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Original Article

The effectiveness of essential oil from *Citrus limon* peel on *Candida albicans* biofilm formation: An experimental *in vivo* study

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الملخص

أهداف البحث: تظهر "المبيضات البيضاء" مقاومة للأدوية المضادة للفطريات. لذلك، من الضروري استخدام المواد المشتقة من المصادر الطبيعية كعامل مضاد للفطريات. يظهر الزيت العطري من قشر الليمون الحمضي قدرة على منع نمو المبيضات البيضاء في المختبر. الغرض من هذه الدراسة هو تحديد مستوى التركيز الأكثر فعالية للزيت العطري من قشر الليمون الحمضي في تثبيط التغيرات الشكلية الخلوية للمبيضات البيضاء وتشكيل الأغشية الحيوية في الجسم الحي.

طرق البحث: تم حقن ذكور فئران ويستار يتراوح وزنها بين 200 و 300 جرام بالمبيضات البيضاء لمدة 48 ساعة ثم أعطيت جرعة واحدة من ميثيل بريدنيزولون عن طريق الفم كمثبط للمناعة. تم وضع زيت عطري من قشر الليمون الحمضي في شكل هلام وبثلاث تركيزات مختلفة (0.39٪ ، 0.78٪ و 1.56٪) مرتين في اليوم لمدة يومين. تم التضحية بالجرذان بعد 48 ساعة ثم تم تحضير أنسجة الغشاء المخاطي الحنكي لفحصها باستخدام المجهر الإلكتروني الماسح للكشف عن التغيرات الشكلية الخلوية وتشكيل الأغشية الحيوية للمبيضات البيضاء.

النتائج: أظهر الزيت العطري من قشر الليمون الحمضي تركيز 1.56٪، بالمقارنة مع 0.78٪ و 0.39٪، أقوى قدرة على تثبيط تكون المبيضات البيضاء. عطل الزيت العطري من قشر الليمون الحمضي حصول التغيرات الشكلية الخلوية. تم عرض الخلايا التي لم تكن سليمة أو مستعمرة، وظهرت الشعيرات حول الخلايا ناعمة، واختفت طبقة الأغشية الحيوية، ولم يحدث تكوين خيطان.

الاستنتاجات: تأثير الزيت العطري من قشر الليمون الحمضي على التغيرات الشكلية الخلوية وتكوين الأغشية الحيوية يتأثر بتركيزه، وأظهر الزيت العطري من قشر الليمون الحمضي بنسبة 1.56٪ أقوى قدرة على تثبيط التغيرات الشكلية الخلوية وتكوين الأغشية الحيوية. تسلط هذه النتائج الضوء على الزيت العطري لقشر الليمون الحمضي كمرشح مضاد للفطريات لعلاج داء المبيضات.

الكلمات المفتاحية: ليمون حمضي؛ زيت عطري؛ المبيضات البيضاء؛ غشاء حيوي؛ مضاد للفطريات

Abstract

Objectives: *Candida albicans* is resistant to commercial antifungal agents. Therefore, it is desirable to use material derived from natural sources as an antifungal agent. Essential oil from *Citrus limon* peel is able to inhibit the growth of *C. albicans in vitro*. The purpose of this study was to determine the most effective concentration of essential oil from *C. limon* peel with regards to the inhibition of *C. albicans* cyto-morphometric changes and biofilm formation *in vivo*.

Methods: Male Wistar rats weighing 200–300 g were inoculated with *C. albicans* for 48 h and then given a single dose of oral methylprednisolone as an immunosuppressant. Essential oil from *C. limon* peel, in a gel form and at three different concentrations (0.39%, 0.78% and 1.56%), was applied twice a day for 2 days. The rats were killed after 48 h and then palatal mucosa tissues were prepared and examined with a scanning electron microscope (SEM) with regards to *C. albicans*, cyto-morphometric changes and biofilm formation.

Results: Essential oil from *C. limon* peel at a concentration of 1.56% showed the strongest ability to inhibit *C. albicans* growth when compared to 0.78% and 0.39%.

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At a concentration of 1.56%, essential oil from *C. limon* peel disrupted cyto-morphometric changes; cells that were neither in intact nor colonised were evident, the filaments around the cells were smooth, the layer of biofilm had disappeared and there was no evidence of hyphae formation.

