

Antifungal effect of *Citrus reticulata* essential oil and extracts on *Candida* species

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Abstract

Objective: This in vitro study evaluated the antifungal activity of *Citrus reticulata* (tangerine) peel essential oil and extracts against various *Candida* species.

Methods: The essential oil and aqueous and alcoholic extracts of *Citrus reticulata* were prepared at 50% and 90% concentrations. *Candida albicans*, *Candida glabrata*, and *Candida krusei* were cultured on Sabouraud dextrose agar with turbidity standardized to 0.5 McFarland. Growth inhibition zones were measured and compared to 0.2% chlorhexidine (positive control) and normal saline (negative control). The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the 90% essential oil were also determined against the microorganisms.

Results: The 50% essential oil and both 50% and 90% concentrations of aqueous and alcoholic extracts showed no antifungal activity. The 90% essential oil produced inhibition zones of 6.5 ± 0.5 mm for *C. albicans*, 10.5 ± 0.5 mm for *C. glabrata*, and 10.0 ± 1.0 mm for *C. krusei*, all significantly smaller than those of chlorhexidine (17.0 ± 0.2 mm, 15.0 ± 0.3 mm, and 17.0 ± 0.2 mm, respectively; $P < 0.05$). MIC values were 1/8 for *C. albicans*, 1/32 for *C. glabrata*, and 1/4 for *C. krusei* (based on 90% concentration). The essential oil was fungicidal only against *C. albicans* (MFC: 1/2 concentration) and fungistatic against the other two species.

Conclusions: The 90% *Citrus reticulata* essential oil effectively formed inhibition zones against *C. albicans*, *C. glabrata*, and *C. krusei*, although to a lower extent than chlorhexidine. The 90% *Citrus reticulata* essential oil had a fungicidal effect only on *C. albicans*.

Keywords: Antifungal agents, *Citrus reticulata*, *Candida albicans*, *Candida glabrata*, *Candida krusei*, Oral candidiasis

Introduction

Candida species are common members of the human mycobiome but can become opportunistic pathogens, particularly in immunocompromised individuals. The most important cause of oral candidiasis is *Candida albicans*, which naturally resides in the gastrointestinal tract and mucosal surfaces of the body (1). Even a small number of *C. albicans* can cause thrush in infants (1).

Among non-*albicans* *Candida* species, *Candida glabrata* and *Candida krusei* are notable for their involvement in oral candidiasis, distinct mechanisms of pathogenicity, and resistance to antifungal agents.

C. glabrata is a significant cause of oral candidiasis, particularly in immunocompromised individuals. It is known for its ability to colonize and infect mucosal surfaces, often leading to oropharyngeal candidiasis (2). Unlike *C. albicans*, *C. glabrata* can survive within macrophages without eliciting strong immune responses (3). Its ability to adhere to epithelial cells and form biofilms plays a key role in its persistence in the oral cavity (4).

C. krusei is another non-*albicans* *Candida* species implicated in oral candidiasis, particularly in critically ill and immunocompromised patients. It is inherently resistant to fluconazole, which enhances its ability to colonize and infect oral tissues (5, 6). It produces hydrolytic enzymes, such as phospholipases and proteinases, which facilitate host tissue invasion (6).

Nystatin is the first choice for treating oral candidiasis and is available as a 100,000-unit suspension in the Iranian market. Polyene antifungals, such as nystatin, bind to fungal sterols in the cell membrane, alter membrane permeability, and ultimately cause leakage of intracellular components. Moreover, these drugs impair fungal adhesion and inhibit the production of

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sterols, which are essential components of *Candida* cell membranes (7). Polyene antifungals have minimal gastrointestinal absorption and are generally non-toxic and non-allergenic, making them well-tolerated even during prolonged administration (8). However, high doses of polyene antifungals can cause nausea and vomiting (8). Additionally, their bitter taste may lead to low patient compliance over time (8, 9).

Chlorhexidine is primarily an antibacterial agent and also exhibits antifungal properties. It has demonstrated superior inhibitory effects on *C. albicans* compared to the fluorine mouthwash (10). Chlorhexidine is effective against *Candida* infections, sometimes outperforming nystatin, especially against species like *C. krusei* and *C. glabrata* (7). However, its long-term use is limited by several significant shortcomings such as cosmetic concerns and adverse reactions (7).

Studies have identified herbal alternatives with antifungal properties that are comparable to those of commonly used antifungal agents such as nystatin and chlorhexidine. These herbal agents may enhance patient compliance with prescribed therapy. Essential oils are strong-smelling liquids extracted from plants. They are made from parts like flowers, leaves, or peels. These hydrophobic liquids have the smell of the plant they come from because they contain special natural chemicals. They are frequently added to foods for flavoring and are also utilized in the pharmaceutical industry to mask the unpleasant taste of medications (11). Essential oils are typically obtained through distillation or cold pressing.

