

IN VITRO INHIBITORY TEST OF CITRONELLA ESSENTIAL OIL (*Cymbopogon nardus*) AGAINST THE FUNGAL *Candida albicans* ATCC 10231 CAUSATIVE AGENT OF CANDIDIASIS DISEASE

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ABSTRACT

The citronella plant is a plant that is believed to have many benefits for health. This citronella plant produces essential oil which is usually used as an antifungal. This study aims to determine the potential of essential oils in inhibiting *Candida albicans*, to know the *Minimum Inhibitory Concentration* (MIC) of citronella essential oil, and to determine the *Lethal Concentration 50* (LC₅₀) of citronella essential oil. This study used a complete randomized design with four replicates. Treatment of essential oil inhibition test by disc diffusion method on Saboraud Dextrose Agar (SDA) media with essential oil concentrations of 5% (v/v), 10% (v/v), 15% (v/v), 20% (v/v), and 25% (v/v). The data obtained was then analyzed using ANOVA and coupled with the Duncan test. The results of the study showed that the data of all concentration treatments of citronella essential oil had a significantly different effect ($P < 0.05$).

1. Introduction

Fungal infections are infections that are often found in Indonesia. This fungal infection occurs supported by tropical climate conditions in Indonesia. In addition, the causes of these infections are improper personal hygiene, sweaty skin conditions, and a lack of knowledge about fungal growth (Puspitasari et al., 2019). A pathogenic fungal that is often the cause of fungal infections is *Candida albicans* (Marbun, 2021). *Candida albicans* is a microbiota mainly in the vagina, respiratory tract, skin, under the fingers and nails, and mucous membranes. This disease is caused by *C. albicans* is usually called candidiasis (Rodiah et al., 2022).

Treatment of candidiasis has been carried out using antibiotics such as myconazole, nistatin, fluconazole, clotrimazole, ketoconazole, and other groups of azols, but unwanted side effects such as gastrointestinal disorders and abnormalities in liver enzymes are frequent (Marbun, 2021; Ningsih et al., 2017). To overcome these unwanted side effects, it is necessary to explore natural antifungal drugs, one of which is essential oil from the citronella plant (*Cymbopogon nardus*) (Ningsih et al., 2017; Lely et al., 2018).

There have been several studies reporting the inhibitory power of citronella essential oil against the fungal *Candida albicans*, but more detailed research is still needed with the addition of the *Minimum Inhibitory Concentration* (MIC) test and the *Lethal Concentration 50* (LC50) test of citronella essential oil (*Cymbopogon nardus*) against the fungal *Candida albicans* ATCC 10231.

2. Methodology

2.1. Time and place of research

The research was carried out in the Microbiology Laboratory of the Biology Study Program, Faculty of Mathematics and Natural Sciences, Udayana University. The research was carried out from December 2023 to March 2024.

2.2. Tools and materials

The materials used in this study were citronella essential oil, *Candida albicans* ATCC 10231 isolate, *Saboraud Dextrose Agar* (SDA), tween 80, aquadest, Petri cup, cotton swab, disc paper, sulfuric acid, barium chloride (BaCl₂), fluconazole antibiotics, alcohol, lactophenol blue staining, cotton, and spritus. The tools used are Oses, tweezers, incubators, hotplates, scales, microscopes, prepared glass, prepared cover glass, eppendorf tubes, droppipettes, reaction tubes, calipers, matches, stationery, erlenmeyer, vortex, autoclave, droppers, micropipettes, glass bottles, Bunsen, and laminars.

2.3. Methods

Research design

The experimental study used a Complete Randomized Design (CRD) with 7 treatments and 4 replicates. The treatment included concentrations of citronella essential oil extract 5% (v/v), 10% (v/v), 15% (v/v), 20% (v/v), 25% (v/v) with Tween 80 solvent with a concentration of 2%, a positive control (fluconazole 0.1 µg/µL), and negative control (Tween 80 with a concentration of 2%).

