

Effects of citrus limon essential oil (*Citrus limon L.*) on cytomorphometric changes of *Candida albicans*

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ABSTRACT

Background: The most common fungal infection found in oral cavity is oral candidiasis, largely caused by *Candida* species, particularly *Candida albicans* (*C. albicans*). *Candida* infection can get worse since it is difficult to be treated and resistant with antifungal drugs. Therefore, new drugs and compounds as well as alternative therapies involving natural sources that have antifungal activities have continually been developed. Limonene, β -pinene, and γ -terpinene contained in *Citrus limon* essential oil have been known to have quite good antifungal activities against *C. albicans*. **Purpose:** This research aimed to examine and analyze the effects of *Citrus limon* essential oil on cytomorphometric changes of *C. albicans*. **Method:** The research used post test only control group design. Based on the results of the pre-elementary research on antifungal activities of *Citrus limon* essential oil against *C. albicans*, *Citrus limon* essential oil used in this research was on concentrations of 1.56%, 1.37%, 1.17%, 0.98%, and 0.78%. *Citrus limon* essential oil by *C. albicans* inoculum and incubated for 24 hours and 48 hours. After the incubation, those *C. albicans* cells were fixed, dried, and then observed using a scanning electron microscopy. **Result:** The most effective concentrations of *Citrus limon* essential oil triggering cytomorphometric changes of *Candida albicans* were at 1.37% and 1.56% with the incubation period of 48 hours. **Conclusion:** *C. albicans* can undergo necrosis process through cytomorphometric changes after the administration of *Citrus limon* essential oil at concentrations of 1.56% and 1.37% with the incubation period of 48 hours.

Keywords: *Citrus limon*; *Candida albicans*; necrosis; cytomorphometric changes

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INTRODUCTION

Candida is an opportunistic organism in oral cavity, triggering no disease in healthy people, but leading to an infection in the body with low immune. *Candida albicans* (*C. albicans*) is a commensal organism colonizing on the skin and mucosal tissue of gastrointestinal and genitourinary tracts. When there is an imbalance between *C. albicans* and other oral microbial components, *C. albicans* will proliferate, colonize, and invade mucosal tissues to trigger opportunistic infection.¹ *C. albicans* infection in people with HIV/ AIDS is a type of infection most commonly found at around 70-80% and also considered as a major cause of oral candidiasis, followed by *Candida guilliermondii* approximately at around 11.11%.² In recent

years, many reported cases of oral candidiasis infections are caused by *Candida glabrata*.³ Population of *Candida glabrata* is almost half of the total population of non-*C. albicans*.⁴ However, *C. albicans* is still considered as the largest species of oral candidiasis in both HIV/ AIDS patients and other immunocompromised patients.²

Antifungal compounds widely used in the medical field for the treatment of fungal infections are derived from the polyene group, such as nystatin, amphotericin, and natamisin, as well as from the azole group, such as imidazole and triazole. Nevertheless, this conventional treatment of fungal infection is not considered to be beneficial in fungal infections treatment that have been resistance to antifungal compounds.⁵

Lemon rinds, contain essential oil, formed within the endoplasmic reticulum of the plant cells and then obtained from steam distillation or extraction process of the fruit, flowers, wood, roots, leaves, and seeds of the plant.⁷ The essential oil has anti bacterial, anti-oxidant, and anti-fungal functions. The essential oil contained on the outer part (pericarp) of lemon rinds is largely composed of limonene (90%), citral (5%), terpinol, linodol α -pinene, camphene, β -pinene, sabinene, myrcene, γ -terpinene, linalool, β -bisabolene, trans- α -bergamotene, and geranyl acetate.⁸ A previous research even reveals that limonene component found in the essential oil has good anti-fungal effects on *Trichophyton rubrum*.⁹

In another previous research, lemon rinds, moreover, contain some anti-fungal compounds classified into terpenoids, namely limonene, β -pinene, and γ -terpinene, which have strong anti-fungal activities against *C. albicans*. Terpenoids inhibit ergosterol synthesis that occurs in the cell membrane of *C. albicans*.¹⁰ Consequently, the synthesis of nucleic acids is disrupted, resulting in increased cell membrane permeability.¹¹ Limonene, β -pinene, and γ -terpinene also can inhibit the metabolism of *C. albicans*, interfering organelles balance. The imbalance in organelles then makes intracellular components of the organelles disrupted as well as DNA damaged, resulting in the death of *C. albicans*.¹² Another previous research even reveals that the extract of lemon rinds combined with 96% petroleum ether has inhibitory effects on the growth of *C. albicans* in vitro.¹⁰