Tangerine (*Citrus reticulata*) contains a high proportion of terpenes, which can make up over 90% of its essential oil. Terpenes are a diverse group of natural compounds with the general formula $(C_5H_8)_n$. They are widely used in medicine, food, cosmetics, and perfumes due to their biological activities. Some terpenes have demonstrated antifungal, antibacterial, anti-inflammatory, and calming effects. The peel of *Citrus reticulata* is particularly rich in specific terpenes such as S-limonene, α -pinene, α -myrcene, and cis-terpinene (12, 13), which have shown strong antioxidant, antibacterial, and anticancer properties (14). In addition, polymethoxyflavones derived from *Citrus reticulata* peel have exhibited antimicrobial effects by disrupting fungal cell membrane integrity and reducing chitin production, a key component of the fungal cell wall (15).

Several studies have investigated the antibacterial activity of *Citrus reticulata* against pathogens like *Escherichia coli*, *Pseudomonas aeruginosa*,

Staphylococcus aureus, and *Salmonella enterica* (14, 16). However, a few studies have evaluated the antifungal effects of *Citrus reticulata* on fungal species, particularly *C. glabrata*, and *C. krusei*. Therefore, the present study aimed to assess the antifungal activity of *Citrus reticulata* essential oil, aqueous extract, and alcoholic extract at two concentrations (50% and 90%) against *C. albicans*, *C. glabrata*, and *C. krusei*.

Materials and methods

The protocol of the present in-vitro study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.DRC.REC.1398.070).

Study design

In this study, three forms of *Citrus reticulata* were tested:

- Essential oil, which is a concentrated, volatile oil extracted from the plant peel using distillation. It contains aromatic compounds like terpenes.
- Aqueous extract, made by soaking or boiling plant material in water to extract water-soluble compounds.
- Alcoholic extract, prepared by soaking the plant material in alcohol (usually ethanol) to extract alcohol-soluble compounds, including many active plant chemicals.

Each preparation was tested at two concentrations (50% and 90%) to assess its antifungal activity against *Candida albicans*, *Candida glabrata*, and *Candida krusei*.

Preparation of *Citrus reticulata* essential oil

The peels of *Citrus reticulata* were dried under laboratory conditions at 24 °C with ventilation for one week, then ground into a fine powder using an electric grinder. Forty grams of the powdered peel were mixed with 650 ml of distilled water and subjected to hydro-distillation in a Clevenger apparatus (Aeman Lab, Tehran, Iran) at 100 °C for four hours. The extracted essential oil was dehydrated using sodium sulfate and stored in a 2 ml glass vial with an aluminum cap at 4 °C until use (17).

Due to the low solubility of *Citrus reticulata* essential oil in water, a 3% Tween 80 surfactant (Iranian Institute of Research & Development in Chemical Industries, Karaj, Iran) was used to aid its dispersion in the agar well diffusion method. Therefore, the essential oil was diluted in 3% Tween 80 to obtain final concentrations of 50% and 90%.

Preparation of aqueous and alcoholic extracts of Citrus reticulata

To prepare the aqueous extract, powdered *Citrus reticulata* peel was mixed with distilled water at a 1:6 ratio (w/v) and soaked for several hours to allow water-soluble compounds to dissolve. The mixture was then filtered using a Büchner funnel and specialized filters. The filtrate was concentrated using a rotary evaporator for six hours.

To prepare the alcoholic extract, the peel powder was mixed with ethanol at the same 1:6 ratio (w/v) and soaked under similar conditions. After filtration using a Büchner funnel and vacuum pump, the ethanol was removed using a rotary evaporator for six hours (17).

The aqueous and alcoholic extracts were prepared without surfactant at concentrations of 50% and 90%.

Fungal suspension preparation

Fungal strains were obtained from the Iranian Industrial Microorganism Collection Center (Tehran, Iran). *Candida albicans* (PTCC 5027) was cultured on Sabouraud Dextrose (SabDex) medium (Merck, Germany) at 37 °C for 24 hours, while *C. glabrata* (PTCC 5295) and *C. krusei* (PTCC 5297) were cultured at 30 °C on the same medium. To prepare uniform fungal suspensions with standardized concentrations, the turbidity was adjusted to a 0.5 McFarland standard, equivalent to approximately 1.5×10^8 colony-forming units (CFU)/ml.

Inhibition zone measurement

The agar well diffusion method was used to evaluate the antimicrobial activity of *Citrus reticulata*. All procedures were carried out under sterile conditions in a Class II laminar flow hood.