Conclusion: The effect of essential oil from *C. limon* peel on cyto-morphometric changes and biofilm formation was concentration-dependent. Essential oil from *C. limon* peel at a dose of 1.56% showed the strongest ability to inhibit cyto-morphometric changes and biofilm formation. These findings demonstrate that essential oil of *C. limon* peel is a potential antifungal candidate for the treatment of candidiasis.

Keywords: Antifungal; Biofilm; *Candida albicans*; *Citrus limon*; Essential oil

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Introduction

Oral candidiasis is an opportunistic infection that affects the oral mucosa; the majority of cases are caused by *Candida albicans* yeast. The pathogenesis of oral candidiasis is not fully understood although several predisposing factors are known to be responsible for moving *Candida* from the saprophytic stage into the parasitic stage.[1] Since 1995, species of *Candida* have become the fourth largest cause of nosocomial infections in the bloodstream with an average mortality rate of 39%. In intensive care units, *Candida* infection is the third most common type of nosocomial infection with an average mortality rate of 47%.[2]

Oral candidiasis is generally treated with antifungal agents, such as fluconazole, echinocandins or polyene. However, antifungal therapy may cause various side effects, such as high toxicity levels, recurrences or pharmacodynamic interactions; therapy may also trigger drug resistance. In addition, the main species of *Candida*, including *C. albicans, Candida tropicalis, Candida parapsilosis* and *Candida glabrata*, have the ability to form biofilm layers that increase resistance to antifungal agents.[3] The biofilm of *C. albicans* consists of biological material from specific cells of microorganisms that form a layer of an extracellular matrix that exerts a specific protective function during the growth of *C. albicans*, increasing resistance towards destructive agents.[4]

Some natural ingredients have been studied to overcome this resistance to antifungal drugs. One of the natural ingredients that has been studied is essential oil derived from *Citrus limon* peel. Aside from this oil being readily available and inexpensive, the terpenoid content in this essential oil is particularly high. Terpenoids are known to have high antifungal properties and significant levels of coumarin, flavonoids, linalool and carotene and terpenoids have been widely studied and developed as natural antimicrobial and antioxidant agents.[5]

Until now, various concentrations of essential oil from *C. limon* peel have been investigated for their ability to inhibit the development of *Candidiasis*; however, these experiments have generated inconsistent results. As evidenced by DNA fraction analysis, essential oil from *C. limon* peel derived from *n*-hexane fraction solvent was proven to kill *C. albicans* cells.[6] Other research demonstrated that a dose of 0.78% essential oil from *C. limon* peel was the minimum inhibitory concentration (MIC) and that a dose of 1.56% essential oil from *C. limon* peel was the minimum fungicidal concentration (MFC) for *C. albicans in vitro*.[7] In our previous research, essential oil from *C. limon* peel at doses of 1.56% and 1.37% were both able to kill *C. albicans* cells through cyto-morphometric changes.[8] Based on this previous data, we hypothesised that higher concentrations of essential oil from *C. limon* peel concentration would be directly related to the reduction in number of *C. albicans*. To strengthen this hypothesis, it was necessary to conduct further research *in vivo* to determine the most effective concentration for the inhibition of *C. albicans*, cyto-morphometric changes and biofilm formation *in vivo*.

Materials and Methods

Study design

This study was a laboratory experimental study that used a post-test only control group design using Wistar rats (*Rattus norvegicus*).

Preparation of essential oil from C. limon peel

The *C. limon* used in this study was obtained from the Citrus and Subtropic Fruit Research Institute (BALITJESTRO), Batu, East Java, Indonesia. The essential oil was produced using the process described by Ganesha et al.[9] The essential oil from *C. limon* peel was extracted by steam distillation and turned into gel with 3% CMC-Na,[10] producing three separate concentrations of 0.39%, 0.78% and 1.56%.[10]

Animal preparation and C. albicans inoculation

The samples consisted of 24 male Wistar rats, each approximately 3 months of age and weighing 200–300 g. Prior to inoculation, the animals were housed in individual cages with controlled temperature (27 °C) and artificial lighting on a 12-h-on/12-h-off cycle with free access to water and standard food.

Cultures of *C. albicans* were obtained from swab results of oral candidiasis lesions taken from patients with stage 4 HIV/AIDS, which were then inoculated into Sabouraud broth medium and cultured at the Microbiology Laboratory, Faculty of Dentistry, Airlangga University, Surabaya. For inoculation, the Wistar rats were anesthetised intramuscularly with 10% ketamine and then inoculated with *C. albicans* standard Mc-Farland 1 on the palatal mucosa. Once the subjects were unconscious, they were given a single dose of

oral methylprednisolone (0.05 mg/g of body weight) through a nasogastric tube. The inoculation was performed over a period of 48 h.

Essential oil from C. limon peel treatment

After 48 h of *C. albicans* inoculation, essential oil from *C. limon* peel was applied every 12 h for a period of 48 h. A cotton swab was used to apply the oil to the palatal mucosa area of each animal. The animals were divided randomly into the following four groups.

1. A control group (four animals and no treatment),
2. Group A (four animals receiving treatment with essential oil from *C. limon* at a dose of 0.39%),
3. Group B (four animals receiving treatment with essential oil from *C. limon* at a dose of 0.78%),
4. Group C (four animals receiving treatment with essential oil from *C. limon* peel at a dose of 1.56%).

Tissue preparation

Each animal was decapitated after the treatment period and each animal's palatal mucosa was biopsied and fixed with 2% glutaraldehyde at a temperature of 4 °C. The samples were then washed three times with phosphate buffer solution (pH 7.4), each for 5 min at 4 °C, and then treated with 1% osmic acid post-fixation solution for 1−2 h at 4 °C. Samples were re-washed three times with phosphate buffer solution (pH 7.4), each for 5 min at 4 °C and then dehydrated with a graded series of alcohol (30%, 50%, 70%, 80%, 90% and absolute) up to two times, each for 15−20 min. Alcohol dehydration at 30%−70% was carried out at 4 °C, and 80% alcohol dehydration was carried out at room temperature and was then replaced with absolute amyl acetate as a preservative while drying. The samples were then dried using a critical point drying (CPD) tool and then attached to a stub (holder) using special glue. Each sample was coated with a vacuum evaporator and a coating material made from either pure gold or carbon.

C. albicans cyto-morphometric and biofilm analysis

Palatal mucosa tissue samples were examined with an electron microscope (JEOL JSM-T100, MA, Massachusetts, USA) at magnifications of 500× and 2000×. The results of the study are presented in the form of photographs and data analysis showing the number of *C. albicans* colonies in the biofilms and cyto-morphometric changes in the size and shape of *C. albicans* cells in biofilms. The sizes and shapes were mea-sured with a comparison scale and are expressed in microns (μm).

Statistical analysis

Data were collected and normality was tested with the Shapiro−Wilk test. The Levene test was used to assess data for homogeneity. MANOVA multivariate tests, namely the Gomes Howell post-hoc test and the Bonferroni test, were used to analyse the significance of the data. The software analysis used was Statistical Product and Service Solution (SPSS) (IBM SPSS Statistic 24 for Mac, Armonk, New York, USA) All data were considered significant if $p < 0.05$.

Results

Biofilm analysis of C. albicans

Calculations involving *C. albicans* colonies on biofilms were performed using images acquired by SEM at 500× magnification. The control group presented in Figure 1A shows a solid biofilm, resembling a mesh/web of rough-