Rejuvenation and confirmation of *Candida albicans* ATCC 10231

Isolate of the fungal *Candida albicans* ATCC 10231 was obtained from the Microbiology Laboratory of the Biology Study Program, Faculty of Mathematics and Natural Sciences, Udayana University. Rejuvenation was carried out by taking one Ose of *C. albicans* fungal culture using sterile Ose, then planted on an inclined natural resource medium by scratching, then incubated in an incubator with a temperature of 37°C for 24 hours (Rodiah et al., 2022). Confirmation of *C. albicans* fungi was carried out by macroscopic and microscopic observations.

Preparation of citronella essential oil concentrate

Essential oils are obtained from the Sundari Essential Oil collection. Citronella essential oil with a concentration of 100% is then diluted with Tween 80 with a concentration of 2%. This dilution is adjusted to the concentration treatment to be used. The first dilution is done to make a stock concentration of 50% (v/v). The stock concentration is then diluted with the same solvent to obtain concentrations of 5% (v/v), 10% (v/v), 15% (v/v), 20% (v/v), and 25% (v/v).

Manufacture of standard solution 0.5 McFarland

A 1% sulfuric acid solution of 9.95 mL was put into the test tube then 0.05 mL of 1% barium chloride solution was added. The test tube containing the mixture of the two solutions is then shaken until homogeneous. A solution of 0.5 McFarland is equivalent to 1×10^8 cells/mL (Aviany and Pujiyanto, 2020).

Manufacture of *Candida albicans* suspension

C. albicans cultures in oblique agar media were taken with Ose needles and then suspended into tubes containing NaCl, then homogenized and adjusted to the same turbidity level as McFarland's solution (Retnaningsih and Dayanti, 2017).

Antifungal activity test *Candida albicans* ATCC 10231

This test is carried out by the disc diffusion method. *Saboraud Dextrose agar* media is poured into a Petri dish and left to solidify. After that, the suspension of the *Candida albicans* fungal was taken, then swabs were carried out in a streak on the SDA medium. Each disc paper was dripped with various concentrations of citronella essential oil as much as 20 µL and the positive control, namely Fluconazol, and the negative control, namely Tween 80 with a concentration of 2%, then placed on the natural resources medium that already contained the suspension. The treatment was repeated four times. After that, it is incubated for 24 hours at a temperature of 37°C. After that, observations and measurements were made of the diameter of the resistance zone formed and measured using a caliper. The calculation of the diameter of the inhibition zone is carried out by measuring the length of the diameter vertically and horizontally (Jumardin and Masnawati, 2015). According to Winastri

et al. (2020), the category of inhibition zone diameter is divided into four categories presented in Table 1.

Table 1. Category of Diameter of the Resistance Zone

Diameter	Category of Inhibition Zone
≤ 5 mm	Weak
6-10 mm	Keep
11-20 mm	Strong
≥ 21 mm	Very powerful

Minimum Inhibitory Concentration (MIC) Test of Citronella Essential Oil in *Candida albicans* ATCC 10231

At this stage of the MIC test, a concentration of 5% (v/v) citronella essential oil was prepared as a primary stock, which was then diluted to concentrations of 1% (v/v), 2% (v/v), 3% (v/v), and 4% (v/v). MIC testing has the same stages as essential oil inhibition testing (Jumardin and Masnawati, 2015).

Lethal Concentration 50 Test (LC₅₀) of citronella essential oil (*Cymbopogon nardus*) against *Candida albicans* ATCC 10231

The method used in the LC₅₀ test is the pour cup method. 1 mL of Eppendorf was prepared, then filled with 100 μ L of fungal suspension and added a mixture of essential oil concentrations in the previous MIC test and Tween 80 solvent with a concentration of 2% was added until it reached a final volume of 1 mL. The number of cells tested was 10³ cells/mL that had been diluted from 10⁸ cells/mL. Eppendorf tubes are homogenized using vortex, then left for 15 minutes at room temperature, this is done to provide contact time between essential oils and fungal. The solution in Eppendorf is poured entirely into Petri dishes and *Saboraud Dextrose Agar* (SDA) media is added. The medium is waited for to solidify, after which the Petri dish is incubated for 24 hours at a temperature of 37°C. The number of colonies growing is observed and counted manually assuming 1 colony comes from 1 cell. The results of the calculation were then plotted on a curve that showed In Vitro Inhibitory Test of Citronella Essential Oil (*Cymbopogon Nardus*) against the Fungal *Candida albicans* ATCC 10231 Causative Agent of Candidiasis Disease of test fungal cells and determined the regression equation (Tanya et al., 2021).

2.4. Data analysis

All quantitative data obtained were analyzed quantitatively using Analysis of Variance (ANOVA) using the SPSS application and excel application.

3. Results and Discussion

3.1. Result

***Candida albicans* ATCC 10231 fungal confirmation test**

The results of the macroscopic confirmation test of the fungal *Candida albicans* on Sabouraud Dextrose Agar (SDA) media showed that the colonies that grew were yellowish-white, spherical in shape, and had a sour odor. *C. albicans* fungal colonies on Sabouraud Dextrose Agar can be seen in Figure 1.

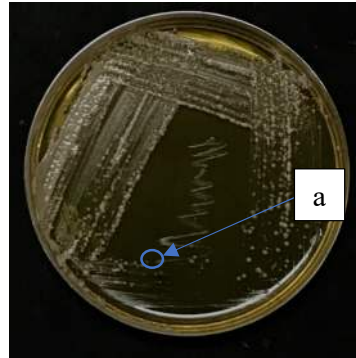


Figure 1. *C. albicans* ATCC 10231 on Sabouraud Dextrose Agar after incubation for 24 hours at 37°C, (a) colonies *C. albicans*

The results of microscopic observations of *C. albicans* ATCC 10231 using a microscope with a magnification of 40x, showed that the fungal is oval in shape with bud cells that remain attached to the stem cell so that it looks like a pseudomycelium. *C. albicans* ATCC 10231 can be seen microscopic in Figure 2.

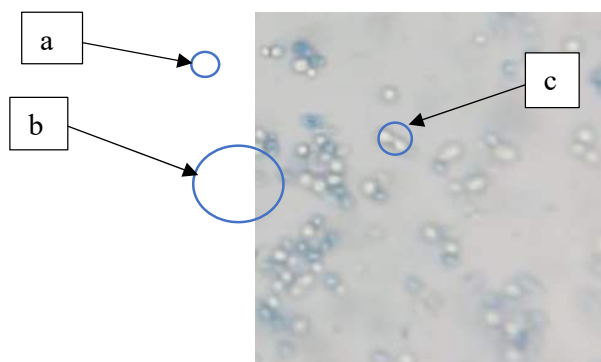


Figure 2. Observation Results *C. albicans* ATCC 10231 with the addition of *Lactophenol Blue* at 40x magnification, (a) Microscopic shape of the cell *C. albicans*; (b) Pseudomycelium; (c) Shape of the bud cell *C. albicans* that attach to the stem cell

Test of inhibition of citronella essential oil against *Candida albicans* ATCC 10231

The results of the inhibition test of citronella essential oil against *Candida albicans* ATCC 10231 showed that citronella essential oil had the ability to inhibit the growth of *C. albicans* ATCC 10231 fungal. The highest inhibition was found in essential oils with a concentration of 25% (v/v) with an

average inhibition zone diameter of 18.5 mm. The smallest inhibitory power was found in essential oils with a concentration of 5% (v/v) with an average inhibitory zone of 3.6 mm. The negative control using Tween 80 with a concentration of 2% did not form an inhibitory zone while the positive control using Fluconazole 0.1µg/µL resulted in an inhibitory zone diameter of 22.7 mm. The results of the inhibition test data are presented in Table 2.

Table 2. Results of the Test of the Inhibition of Citronella Essential Oil against *C. albicans* ATCC 10231

Concentration Treatment	Standard Deviation ± Inhibition Zone Diameter*
Negative control (v/v)	0a
5% (v/v)	3.6±0.24b
10% (v/v)	4.7±0.31c
15% (v/v)	8.8±0.35d
20% (v/v)	14.4±0.32e
25% (v/v)	18.5±0.20f
Positive control (w/v)	22.7±0.47g

Note: The values in Table 2 ± standard deviation are the average of 4 repetitions. Different letter notations showed a significant difference in mean value (P<0.05) based on the Duncan test after the analysis of various fingerprints (ANOVA).

Minimum Inhibitory Concentration (MIC) Test of Citronella Essential Oil against *Candida albicans* ATCC 10231

The results of the *Minimum Inhibitory Concentration* (MIC) test of citronella essential oil against *C. albicans* fungal ATCC 10231 showed that the smallest concentration that was able to inhibit the growth of *C. albicans* fungal was a concentration of 2% (v/v) with an average inhibition zone diameter of 0.63 mm. The complete MIC test data results are presented in Table 3.

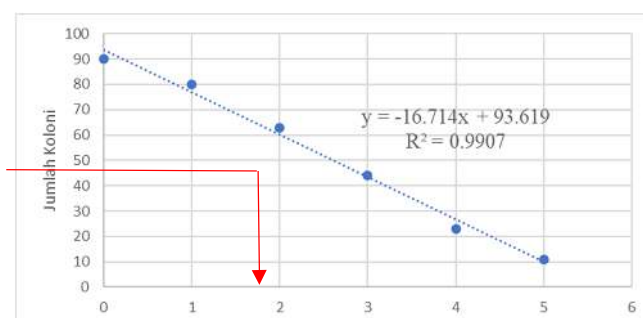
Table 3. Test Results *Minimum Inhibitory Concentration* (MIC) Citronella Essential Oil against *Candida albicans* ATCC 10231

Concentration Treatment	Standard Deviation ± Inhibition Zone Diameter*
1% (v/v)	0.00±0.00
2% (v/v)	0.63±0.32
3% (v/v)	1.25±0.20
4% (v/v)	2.20±0.23
5% (v/v)	3.81±0.42

Note: The values in Table 3 ± standard deviation are the average of 4 repetitions.

Lethal Concentration Test (LC₅₀) of citronella essential oil against *Candida albicans* ATCC 10231

LC₅₀ test of citronella essential oil in *Candida albicans* fungal ATCC 10231 uses concentrations of 5% (v/v), 4% (v/v), 3% (v/v), 2% (v/v), and 1% (v/v). Based on the results of the regression equation, the linear equation $y = -16.714x + 93.619$ was obtained so that the LC₅₀ value of 2.91% was obtained. Figure 3. showed the relationship between the number of fungal colonies and the concentration of citronella essential oil.



In Vitro Inhibitory Test of Citronella Essential Oil (*Cymbopogon Nardus*) against the Fungal *Candida albicans* ATCC 10231 Causative Agent of Candidiasis Disease

Figure 3. Test Results of LC₅₀ Citronella Essential Oil against *Candida albicans* ATCC 10231

3.2. Discussion

The results of the confirmation test of *Candida albicans* ATCC 10231 microscopic using Saboraud Dextrose Agar (SDA) media showed that the colonies that grew were yellowish-white, spherical in shape, and had a sour smell. Microscopic observation of *C. albicans* with the addition of Lactophenol Blue using a 40x magnification microscope showed that *C. albicans* ATCC 10231 cells were oval in shape and the shoot cells were attached to the stem cells so that they formed like pseudomycelium. Lactophenol Blue used in microscopic observations contains phenol, cotton blue, lactic acid, and glycerol. Phenol functions to kill fungi, cotton blue is used to dye fungal blue, lactic acid is used to maintain the structure of fungi, and glycerol functions to preserve preparations and maintain cell physiology (Irawan et al., 2019; Nurfadillah et al., 2021; Basava et al., 2016).

The results of the inhibition test of citronella essential oil had a significantly different effect ($P < 0.05$) on the growth of *C. albicans* ATCC 10231. Based on the average difference between the treatments after analysis with the Duncan test, there was a real difference ($P < 0.05$) both between negative control, positive control and the concentration of citronella essential oil tested. This is shown by the difference in the inhibition zones formed around the disc paper. The inhibition zone formed indicates that citronella essential oil contains active ingredients that function to inhibit the growth of *C. albicans* in vitro. According to Rahmi et al., (2019) that the formation of the inhibition zone is caused by the presence of active substances from citronella essential oil which is known to have antifungal activity. Wijerathna et al., (2023) reported that the active compounds contained in citronella essential oil are citronelal and geraniol. Furthermore, Kurniawan et al. (2020) stated that the active compounds contained in citronella essential oil based on the results of GC-MS analysis contained citronelal with a percentage of 55.78% and geraniol as much as 17.69%.

According to Wijayanti (2015) and Guimaraes et al., (2019) that geraniol and citronelal are monoterpenoids which are a combination of two isoprene molecules. The citronelal mechanism as an antifungal is to reduce ergosterol levels and cause damage to cell membranes (Sing et al., 2016). Geraniol is not easily soluble in water, but rather soluble in organic solvents. Geraniol also has a characteristic pungent odor (Nugraheni et al., 2021). The mechanism of geraniol as an antifungal is with the lipophilic properties of essential oils so that they adhere to the lipid membrane of the fungal cell, then the permeability of the membrane is disturbed, causing cell apoptosis characterized by DNA fragmentation (Setyawati and Yuliani, 2024; Gaonkar et al., 2018; Leite et al., 2015).

The diameter of the formed inhibition zone determines the category of inhibition strength of the essential oil. The grouping of inhibition strength is divided into several categories, namely ≤ 5 mm is categorized as weak, 6-10 mm is categorized as moderate, 11-20 mm is categorized as strong, and ≥ 21 mm is categorized as very strong (Winastri et al., 2020). Based on the results of the inhibition zones from the research concentration series, it can be categorized that the concentrations of 25% (v/v) and 20% (v/v) are included in the strong category, the concentration of 15% (v/v) is included in the medium category and the concentrations of 10% (v/v) and 5% (v/v) are included in the weak category.

The average diameter of the resistance zone formed in this study increased along with the increase in the concentration of citronella essential oil treated. This phenomenon is suspected to be caused by the higher the concentration given, the more active ingredients contained to inhibit *C. albicans* in vitro (Lani et al., 2021). This is supported by a study conducted by Sulistiawati et al., (2018) regarding pal leaf essential oil (*Myristica fragrans* Houtt) on the growth of *C. albicans* ATCC 10231 fungal which tested concentrations of 10%, 25%, 50%, 75% and 100% resulting in an average diameter of inhibition zones of 8.5 mm, 16 mm, 17 mm, 19 mm, and 23.75 mm, respectively.

This antifungal activity test uses the antibiotic fluconazole as a positive control. Based on the results of the research test, the positive control of fluconazole resulted in an inhibitory zone diameter of 22.7 mm. Positive control was used as a solution to compare the results between raw antifungal drugs and essential oils (Rompas et al., 2022). The mechanism of action of fluconazole as an antifungal is by inhibiting the lanosterol enzyme with 14- α demethylase which has involvement in the process of converting lanosterol to ergosterol, then the process becomes inhibited so that it decreases the production of ergosterol and the accumulation of toxic sterol precursors. This results in damage to the structure and function of the fungal cell membrane so that it interferes with the fluidity of the cytoplasm and cell wall (Afrina et al., 2017; Serra et al., 2018).

