

Research Report

Fungal inhibitory effect of *Citrus Limon peel* essential oil on *Candida albicans*

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ABSTRACT

Background: Oral candidiasis is an opportunistic infections due to *Candida albicans* that often found in people with HIV/AIDS. Anti-fungi, polyne and azole, are used in the treatment of oral candidiasis, but often cause persistence and recurrence. *Citrus Limon peel* contains terpenoids capable of inhibiting the synthesis of ergosterol, a component of the fungal cell wall that helps to maintain cell membrane permeability. Essential oil derived from citrus limon peel, thus, is expected to inhibit the growth of *Candida albicans*. **Purpose:** This research was aimed to know how essential oil derived from citrus Limon peel can inhibit the growth of *Candida albicans*. **Method:** This research was a laboratory experimental research carried out in three phases. First, essential oil was made with cold pressing method, and then the concentration of 100% was diluted to 50%, 12.5%, 6.25%, 3.125%, 1.56% and 0.78%. A test was conducted on the culture of *Candida albicans* in Sabouraud broth, accompanied by control (+) and (-). Second, the dilution of essential oil was conducted to alter the concentration with inhibitory power, from the strongest one to the weakest one, and then it was tested on the culture of *Candida albicans*. Third, spreading was carried out from liquid culture to agar media in order to measure the number of colonies. **Result:** *Candida albicans* did not grow on media with 100% essential oil treatment, but it grew on media with 50% essential oil treatment. In the second phase, dilution of 100%, 90%, 80%, 70%, 60% and 50% was conducted. The growth of *Candida albicans* was found on the treatment media of 60% and 50%. On the agar media, the growth occurred in the cultured medium treated with 70%. **Conclusion:** The minimum inhibitory power of essential oil derived from citrus Limon peel against *Candida albicans* was in the concentration of 80%. Essential oil derived from citrus Limon peel has antifungal effect and potential as a therapeutic agent for oral candidiasis.

Keywords: Oral Candidiasis; HIV/ AIDS; *Candida albicans*; *Citrus Limon*; essential oil

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INTRODUCTION

Candida albicans infection is an opportunistic infection mostly found in oral mucosa. *Candida albicans* is a normal flora of the oral cavity that can be changed into pathogens in patients who are in immunocompromised condition then leading to an infection, known as *candidiasis*. Oral Candidiasis occurs in 50-90% of HIV/AIDS cases, and 90% of patients infected with HIV suffer from this infection.¹

Citrus Limon essential oil, *cedro oil*, is obtained by extracting the peel of *Citrus Limon*. The oil can be extracted from the peel of *Citrus Limon* in three ways, namely:

distillation, pressure (cold pressing) and solvent extraction. *Citrus Limon* essential oil contains more than 90% *terpenoids* potentially powerful as anti-fungi. Terpenoids (the original turpentine of “terpinen”) is a hydrocarbon with the chemical structure of C₁₀H₁₆. This compound is actually contained in oil derived from plant products, serving to give aroma in the plant products.² Terpenoids is also found in a variety of traditional herbal medicine. Various studies report that this compound has pharmacological properties, such as antibacterial and antineoplastic, as well as other various pharmacological functions. Terpenoids plays a role in creating aroma in eucalyptus, giving flavor in

cinnamon, cloves and ginger, as well as producing yellow color in sunflower and red color in tomatoes. Steroids and sterols are biological products derived from terpenoid precursors. Terpenoids when combined with protein in the cell membrane is known as isoprenylation.³

Ergosterol is sterol found in fungi. Ergosterol is also known as a component of fungal cell membranes. Ergosterol is only found in fungal cell membranes, and does not exist in other living things. Thus, it can be concluded that enzymes involved in the synthesis of ergosterol could be the main target of antifungal medicine.⁴ Therefore, this study was aimed to observe the inhibitory power of essential oil derived from *Citrus Limon* peel against the growth of *Candida albicans*. As a result, this research is expected to become useful base in the development of alternative medicines for *oral candidiasis* with herbal ingredients grown in Indonesia.