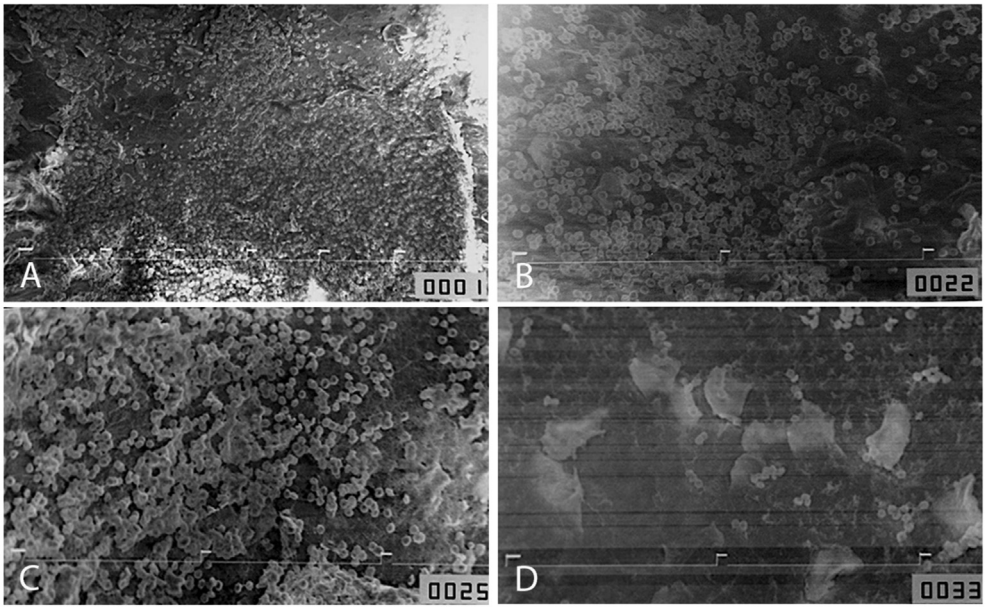

Figure 1: SEM of the *Candida albicans* biofilm at a magnification of 500×. (A) Control, (B) *Citrus limon* essential oil concentration 0.39% (group A), (C) *Citrus limon* essential oil concentration 0.78% (group B), (D) *Citrus limon* essential oil concentration 1.56% (group C).

Table 1: Colony counts and cell sizes of *Candida albicans* in biofilm.

Groups	Colony count	Cell size (μm)
Control	83.33 ± 12.45[a]	2.28 ± 0.25830[a]
Group A	35.67 ± 6.38[a]	2.12 ± 0.19[a]
Group B	15.67 ± 1.86[a]	1.66 ± 0.13[a]
Group C	11.83 ± 1.17[a]	1.17 ± 0.15[a]
p-value	0.000[a]	0.000[a]
	0.001[a]	0.001[a]
	0.021[a]	0.360[b]

Group A, treatment with *Citrus limon* essential oil 0.39%; group B, treatment with *Citrus limon* essential oil 0.78%; group C, treatment with *Citrus limon* essential oil 1.56%.
Data is presented as mean ± standard deviation. The same superscript letter shows significant differences in each group.
[a] Significant differences using the Gomes–Howell test at $p < 0.05$.
[b] Significant differences using the Gomes–Howell test $p > 0.00$.

looking fibres. This appearance represents mature biofilm with a solid *C. albicans* colony; the filamentous appearance indicated the formation of *C. albicans* hyphae.

Group A presented with a solid biofilm, dense and separate colonies, resembling a mesh web of rough-looking fibres. Figure 1B shows that there was slight damage to the mature biofilm but confirms the formation of *C. albicans* hyphae.

Group B presented with a rather dense biofilm with many colonies separated by a thin filamentous formation. Figure 1C illustrates degradation of the *C. albicans* biofilm with reduced density and compaction of *C. albicans* colonies.

In group C, there were only a few separated *C. albicans* colonies with less filamentous formations; furthermore, the *C. albicans* colonies appeared to be thinner than those of group A and group B. Figure 1D illustrates the occurrence of biofilm degradation along with *C. albicans* cells undergoing necrosis; there was no new hyphae formation (see Figure 1D).

The *C. albicans* colony counts for the biofilms care given in Table 1. Group C had the lowest number of colonies when compared to the control group ($p = 0.000$), group A ($p = 0.001$) and group B ($p = 0.021$).

Cyto-morphometric analysis of C. albicans

The cyto-morphometry of *C. albicans* formation was examined by SEM at 2000× magnification. Cyto-morphometric analyses in the control group revealed round cells that were dense and tightly colonised with a dense and rough filamentous appearance at the base of the colonies. The *C. albicans* in the biofilm was in good condition with a high number of round and intact cells, dense and compact colonies and hyphae formation (see Figure 2A).

Cyto-morphometric analysis in group A revealed round cells and intact colonies that had started to separate but maintained a filamentous appearance around the base. Figure 2B shows that the *C. albicans* were still in good condition with round, intact cells but also show that damage had begun to appear in the *C. albicans* biofilm, marked by separate colonies.

The cyto-morphometric appearance in group B presented with flat, oval-shaped cells; the colonies were separated and there was a clear lack of structure. The filaments were less formed and were smooth in appearance. Figure 2C shows damage in the biofilm and that the damage incurred by *C. albicans* was characterised by cyto-morphometric changes in cell shape.

In group C, the cyto-morphometric of *C. albicans* revealed cells that were neither intact nor colonised with fewer filaments around them; the cells also appeared smooth. Figure 2C shows damage to the *C. albicans* and the biofilm marked by changes in cell shape, a disappearance of the biofilm layer and no new cell or hyphae formation.

A comparison of *C. albicans* cell sizes is given in Table 1. The fewest cyto-morphometric changes in *C. albicans* were

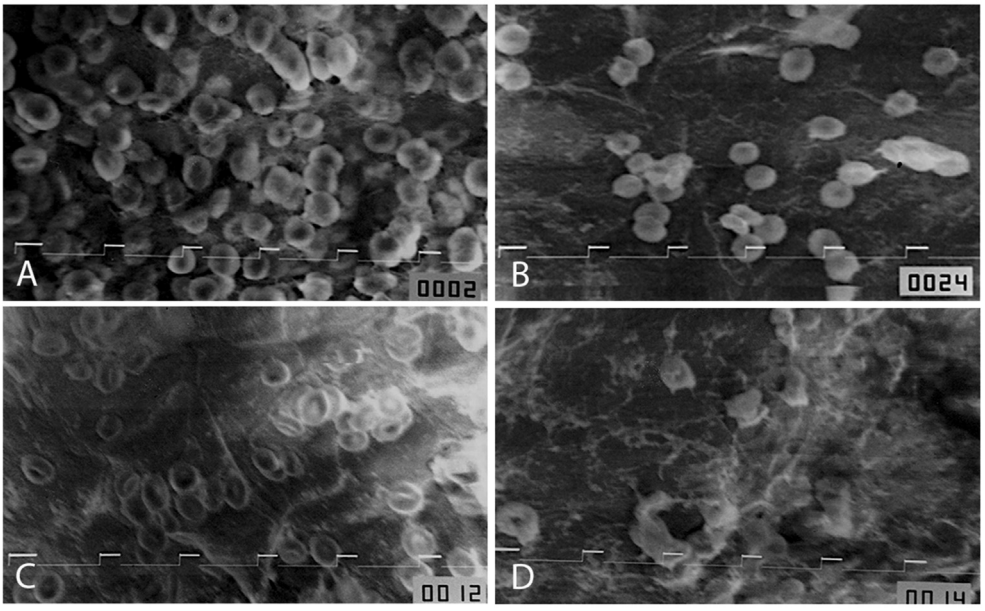

Figure 2: The size of *Candida albicans* cells, as determined by SEM at a magnification of 2000×. (A) Control, (B) *Citrus limon* essential oil concentration 0.39% (group A), (C) *Citrus limon* essential gel concentration 0.78% (group B), (D) *Citrus limon* essential oil concentration 1.56% (group C).

found in group C (1.17 ± 0.15); the cyto-morphometric changes in the control group were significantly more extensive than both group B and group C (both p = 0.000).

Discussion

Research on the effects of essential oil from *C. limon* peel has never been carried out on animals. The concentration of essential oil from *C. limon* peel used in this study was based on previous research stating that the minimum inhibitory concentration on *C. albicans* biofilm was (0.78%) and the fungicidal activity against *C. albicans* was 1.56%.[8] The essential oil from *C. limon* peel used in this study was provided in a gel form to facilitate application because this material immediately melts when it comes into contact with the mucosa where it forms a layer that functions as a covering agent. In addition, the absorption rate of the gel by the mucosa is better than the absorption rates of other semi-solid forms.[11]

This study provides evidence that essential oil from *C. limon* peel not only induces cyto-morphometric changes but also affects biofilm formation. Essential oil from *C. limon* peel contains citral, a substance that is known to inhibit the S-phase in the cell cycle and reduce the DNA replication of *C. albicans*. Citral also inhibits the synthesis of ergosterol, the largest lipid component in the cell membranes of *C. albicans*. If ergosterol synthesis is inhibited, there will be an imbalance in membrane permeability. Cytoplasmic protein will experience denaturation, and the respiratory function and proliferation of *C. albicans* cells will then be disrupted. Disruption to cellular function prevents *C. albicans* from proliferating and inducing cyto-morphometric changes.[12,13] In this study, this effect increased as the concentration of *C. limon* peel increased. Essential oil from *C. limon* peel at a dose of 1.56% provided the maximum effect in terms of the disruption of cyto-morphometric changes, presenting cells that lacked shape, structure and colonisation. Essential oil from *C. limon* peel at a dose of 1.56% also resulted in fewer filaments; in addition, the filaments were smooth in appearance, the biofilm layer had disappeared, and no new hyphae formation was observed.

While inhibiting cyto-morphometric changes, essential oil from *C. limon* peel was also able to inhibit and degrade biofilm formation. Limonene was responsible for this effect as it can damage the structure of the extracellular matrix of *C. albicans* biofilm by damaging the chitin bond in the 1,3-β-glucan composition and inhibit chitinase synthesis in the formation of the extracellular matrix, thus disrupting the *C. albicans* cyto-morphometric changes that lead to hyphae formation.[14] A significant level of chitin is formed during the cyto-morphometric changes that lead to the formation of hyphae. When adhesion occurs and initiates hyphae morphogenesis, *C. albicans* requires a supportive environment for cyto-morphometric changes into pathogens. Single yeast develop when the pH is less than 6, and hyphae form when the pH is more than 7.. During hyphae formation, *C. albicans* undergo adaption by increasing the uptake of amino acids, amines, polyamine and glucose. These molecules then break the substrate intracellularly with the help of Dur1,2 urea amidolase and releases the product (ammonia) through the ammonia transport outward (*Ato*) protein. This activity creates alkalinisation to support the cyto-morphometric changes that lead to hyphae formation. Limonene undergoes oxygenation metabolism that is dominated by three elements: perillic acid, dihydro-perillic acid and limonene-1,2-diol. These elements can neutralise the pH of the mucosa. Therefore, this does not support the adhesion of *C. albicans*. This activity is inhibited by limonene metabolite elements by neutralising alkalinisation of pH and decreasing Dur1,2 urea amidolase activity through oxygenase activity. If there is no supporting extracellular environment, chitin synthesis is inhibited and cyto-morphometric changes of the hyphae fails.[15,16] The inhibition and degradation of biofilm formation was induced by essential oil from *C. limon* peel at a dose of 1.56%, as evidenced by the formation of fewer colonies with thin filamentous formations. This finding was confirmed by the observation of biofilm degradation by *C. albicans* cells undergoing necrosis and no new hyphae formation.

This study provided evidence relating to the essential oils derived from *C. limon* peel. *C. limon* inhibits *C. albicans* and is known to inhibit *C. glabrata, Candida krusei, C. orthopsilosis, C. parapsilosis* and *C. tropicalis*.[17–19]

Although, we demonstrated that essential oil from *C. limon* peel has an effect on *C. albicans* biofilm formation and degradation, there were some limitations to this study that need to be considered. First, this study was preliminary; we still need to prove the effectiveness of *C. limon* peel on *C. albicans* before it can be compared with the currently available anti-fungal drugs. Second, no specific active ingredient has been identified in the essential oil from *C. limon* peel that causes the degradation of *C. albicans* biofilm. However, our findings can be used as a basis for developing further research on the antifungal potential of essential oil from *C. limon* peel and its phytocomponents against *C. albicans*.

This study confirmed that the effectiveness of essential oil from *C. limon* peel on cyto-morphometric changes and biofilm formation is influenced by its concentration. Essential oil from *C. limon* peel at a dose of 1.56% showed the strongest ability to inhibit cyto-morphometric changes and biofilm formation. This finding highlights the potential of essential oil from *C. limon* peel as an antifungal candidate for the treatment of candidiasis. The integration of essential oil from *C. limon* peel into other drug delivery systems can be adapted to the mechanisms of candidiasis treatment as required.

Conclusion

The effect of essential oil from *C. limon* peel on cyto-morphometric changes and biofilm formation is influenced by its concentration. Essential oil from *C. limon* peel at a dose of 1.56% showed the strongest ability to inhibit cyto-morphometric changes and biofilm formation. Based on our findings, essential oil of *C. limon* can eradicate *C. albicans* biofilm and has potential as an antifungal agent for the treatment of oral candidiasis.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

This study was approved have and supervised by the Research Ethical Clearance Commission, Universitas Airlangga Faculty of Dental Medicine (No. 063/HRECC.-FODM/V/2017).

Authors contributions

DR: conceptualisation, methodology, writing and review and editing; RT: investigation, data curation, formal analysis and writing (original draft); DSE: conceptualisation and supervision; AEP: conceptualisation and supervision. All authors critically reviewed and approved the final draft and is responsible for the content and similarity index of the manuscript.

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