Cell necrosis is morphologically characterized by increased cell volume (oncosis), organelle swelling, and plasma membrane rupture, followed by intracellular component secretion. Some ingredients even can trigger the death of fungal cells, such as H₂O₂, acetic acid, as well as some metals and materials/ anti-fungal drugs. Anti-fungal ingredients at low concentrations can lead to apoptosis, but at high concentrations can cause necrosis as a result of radical damage to the cellular structure and integrity.¹³

A research conducted by Kim et al on the death process of *C. albicans* given Amphotericin B and flucytosine shows that the morphology of *C. albicans* cells undergo the process of death through cell membrane damage.¹⁴ Similarly, a research conducted by Dai *et al.*¹² finds that the administration of the root extract of *Scutellaria baicalensis* on *C. albicans* can trigger apoptosis in the cells of *C. albicans*, leading to death. Hao *et al.*¹⁶ argues that *Casposungin* containing antifungal activities can trigger apoptosis and necrosis on the cells of *C. albicans*. Equol as a soy isoflavone also has antifungal activities against *C. albicans* by triggering the ultrastructural changes of the cells. For those reasons, this research aimed to analyze the effects of *Citrus limon* essential oil on cytomorphometric changes (necrosis process) of *C. albicans* using a scanning electron microscopy (SEM).

MATERIALS AND METHOD

This research was a laboratory research with a purely experimental approach. This research was conducted to observe changes in cell size and morphology (cytomorphometrics) of *C. albicans* treated with the essential oil of lemon rinds by using a SEM.

Based on results of the preliminary research, the essential oil of lemon rinds has inhibitory effect at a concentration of 0.78%, and fungicidal effect at a concentration of 1.56%. Therefore, in this research the essential oil of lemon rinds used was at five different concentrations, namely 1.56%, 1.37%, 1.17%, 0.98%, and 0.78%. Those five groups of different concentrations as well as a control group without any treatment were incubated for 24 hours. Next, there were also five groups of different concentrations, namely 1.56%, 1.37%, 1.17%, 0.98%, and 0.78%, as well as a control group without any treatment, incubated for 48 hours.

After that, fixation process and drying were performed, and then observation was conducted using a SEM at a magnification of 3500x. The images resulted from the observation using a SEM were calibrated to measure the cell size of *C. albicans*, six of which were taken from each treatment group. The data obtained then were processed using a statistical analysis, a One-way ANOVA to determine differences between groups, followed by LSD test.¹⁷

RESULTS

Based on results of the measurement of the cell size of *C. albicans*, the mean and standard deviations of the cell size of *C. albicans* were obtained from each group. The normality of the data then was analyzed using Kolmogorov-Smirnov test. Result of the Kolmogorov-Smirnov test on the groups with the incubation period of 24 hours showed a significance value of 0.05. This result indicated that the data in each group were distributed normally. Meanwhile, result of the homogeneity test using Levene's test on the groups with the incubation period of 24 hours showed a significance value of 0.193. This result illustrated that the data were homogeneous. Next, result of the One-way ANOVA test on the groups with the incubation period of 24 hours showed a significance value of 0.583 ($p > 0.05$). This result demonstrated that there was no significant difference between the control group and the five groups of different concentrations with the incubation period of 24 hours.

Result of the Kolmogorov-Smirnov test on the groups with the incubation period of 48 hours showed a significance value 0.05. This result indicated that the data in each group were distributed normally. Result of the homogeneity test using Levene's test on the groups with the incubation period of 48 hours showed a significance value of 0.073. This result illustrated that the data were homogeneous. Result of the

Table 1. Results of the LSD test on the five groups of different concentrations with the incubation period of 48 hours

Group	(1.56%)	(1.37%)	(1.17%)	(0.98%)	(0.78%)	Control
(1.56%)	-	0.855	0.097	0.045*	0.015*	0.014*
(1.37%)		-	0.136	0.066	0.022*	0.021*
(1.17%)			-	0.079	0.386	0.377
(0.98%)				-	0.619	0.606
(0.78%)					-	0.986
Control						-

Note: * There was a significant difference

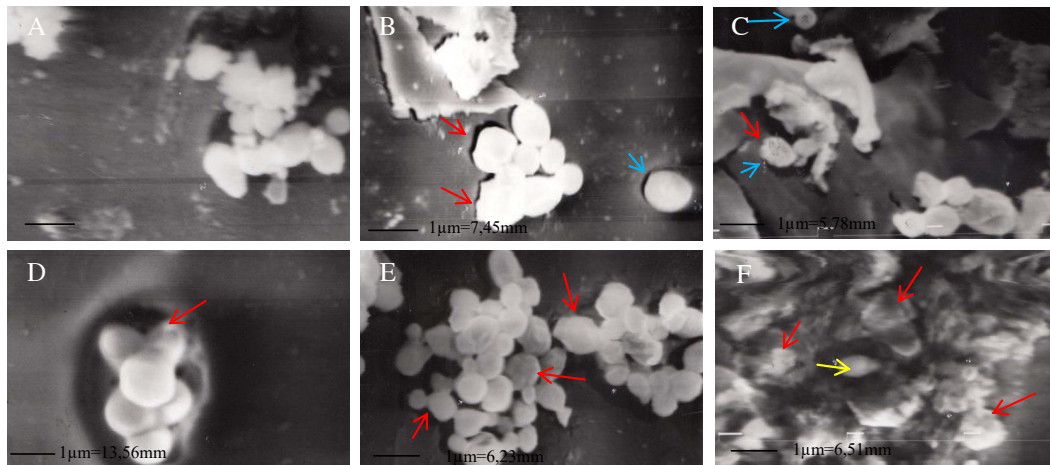


Figure 1. The cells of *C. albicans* were exposed to the essential oil of lemon rind with the incubation period of 24 hours. (A) control: the surface of cells was smooth, round or oval, and colonizing; (B) the group with the concentration of 0.78%: the surface of some cells was rough and not round perfectly (→). Some cells were separated from colonies (→); (C) the group with the concentration of 0.98%: the surface of cells was rough and not round perfectly (→). Some cells were separated from colonies (→); (D) the group with the concentration of 1.17%: the surface of some cells was rough, not round (→), and not colonizing (→); (E) the group with the concentration of 1.37%: the surface of cells was rough and not round (→); (F) the group with the concentration of 1.56%: the surface of cells was rough and not round perfectly (→). Some cells were separated from colonies (→).

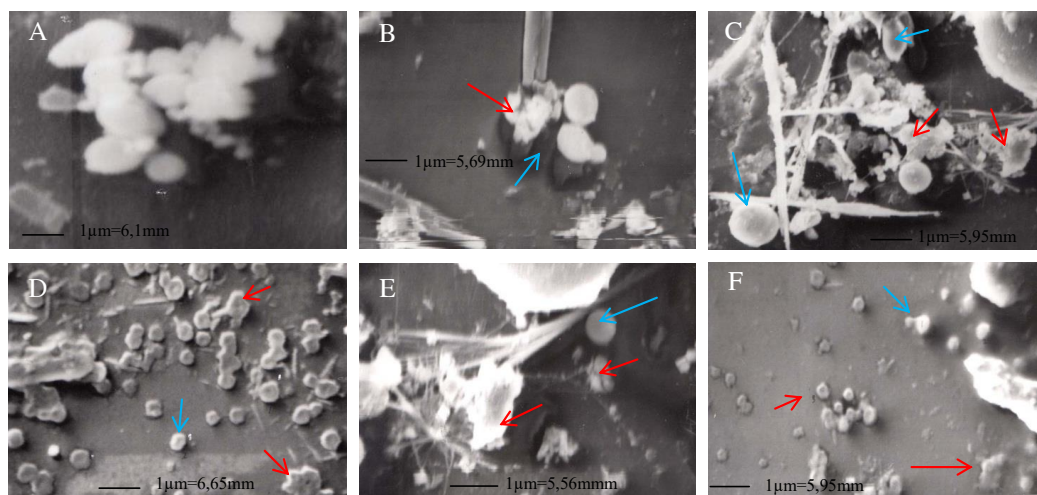


Figure 2. The cells of *C. albicans* were exposed to the essential oil of lemon rind with the incubation period of 48 hours. (A) the control group: the surface of the cell was smooth and round in colonies; (B) the group with the concentration of 0.78%: the surface of few cells was rough, not round (→), and not colonizing (→); (C) the group with the concentration of 0.98%: the surface of the cells was rough and not round (→). Some cells were separated from colonies (→); (D) the group with the concentration of 1.17%: the surface of the cells was rough, not round (→), and not colonizing (→); (E) the group with the concentration of 1.37%: the surface of the cells was rough, stood out, not round (→), and not colonizing (→) (F) the group with the concentration of 1.56%: The surface of the cells was rough, stood out, not round (→), and not colonizing (→).

One-way ANOVA test on the groups with the incubation period of 48 hours showed a significance value of 0.038 ($p < 0.05$). This result demonstrated that there was a significant difference between the control group and the five groups of different concentrations with the incubation period of 48 hours. Results of the LSD test can be seen in Table 1.

Based on Table 1, there were significant differences between the group with the concentration of 0.98% and the group with 1.56%, between the group with the concentration of 0.78% and the group with the concentration of 1.56%, between the group with the concentration of 0.78% and the group with the concentration of 1.37%, between the control group and the group with the concentration of 1.56, as well as between the control group and the group with the concentration of 1.37%. Consequently, it can be said that the essential oil of lemon rinds in the treatment groups with the concentrations of 1.56% and 1.37% had a significant change in the cell size of *C. albicans* compared to the control group and the other treatment groups with the concentrations of 1.17%, 0.98% and 0.78%.

Moreover, results of the observation using a SEM on all the groups of different concentrations, 1.56%, 1.37%, 1.17%, 0.98%, 0.78%, and the control group with both of the incubation periods, 24 hours and 48 hours indicate the morphological changes in the cell wall.

DISCUSSION

C. albicans are often resistant to antifungal therapy, such as fluconazole and amphotericin. Thus, another more effective antifungal therapy needs to be developed as an alternative by considering the death process of *C. albicans* cells. Consequently, there are so many researches focused on alternative materials containing better antifungal activities. Hydrophobic molecules composing of essential oil are known to be able to attack ergosterol in fungal cell membranes. They will trigger changes in membrane permeability as well as damage to the membrane, and then ultimately the cells of the fungi will be secreted, resulting in cell death. Essential oil molecules can also interfere with the enzymes bound to the fungal cell membranes, thereby disrupting the formation of cell membranes. In other words, the essential oil can kill and inhibit the growth of fungi.¹⁸

Based on results of the GCMS test, the essential oil of lemon rinds contained limonene (19.79%), β -pinene (1.06%), and γ -terpinene (0.45%). Biological activities of the essential oil of lemon rinds are related to monoterpenes compounds characterized by high concentrations of limonene, β -pinene, γ -terpinene, and linalool contained in the components of the essential oil.¹⁹ Limonene can trigger interference to the cell membrane of *C. albicans*, resulting in secretion of the cellular components. Limonene can also change the structure of methylesterification from pectin, a major component of the fungal cell wall. Such changes in the structure of pectin are associated with changes in cell adhesion and plasticity, pH and ionic content of the cell

wall, as well as effects of *C. albicans* growth, membrane integrity, and permeability.²⁰

β -pinene, can trigger both disruption of the cell membrane of *C. albicans* as well as interference to the functioning of mitochondria. β -pinene can also interfere with the movement of ions H^+ and K^+ in the cells of *C. albicans*, resulting in interference to the function of mitochondria in providing energy for cells in the form of ATP. β -pinene as an oxidant can increase permeability of the mitochondria and ATPase inhibitors that can block the formation process of energy by mitochondria.²¹

γ -terpinene, can interfere with the synthesis of ergosterol in *C. albicans*. Ergosterol plays an important role in the cell growth of *C. albicans*. Ergosterol, a predominant lipid molecule in fungal cells, serves to regulate fluidity of membrane as well as permeability and activity of membrane-bound enzyme. Terpinene also can interfere with the synthesis of proteins that can interfere with metabolic processes in the nucleus of *C. albicans* cells.²²

The essential oil of lemon rinds contains anti-fungal compounds that shows a variety of biological activities. The essential oil also can affect changes in the colony and morphology of *C. albicans* cells by lowering their enzymatic activities and reducing their ability to assimilate through the active components contained, such as pinene and γ -terpinene.²³ The antifungal mechanism of the essential oil together with its monoterpen compounds can generate toxic effects on the membrane structure and functions of *C. albicans*.^{24,22} In fungal cells, α -pinene and β -pinene can disrupt the integrity of cells, inhibit respiration and ion transport process, and increase the permeability of membrane.²² Apart from its cytotoxic effects, γ -terpinene activities through interactions with the cell membrane can lead to loss of ATP synthesis capacity required for setting the cell functions.²⁵

Based on results of the One-way ANOVA test on the those five groups of different concentrations with the incubation period of 24 hours, there was no significant difference between those five groups of different concentrations and the control group. This indicates that there was no difference in the size of *C. albicans* cells between those five groups of different concentrations with the incubation period of 24 hours and the control group. There was no significant change in the size of *C. albicans* cells after treated by the administration of the lemon rind essential oil at the concentrations of 1.56%, 1.37%, 1.17%, 0.98%, and 0.78% incubated for 24 hours in Liquid Sabourth Dextrose media.

Based on results of the One-way ANOVA test on those five groups of different concentrations with the incubation period of 48 hours, there were significant differences between those five groups of different concentrations and the control group. This illustrates that there were significant differences in the size of *C. albicans* cells between those five groups of concentration with the incubation period of 48 hours and the control group. There was a significant change in the size of *C. albicans* cells after treated by

the administration of the lemon rind essential oil at the concentrations of 1.56%, 1.37%, 1.17%, 0.98%, and 0.78% incubated for 48 hours in Liquid Sabourth Dextrose media.

Based on the results of the One-way ANOVA test showed that there were some groups with the concentrations of 1.37% and 1.56% had higher significant values than the other groups. This means that the essential oil of lemon rinds at the concentrations of 1.37% and 1.56% can cause a significant change in the cell size of *C. albicans* compared to the control group. The mean cell size of *C. albicans* in the control group was 6.96 μm , while the mean cell size of *C. albicans* in the group with the concentration of 1.56% was 10.91 μm and 10.63 μm in the group with the concentration of 1.37%. All the treatment groups with the incubation period of 48 hour had greater changes in the cell size of *C. albicans* than the control group, especially in the groups with the concentrations of 1.37% and 1.56%.

The results of the observation using a SEM showed that the cell size of *C. albicans* in the groups with the essential oil of lemon rinds changed into the larger ones. The surface of those *C. albicans* cells was rough because the cell wall was wrinkled, swollen, and stood out, indicating of damage to the cell wall of *C. albicans* due to swelling of organelles inside the cells, resulting in an increase in volume suppressing the cell wall. As a result, the cell wall cracked. Some of the fungal cells were also scattered and did not form colonies. It means that the essential oil could make permeability change, triggering osmotic imbalance. Thus, there were curves on the damaged cell wall. Changes in the cell size occur during the process of necrosis indicate the cell necrosis process occurs slowly and involves damage to the cell wall.²⁶

The use of a SEM in this research was to illustrate dimensional effects of the essential oil of lemon rinds against *C. albicans* cells. *C. albicans* cells experienced stiffness, and then clump together before they were totally destroyed by the essential oil. The cell walls became thick after the treatment due to the accumulation of membrane components surrounding the cell wall surface. This cell wall thickening can also be caused by increased leaks of amino acid transmembrane and other cytoplasmic components, while the peripheral cytoplasm becomes porous looked as indentations, so lowering the density of the cytoplasm, indicating necrosis.²⁷

The use of incubation period variables, 24 hours and 48 hours, aimed to determine the effective time required by the essential oil of lemon rinds to affect *C. albicans*. Incubation period can affect fungicidal activities of antifungal compounds against *C. albicans*.²⁸

Based on results of the observations on the necrosis process of *C. albicans* using a SEM, there was no significant difference in the size of *C. albicans* cells between the five groups of different concentrations (1.56%, 1.37%, 1.17%, 0.98%, and 0.78%) with the incubation period of 24 hours and the control group. This means that the essential oil of

lemon rinds with the incubation period of 24 hours did not demonstrate antifungal activities against *C. albicans*. The results of the observations on the necrosis process of *C. albicans* using a SEM also showed that there were morphological differences between the five groups of different concentrations (1.56%, 1.37%, 1.17%, 0.98%, and 0.78%) with the incubation period of 24 hours and the control group. In the control group, the cells of *C. albicans* appeared round and smooth, as well as in the form of colonies. Meanwhile, in the five groups of different concentrations (1.56%, 1.37%, 1.17%, 0.98%, and 0.78%), the cells of *C. albicans* did not appear in colonies or round, but seemed rough or shriveled, and dispersed without forming any colonies. Such changes in the morphology of *C. albicans* cells that looked rough and shriveled were actually related to cellular physiological stress triggered by anti-fungal compounds, resulting in apoptosis process.¹⁴

There were differences in the size of *C. albicans* cells between the five groups of different concentrations (1.56%, 1.37%, 1.17%, 0.98%, and 0.78%) with the incubation period of 48 hours and the control group. Results of the LSD test reveal that the significant differences in the size of *C. albicans* cells were found between the control group and the groups with the concentrations of 1.56% and 1.37% compared to the other groups with the concentrations of 1.17%, 0.98%, and 0.78%. This indicates that the essential oil of lemon rinds with the incubation period of 48 hours could trigger a significant necrosis process against *C. albicans*, mainly on the groups with the concentrations of 1.56% and 1.37%. It can be concluded that the essential oil of lemon rinds at concentrations of 1.56% and 1.37% with an incubation period of 48 hours can trigger cytomorphometric changes, especially changes in the morphology and size of *C. albicans* cells (characterized by necrosis).

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