Negative control testing using Tween 80 solvent with a concentration of 2% found no inhibition zones around the disc paper. This shows that the use of negative control has no effect on the inhibitory test as an antifungal. Therefore, the inhibition that is formed is not affected by the solvent used, but rather the inhibition is affected by the essential oils used. The use of Tween 80 with a concentration of 2% as a solvent is due to the fact that the solvent is able to increase the solubility of insoluble or slightly soluble materials in the dispersion medium by reducing the interface voltage between the two phases of the unmixed liquid (Awaludin et al., 2019; Katrin et al., 2015).

The *Minimum Inhibitory Concentration* (MIC) test of citronella essential oil against *C. albicans* ATCC 10231 aims to determine the minimum concentration as an antifungal (Salasa et al., 2019). Based on the data of Table 4, the minimum concentration of essential oils that can inhibit the growth of *C. albicans* ATCC 10231 is a concentration of 2% (v/v) with an average diameter of 0.63 ± 0.32 mm. Another case is the research conducted by Salasa et al. (2019) who tried to examine kecombrang leaf extract, it was proven that the smallest concentration was at a concentration of 6.25% which was able to inhibit *C. albicans*. Likewise, what Ramschie et al. (2017) did obtained the minimum concentration of noni leaf extract (*Morinda citrifolia* L.) against *C. albicans* was at a concentration of 12.5%. The difference in each inhibition zone is suspected to be due to the type of active ingredient content of each plant is different, where the content of the active ingredient of a plant is also influenced by several factors.

Factors that affect the content of active ingredients of a plant, one of which is the location where the plant grows, where the influence of the location consists of climate, temperature, rainfall, duration of sunlight, soil characteristics and nutrients in the soil (Gizaw et al., 2022; Astuti et al., 2014). Research by Rahmawati et al. (2023) stated that plants grown in lowlands have more active compound content compared to plants grown in highlands. This happens because rainfall in the lowlands is smaller than in the highlands, causing plants to experience a lack of water which has an impact on the enzymes that play a role in the synthesis of compounds in plant essential oils. In addition, the active compounds of citronella essential oil are also influenced by the age of the plant, the method of isolation of the essential oil itself, packaging and storage time (Marwati et al., 2021; Kurniawan et al., 2020).

In this study, *Lethal Concentration 50* (LC₅₀) of citronella essential oil was tested against the fungal *C. albicans* ATCC 10231 which aimed to determine the acute toxicity of citronella essential oil which kills 50% of the fungal *C. albicans* ATCC 10231. The smaller the LC₅₀ value, the more toxic the compounds contained in the citronella essential oil (Aprilyanie et al., 2023). According to Ningdyah et al. (2015) the toxicity category of a substance for LC₅₀ concentrations ≤ 30 ppm has a very high toxicity category level, LC₅₀ concentrations ≥ 31 ppm and LC₅₀ ≤ 1000 ppm then it has a moderate toxicity category level, and if LC₅₀ > 1000 ppm has a low toxicity category level. The concentration that causes *C. albicans* ATCC 10231 to experience 50% mortality was obtained from the analysis of a linear regression equation that has a relationship between the cell density of the test fungal and the concentration of citronella essential oil. Based on the results of the research that has been carried out, it was obtained that the LC₅₀ value was at a concentration of 2.91% (29,100 ppm). The results showed that the concentration of citronella essential oil against *C. albicans* had a low toxicity category to be used as an antifungal.

4. Conclusion

Based on the research that has been carried out, the conclusions that can be obtained are:

1. Citronella essential oil (*Cymbopogon nardus*) has the potential to inhibit the fungal *Candida albicans* ATCC 1023.

2. *The Minimum Inhibitory Concentration* (MIC) of citronella essential oil (*Cymbopogon nardus*) against the fungal *Candida albicans* ATCC 10231 is a concentration of 2% with an average inhibition zone diameter of 0.63 ± 0.32 mm.
3. The *Lethal Concentration 50* (LC₅₀) value of citronella essential oil (*Cymbopogon nardus*) against the fungal *Candida albicans* ATCC 10231 was at a concentration of 2.91%.

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