MATERIALS AND METHODS

This research was a laboratory experimental research with a post-test only control group design. Extraction of essential oil derived from *Citrus Limon* was carried out with cold pressing method. A total of 0.5 kg of *Citrus Limon* peels produced essential oil 10 ml with a concentration of 100%. The essential oil with the concentration of 100% was then diluted into a concentration of 50%, 12.5%, 6.25%, 3.125%, 1.56%, and 0.78%. Dilution was carried out to obtain a concentration of 90%, 80%, 70% and 60%. The extraction and dilution were conducted in the Laboratory of Phytochemistry, Faculty of Pharmacy, Universitas Airlangga.

Candida albicans specimens were obtained from *oral candidiasis* lesions in HIV/AIDS patients hospitalized in the Inpatient Care Unit of Intermediates Infectious Diseases in Dr. Soetomo Hospital. The samples were 20–45 years old male or female patients diagnosed with HIV/AIDS as well as *oral candidiasis*. *Candida* culture was performed on *Sabouraud broth*, then incubated for 48 hours at a temperature of 37° C in the Laboratory of Microbiology, Faculty of Dentistry, Universitas Airlangga, and identified in the Balai Besar Laboratorium Kesehatan Surabaya with sugar fermentation test method, germ tube test, and chlamydospores test on cornmeal agar. After pure culture was obtained, and it was randomly divided for treatment with the provision of essential oil with different concentrations.

RESULTS

In the first phase, the effect of *Citrus Limon* essential oil with the concentration of 50%, 12.5%, 6.25%, 3.125%, 1.56% and 0.78% against *Candida albicans* cultured in *Sabouraud broth* was observed to know the sterility of the essential oil. *Candida albicans* culture without treatment

was as the positive control, while *Candida albicans* culture with *Citrus Limon* essential oil treatment with the concentration of 100% was as the negative control.

On the tube treated with *Citrus Limon* essential oil with the concentration of 100% (as the negative control), the turbidity in the broth media increased compared to that in the positive control. It indicates that there was no *Candida albicans* growth. On the tubes treated with *Citrus Limon* essential oil with the concentration of 50%, 25%, 12.5%, 6.25%, 3.12%, 1.562% and 0.78%, the turbidity in the broth media increased. It means that there were *Candida albicans* grown (Table 1). In this phase, the minimum inhibitory power of *Citrus Limon* essential oil against the growth of *Candida albicans* was still not determined since the range between 100% and 50% was too wide. Therefore, this research continued to the second phase, in which the dilution of *Citrus Limon* essential oil was conducted by using 100% essential oil and corn oil as diluent to obtain a concentration of 100%, 90%, 80%, 70%, 60% and 50%.

The results of the test on *Candida albicans* cultures during this phase were represented in Table 2. Based on the data in Table 2, there was no *Candida albicans* growth in the culture tubes treated with *Citrus Limon* essential oil with

Table 1. The growth of *Candida albicans* in broth media during the first phase of the treatment

Tube No.	Concentration	Colony growth
1	100 % <i>Citrus Limon</i> essential oil	(-)
2	50 % <i>Citrus Limon</i> essential oil	(+)
3	25 % <i>Citrus Limon</i> essential oil	(+)
4	12.5 % <i>Citrus Limon</i> essential oil	(+)
5	6.25 % <i>Citrus Limon</i> essential oil	(+)
6	3.125 % <i>Citrus Limon</i> essential oil	(+)
7	1.5625 % <i>Citrus Limon</i> essential oil	(+)
8	0.78125 % <i>Citrus Limon</i> essential oil	(+)
9	Positive control	(+)
10	Negative control	(-)

Table 2. The growth of *Candida albicans* in *broth* media during the second phase of the treatment

Tube No.	Concentration	Colony growth
1	100 % <i>Citrus Limon</i> essential oil	(-)
2	90 % <i>Citrus Limon</i> essential oil	(-)
3	80 % <i>Citrus Limon</i> essential oil	(-)
4	70 % <i>Citrus Limon</i> essential oil	(-)
5	60 % <i>Citrus Limon</i> essential oil	(+)
6	50 % <i>Citrus Limon</i> essential oil	(+)
7	Positive control	(+)
8	Negative control	(-)

Table 3. The growth of *Candida albicans* colonies in agar media

No.	Group	Number of colonies
1	100 % <i>Citrus Limon</i> essential oil	-
2	90 % <i>Citrus Limon</i> essential oil	0
3	80 % <i>Citrus Limon</i> essential oil	0
4	70 % <i>Citrus Limon</i> essential oil	7
5	60 % <i>Citrus Limon</i> essential oil	12.5
6	50 % <i>Citrus Limon</i> essential oil	-
7	Positive control	76
8	Negative control	0

the concentration of 100%, 90%, 80% and 70%. But, there were *Candida albicans* growth in the culture tubes treated with *Citrus Limon* essential oil with the concentration of 60% and 50%.

This research then continued to the third phase. In this phase, spreading was conducted on the cultures in the Sabouraud dextrose agar media from the treatment tube 2 to 5 by giving *Citrus Limon* essential oil with the concentration of 90%, 80%, 70% and 60%, and then colonies grown were measured. The average number of *Candida albicans* colonies was getting increased as the concentration of *Citrus Limon* essential oil was getting decreased. The growth of *Candida albicans* emerged in Sabouraud Dextrose Agar medium treated with 70% *Citrus Limon* essential oil (7 colonies). Similarly, there were *Candida albicans* colonies grown in the medium treated with 60% *Citrus Limon* essential oil (Table 3). It can be said that the greater the concentration of *Citrus Limon* essential oil is, the lower the number of colonies formed. There was no *Candida albicans* grown in the agar medium 3, in which the spreading of the tube was treated with 80% *Citrus Limon* essential oil, as well as in the agar medium 2 treated with 90% *Citrus Limon* essential oil (Table 3).

DISCUSSION

Oral candidiasis is a common opportunistic infection in patients with HIV-positive diagnosis. *Oral candidiasis* emerges as *erythematous* and *pseudomembranous* in hard palate, soft palate, tongue and buccal mucosa, as well as *angular cheilitis*. *Oral candidiasis* occurs in 50-90% of HIV/ AIDS cases, and an estimated 90% of HIV-infected patients also suffers from this infection during the progression of their disease.¹⁻⁵ Mucosal infection is generally not a directly life-threatening disease, but when microorganism can manage to penetrate the epithelial and gain access to the blood circulation, *candidemia* can trigger to fungal manifestations of fungal infection in kidneys, heart and brain.⁶

Citrus Limon is widely grown in Indonesia, China and India. In Indonesia, *Citrus Limon* is known as Lemon. The fruit is widely cultivated in farm fields in Batu (Malang, East Java). Lemon has also been widely used for culinary and non-culinary purposes throughout the world. Lemon even is also used for treatment, ie as aromatherapy, antioxidants and antiseptics. Lemon is often used to reduce headaches and migraine, as well as for rheumatism and arthritis treatments. *Citrus Limon* peel juice has also been widely used as a substance mixed with warm water to be used as a mouthwash and to relieve pain due to injuries in the oral cavity.⁷

Essential oil extracted from *Citrus Limon* is also known as cedro oil. The oil has a pungent smell, a refreshing pale yellowish green color and low viscosity. The essential oil derived from *Citrus Limon* peels is a byproduct of the industrial processing of lemons, so *Citrus Limon* essential oil is a relatively inexpensive material easily obtained. *Citrus Limon* essential oil contains various compositions that are useful in body protection, such as limonene, β -pinene and γ -terpinene serving as antifungi.⁸

There are three ways of making essential oil, namely distillation, expression and extraction. To make *Citrus Limon* essential oil derived from its peels, cold pressing method is recommended. The fresh peels of *Citrus Limon* contain a lot of liquid so that essential oil can be produced by cold pressure method. The advantage of the cold pressure extraction method is that it will not damage thermolabile materials contained. Thus, the cold pressure extraction method was chosen to make *Citrus Limon* essential oil in this research since the method is easy, which is by cold squeezing process. Cold squeezing process is to squeeze the peels of *Citrus Limon* with a squeeze over juice filter, which results flow into a sterile tube.⁹ To determine the antifungal potential of *Citrus Limon* essential oil, the growth of *Candida albicans* must be observed.

Candida is an opportunistic organism in oral cavity, causing disease in a healthy host, but the infection can only occur in individuals whose body resistance decreases. *Candida* genus is comprised of more than 200 species, including pathogens such as *C. albicans*, *C. dubliensis*, *C. tropicalis*, *C. parapsilosis*, *C. kefyr*, *C. guilliermondii* and *C. krusei*. *Candida albicans* is a species commonly found on the entire surface of oral cavity, especially on tongue and palate. It is also often found on the surface of the maxillary denture designed in such a way to make negative pressure on the palate mucosa, thereby inhibiting saliva containing immune components enter into the space, resulting in *Candida albicans* growth. *Candida albicans* is often found in the upper jaw of the healthy oral cavity after using maxillary denture for 12 months. *Candida albicans* is also considered as the most pathogenic *Candida* species. *Candida albicans* infection is found in 66% of the healthy maxillary denture users.¹⁰⁻¹²

Oral candidiasis in patients with HIV-AIDS is caused by the decreasing of immune system due to the depletion

of $CD4^+$ leading to a failure in stemming the pathogenicity of *Candida albicans*. Oral candidiasis can occur from the early phase after seroconversion, asymptomatic chronic phase to the advanced phase when it reaches the stage of AIDS. An estimated 90% of HIV-AIDS patients is exposed to oral *Candidiasis* during the progression of the disease, so the infection is closely related to HIV infection.^{1,5} In many countries that have not been able to distribute Highly Active Anti-Retroviral Therapy (HAART) evenly, such as in Indonesia, mucosal *candidiasis* is still the highest cause of morbidity in AIDS patients.^{5,13}

Sabouraud broth and dextrose agar media were used as media for *Candida* growth in this research. These media have acidic concentration (pH 5.6), so the growth of bacteria that are not acid resistant will be inhibited. In Sabouraud media, the growth of *Candida* can be observed after incubation for 24–48 hours.¹⁴

Based on the results of the observation on the growth of *Candida albicans* colonies treated with eight different concentrations of *Citrus Limon* essential oil and on the two control groups respectively replicated six times, there was no *Candida albicans* grown in the treatment group with 100% *Citrus Limon* essential oil. *Candida albicans* growth only occurred in the treatment group with 50% *Citrus Limon* essential oil (Table 1). The wide ranges between these two concentrations underlay this research to continue into the next phase to determine the minimum inhibitory power of *Candida* between the concentration of 100% and 50%. In the second stage, dilution was conducted to obtain the concentration of 90%, 80%, 70% and 60% (Table 2).

The results of the second phase (Table 2) indicate that there were differences of the growth of *Candida albicans* cultures treated differently. In the groups treated with *Citrus Limon* essential oil with the concentration of 100%, 90% and 80%, there was no *Candida albicans* growth, while in the groups treated with *Citrus Limon* essential oil with the concentration of 70%, 60% and 50%, there were *Candida albicans* growth. Furthermore, to clarify the growth of *Candida albicans*, culturing of *Candida albicans* was conducted on Sabouraud dextrose agar media by spreading, and then the number of *Candida albicans* colonies grown was measured (Table 3).

Essential oil contains chemical elements containing hydrocarbon consisting of several compounds, such as terpenoids (isoprenoid), non terpenoids, C13 norterpenoid, phenylpropanoid, ester, lactone, phtalide and isoyhiocnate. *Citrus Limon* essential oil contains antifungal classified into the class of terpenoids, namely limonene and terpinene as monocyclic monoterpinene, and β -pinene as bicyclic monoterpinene. Therefore, the provision of terpenoids will inhibit the synthesis of ergosterol, a component of fungal cell membranes, which plays an important role in regulating cell membrane permeability that keep the liquid inside the fungal cells and other activities, such as enzymes associated with the fungal cells. As a result, the disruption of the synthesis of ergosterol can make the permeability of cell

membranes increased so that the liquid will come out of the cells and the activities of the fungal cells will be inhibited. The provision of terpenoids with high concentration even can cause cellular activities stopped and the cells dead.¹⁵

The results showed that the increasing of the concentration of *Citrus Limon* essential oil could decrease the number of *Candida albicans* colonies. In this research, 80% *Citrus Limon* essential oil expressed the minimum inhibitory power against *Candida albicans*. Minimum fungicidal concentration (MFCs) for amphotericin-B and iatracozazole in this research were better than MFCs in other researches, for Amphotericin-B with the incubation time of 48 and 72 hours (86.4 to 87.7%) and for iatracozazole (91.4 to 93.8%).¹⁶

Finally, it can be concluded that the MFC of *Citrus Limon* essential oil to be antifungal is 80%. It means that *Citrus Limon* essential oil has antifungal properties against *Candida albicans*, so the oil can potentially be developed as a therapeutic agent for oral candidiasis that affects many patients with HIV / AIDS. Therefore, further researches are suggested to conduct a toxicity test on the essential oil if it will be developed as a therapeutic agent.

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