A 100 µL aliquot of fungal suspension, adjusted to 0.5 McFarland turbidity, was evenly spread over the surface of brain-heart infusion (BHI) agar plates. Five wells were then created in each plate using a sterile Pasteur pipette. Three wells were filled with 50 µL of 0.2% chlorhexidine (positive control), normal saline (negative control), and 3% Tween 80 (vehicle control). The remaining two wells in each plate were filled with 50 µL of *Citrus reticulata* samples. Different sets of plates were used to separately assess the essential oil, aqueous extract, and alcoholic extract at both 50% and 90% concentrations.

The inoculated plates were incubated at 37°C for 24 hours. After incubation, the diameter of the inhibition zones was measured in millimeters using a ruler. All

experiments, including each test substance and control, were performed in triplicate for each *Candida* strain to ensure accuracy and reproducibility.

Among the tested substances, only the 90% concentration of *Citrus reticulata* essential oil and 0.2% chlorhexidine produced measurable inhibition zones. Therefore, the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined only for the 90% essential oil.

Determination of minimum inhibitory concentration (MIC)

The MIC of *Citrus reticulata* essential oil was determined for *C. albicans*, *C. glabrata*, and *C. krusei* using the standardized broth microdilution method in a 96-well ELISA microplate. Each test was performed in triplicate. Three rows were designated for essential oil dilutions (one row per replicate), one row for the positive control, and one for the negative control. Each row contained 12 wells.

To begin, 100 µL of brain–heart infusion (BHI) broth was added to all wells. In the first well of each test row, 100 µL of the initial essential oil dilution was added. A two-fold serial dilution was then performed by transferring 100 µL from one well to the next, up to the 12th well. From the last well, 100 µL was removed and discarded, resulting in a range of decreasing essential oil concentrations.

Subsequently, 10 µL of fungal suspension (adjusted to 0.5 McFarland standard) was added to all wells, except those in the negative control. The microplates were incubated at the appropriate temperature for each strain for 24 hours.

After incubation, 20 µL of 0.01% resazurin solution (Sigma-Aldrich, Missouri, USA) was added to each well. Resazurin is a blue dye that acts as an indicator of cellular activity (metabolic activity). If the fungi are alive and active, they reduce resazurin (a blue compound) to resorufin, which is pink. If the fungi are dead or inhibited, no reduction occurs, so the solution remains blue. After the addition of resazurin, the plates were incubated for an additional 2 hours.

The MIC was defined as the lowest concentration of essential oil at which no color change from blue to pink occurred, indicating inhibition of fungal growth.

Determination of Minimum Fungicidal Concentration (MFC)

To determine the MFC of *Citrus reticulata* essential oil for each *Candida* strain, samples from the wells were

Table 1. Mean $\pm$ standard deviation (mm) of the inhibition zone of *Citrus reticulata* essential oil and extracts, 0.2% chlorhexidine, normal saline and Tween 80 on *Candida* species

Fungal species	Essential oil		Aqueous extract		Alcoholic extract		0.2% Chlorhexidine	Normal saline	Tween 80 surfactant	P-value
	50%	90% Mean $\pm$ SD	50%	90%	50%	90%	Mean $\pm$ SD			
<i>Candida albicans</i>	-	6.5 $\pm$ 0.5 ^a	-	-	-	-	17.0 $\pm$ 0.2	-	-	0.001
<i>Candida glabrata</i>	-	10.5 $\pm$ 0.5 ^b	-	-	-	-	17.0 $\pm$ 0.3	-	-	0.004
<i>Candida krusei</i>	-	10.0 $\pm$ 1.0 ^b	-	-	-	-	17.0 $\pm$ 0.1	-	-	0.007
P-value		0.001	-	-	-	-	0.84	-	-	

selected as follows: all blue-colored wells before the MIC well, the MIC well itself, and the first pink-colored well following the MIC (indicating visible fungal growth). Aliquots from each of these wells were streaked onto BHI agar plates. This procedure was performed in triplicate for each sample. Plates were incubated at the appropriate temperature for 24 hours.

The MFC was defined as the lowest concentration of essential oil at which no visible fungal growth appeared on BHI agar, indicating fungicidal activity (18).

Both MIC and MFC values were expressed as fractions of the initial essential oil concentration, based on two-fold serial dilutions.

Results

Table 1 presents the average inhibition zone diameters for different concentrations of *Citrus reticulata* essential oil and 0.2% chlorhexidine against various *Candida* species. The essential oil at 50% concentration, as well as both the aqueous and alcoholic extracts at 50% and 90% concentrations, showed no inhibition zones against any of the fungal strains. