment to the required level of fastidiousness, exactitude, and attention to detail. Sets two and three were consequently performed to the required level of meticulousness and precision. Therefore, the data from sets two and three are reported.

Table 2. Zone Diamaters For 5 Trials For Set 3, Means, and Standard Deviation (SD)

Set 3	Lemongrass Oil	Coconut Oil	Apple Cider Vinegar	1% Butenafine hydrochloride cream	Tea Tree Oil
Trial 1	26 mm	20. mm	21 mm	27 mm	20. mm
Trial 2	26 mm	21 mm	25 mm	31 mm	21 mm
Trial 3	28 mm	19 mm	27 mm	29 mm	23 mm
Trial 4	27 mm	18 mm	29 mm	29 mm	24 mm
Trial 5	28 mm	18 mm	24 mm	32 mm	22 mm
Mean	27 mm	19 mm	25 mm	30. mm	22 mm
SD	1.0	1.3	3.0	1.9	1.6

Table 3. Overall Mean For Each Treatment And Zone Diameter Categories

Treatment	Lemongrass Oil	Coconut Oil	Apple Cider Vinegar	1% Butenafine bydrochloride cream	Tea Tree Oil
Overall mean	28 mm	17 mm	26 mm	30. mm	21 mm
Zone Diameter Category	Susceptible	Intermediate	Susceptible	Susceptible	Susceptible

As is evident from Tables 1 through 3, the zone of inhibition generally followed the trend: 1% Butenafine hydrochloride cream > lemongrass oil ≥ apple cider vinegar > tea tree oil ≥ coconut oil. Based on the defined criteria, the fungus was hence considered susceptible to all the substances tested, except for the coconut oil, where the fungus demonstrated intermediate susceptibility. The percent relative standard deviation (RSD) for all trials in set 3 was < 12% (apple cider vinegar) and that for all trials in set 2 was 36% (coconut oil). Possible reasons for these large RSDs' appear in the discussion section. Figures 1a through 1e below show the zones of inhibition for the various substances studied. Only representative trials are shown. Figure 2 shows the overall mean zone of inhibition diameters for all the substances studied when the data is combined for set 2 and set 3. Figures 3 and 4, show the overall mean zone of inhibition for all the substances studied for sets 2 and 3, respectively.

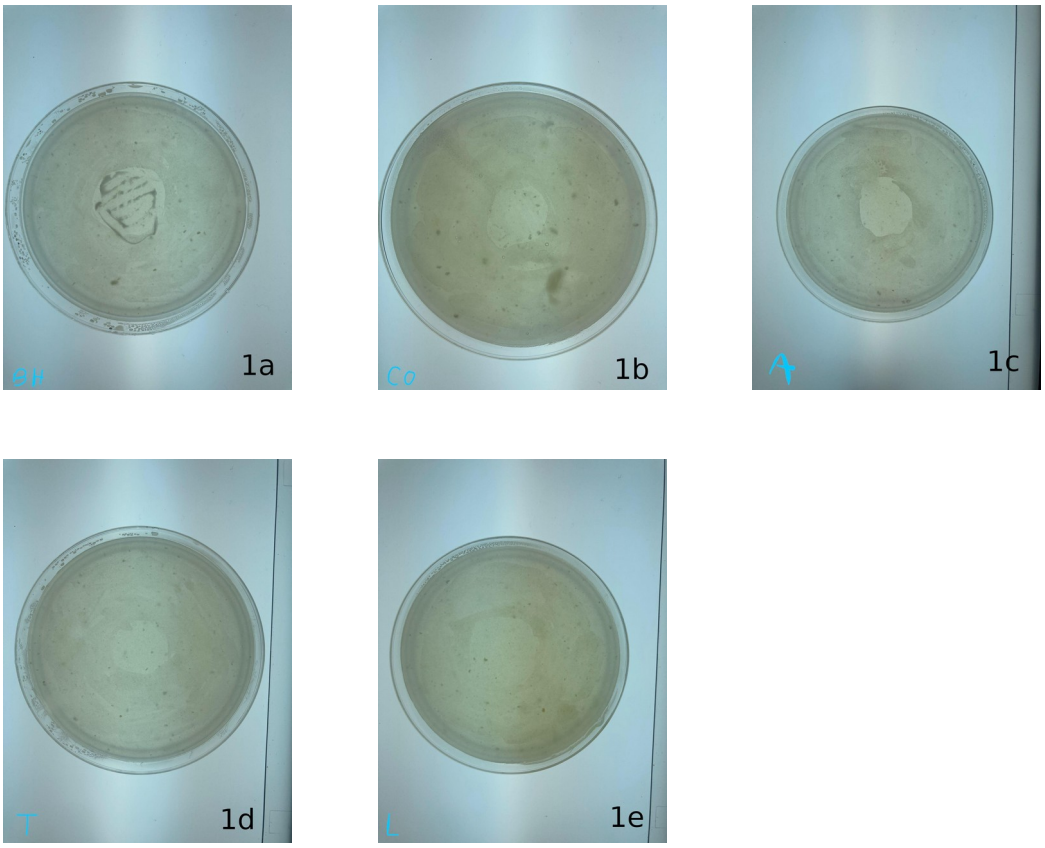

Figure 1. Representative zones of inhibition from set 3, for the various compounds studied. **a**: 1% Butenafine Hydrochloride, Trial 1, 27 mm, **b**: Coconut Oil, Trial 1, 20. mm, **c**: Apple Cider Vinegar, Trial 2, 27 mm, **d**: Tea Tree Oil, Trial 1, 20. mm, **e**: Lemongrass Oil, Trial 3, 28 mm.

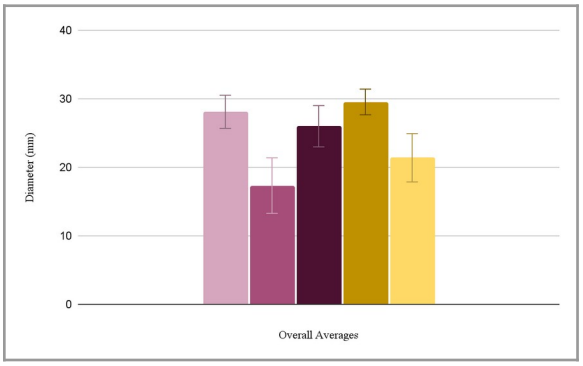

Figure 2. Overall Mean Zone Diameters And Error Bars For Various Antifungal Treatments. The overall mean zone diameter for the lemongrass oil is represented by the light purple bar and has an 8.6% error bar. The medium and dark purple bars represent the mean diameters for the coconut oil and apple cider vinegar with 23% and 12% error bars, respectively. The 1% Butenafine hydrochloride cream and the tea tree oil are represented by the dark and light yellow bars with 6.4% and 17% error bars.

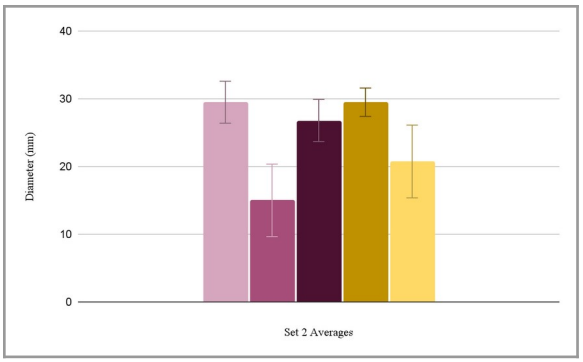

Figure 3. Mean Zone Diameters And Error Bars For Various Antifungal Treatments For Set 2. The mean zone diameter for set 2 for the lemongrass oil is represented by the light purple bar and has an 11% error bar. The medium and dark purple bars represent the mean diameters for the coconut oil and apple cider vinegar with 36% and 12% error bars, respectively. The 1% Butenafine hydrochloride cream and the tea tree oil are represented by the dark and light yellow bars with 7.1% and 26% error bars.

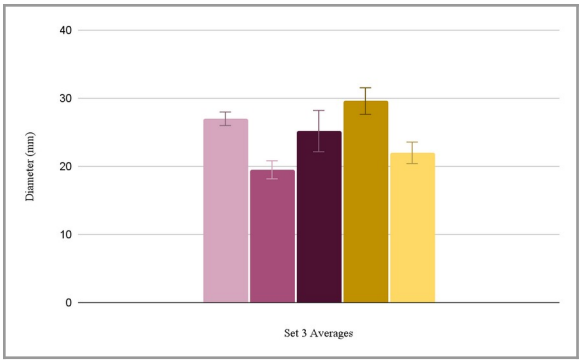

Figure 4. Mean Zone Diameters And Error Bars For Various Antifungal Treatments For Set 3. The mean zone diameter for set 3 for the lemongrass oil is represented by the light purple bar and has a 3.7% error bar. The medium and dark purple bars represent the mean diameters for the coconut oil and apple cider vinegar with 6.8% and 12% error bars, respectively. The 1% Butenafine hydrochloride cream and the tea tree oil are represented by the dark and light yellow bars with 6.6% and 7.2% error bars.

Table 4. Mean inhibition zone diameters and confidence interval for the essential oils

Statistics	Lemongrass Oil	Coconut Oil	Apple Cider Vinegar	1% Butenafine hydrochloride cream	Tea Tree Oil
Mean ± SD	28 ± 2.4	17 ± 4.1	26 ± 3.0	30. ± 1.9	21 ± 3.5
n	9	9	10	9	9
t^* (90%)	2.13	2.13	2.13	2.13	2.13
90% T interval	(26.6, 29.6)	(14.8, 19.9)	(24.3, 27.8)	(28.3, 30.7)	(19.3, 23.6)

Table 5. p values for two-tailed T tests at a 0.05 significance level

p values*	Lemongrass Oil	Coconut Oil	Apple Cider Vinegar	1% Butenafine hydrochloride cream	Tea Tree Oil	Control
Lemongrass Oil	N/A	4×10^{-6}	0.11	0.18	2.6×10^{-4}	0.0
Coconut Oil	4×10^{-6}	N/A	5.7×10^{-5}	0.0	3.6×10^{-2}	3.1×10^{-5}
Apple Cider Vinegar	0.11	5.7×10^{-5}	N/A	7.5×10^{-3}	7.5×10^{-3}	0.0
1% Butenafine hydrochloride cream	0.18	0.0	7.5×10^{-3}	N/A	1.6×10^{-5}	0.0
Tea Tree Oil	2.6×10^{-4}	3.6×10^{-2}	7.5×10^{-3}	1.6×10^{-5}	N/A	1.0×10^{-6}
Positive Control	0.0	3.1×10^{-5}	0.0	0.0	1.0×10^{-6}	N/A

*Significantly different comparators are presented in scientific notation, while significantly similar are presented in decimal notation.

The lemongrass oil produced the largest zone of inhibition of the natural antifungal agents; however, the 1% Butenafine hydrochloride cream was still the most susceptible and showed the highest antifungal activity. The experiment did not work for the first set and no zones were produced, which explains the lack of data for set 1. This could have been due to inexperience in conducting this type of experiment and the yeast being overly stimulated. However, the second set produced valid data, although not all the trials had a clear zone that could be counted. Only the apple cider vinegar produced valid data for all five trials. After taking the means of the five trials for each treatment, the lemongrass oil and the 1% Butenafine hydrochloride cream had the same mean zone of inhibition, *viz.* 30. mm. The coconut oil had the smallest mean diameter, *viz.* 15 mm. The apple cider vinegar had a mean diameter of 27 mm, and the tea tree oil had a mean of 21 mm. The third set of the experiment produced the best and most clear results, and the diameters were visually not as different as they were in the second set. All trials for all treatments produced valid data. After taking the means of the five trials for each treatment, the 1% Butenafine hydrochloride cream produced the largest zone at 29.6 mm, with lemongrass oil producing the second largest zone at 27 mm. The mean for the 1% Butenafine hydrochloride cream stayed approximately the same, while the lemongrass oil mean decreased by 2.5 mm in comparison to set 2. The coconut oil again produced the smallest zone diameter, with a mean of 19 mm, 4 mm more than the mean for set 2. The apple cider vinegar had a mean diameter of 25 mm, and the tea tree oil had a mean of 22 mm. After taking an overall mean of both data sets, the

1% Butenafine hydrochloride cream had the greatest mean of 30. mm. The lemongrass oil had the greatest mean of the natural antifungal agents, 28 mm. The coconut oil had the smallest mean diameter of 17 mm. All of the agents, except for the coconut oil, were placed in the susceptible category, since the diameters were greater than 18 mm. The coconut oil was placed in the intermediate category. Statistical tests were performed on the data using the overall means. A t interval with a 90% confidence level was used to determine the means' accuracy and precision. For example, the confidence interval of the lemongrass oil was (26.6, 29.6). This implied that there was a 90% probability that the mean zone diameter for lemongrass oil would fall between 26.61 mm and 29.61 mm. A two-tailed two-sample t-test with a significance level of 0.05 was also conducted in order to determine if there were any significant differences between the overall means of the substances when they were used at full concentration. All combinations of treatments were statistically significantly different except for the lemongrass oil, the apple cider vinegar, and 1% Butenafine hydrochloride cream. The p-value for the lemongrass oil and the apple cider vinegar was 0.11, which is greater than 0.05. The p-value for the lemongrass oil and the 1% Butenafine hydrochloride cream was 0.18. The null hypothesis, in this case, was that the lemongrass oil had a smaller mean than the other two. The p-value was then doubled to make a two-tailed test. The null hypothesis was rejected because the p-values were greater than 0.05, implying that there was no statistical difference in the mean zones of inhibition of the compared compounds. The results and zone diameters overall did make sense because, according to one experiment testing antimicrobial activity, the mean zone diameter for lemongrass oil was around 27 mm, and the mean zone diameter for this study was 28 mm (4). Also, according to another antimicrobial study, the mean zone of inhibition for apple cider vinegar was around 25 mm, while the mean zone diameter in this experiment was 26 mm (5).

Limitations

A possible source of error could be that the oils moved around the Petri dishes too much and spread across the yeast. This could have caused the zones of inhibition to be larger than they should have been. The agar was also not sterilized for 20 minutes at 121 °C, which could allow rogue fungal growth; however, a negative control was used in order to ensure no outside bacteria was grown. A final possible source of error could be that the drops were not all the same size. This could have been due to the variations in the pipet diameter and the fact that not all droplets were the same size. In the future, the natural substances could be diluted to equivalent minimum inhibitory concentrations (MICs) so they could be compared against each other. This study, however, was an approach to determine whether the oils were effective as antifungal agents when used at their full concentration levels. Also, in the future, different types of yeast could be used, such as *Candida albicans*, and more natural oils could be tested.

Conclusion

The lemongrass oil produced the largest mean inhibition zone diameter (28.11 mm) compared

to the other three natural antifungal agents. The 1% Butenafine hydrochloride cream produced the largest overall mean zone diameter of 30. mm. Therefore, the 1% Butenafine hydrochloride cream was substance to which the fungus was the most susceptible overall. However, the lemongrass oil was the most susceptible out of the non-medical treatments. A possible explanation for why the lemongrass oil turned out to be the best natural antifungal treatment could be because it contains a bioactive component called citral, which has antifungal properties (6). The fungus was most susceptible to the 1% Butenafine hydrochloride cream overall because it is a clinically tested medical treatment that was designed to fight yeast overgrowths. However, according to the t-test results, there was not a significant difference between the mean diameters for the lemongrass oil and the 1% Butenafine hydrochloride cream. It is important to note that, although no statistical difference was found between the mean zone diameters for the lemongrass oil with the apple cider vinegar and 1% Butenafine hydrochloride cream, this does not mean that the natural substances are as effective as the medical cream. All of the natural substances were used and tested at their full concentrations, which means that the ratios above the oils' MICs were different. For example, the MICs of the apple cider vinegar and the 1% Butenafine hydrochloride cream at full concentration are both 100 times more than that of the coconut oil at its full concentration (7). It would therefore be incorrect to conclude that one oil has better antifungal properties than another, due to this limitation. However, the results of this study did show that, when these natural substances were used at full concentration, the fungus was susceptable to all of them except for the coconut oil. Therefore, they could be considered useful in fighting yeast growth without any unwanted side effects, that may appear when using the cream. Some of the side effects that may occur when using the 1% Butenafine hydrochloride cream include blistering, burning, itching, swelling, and redness. Although about 15% of people are sensitive to essential oils, the chances of having an allergic reaction may be significantly reduced if diluted oils are used. If the oils are diluted, this could require, for example, more time to inhibit the growth of yeast, and reapplications may be necessary. However, this process is common with any treatment, and natural oils are less likely to produce the side effects that many approved treatments can produce.

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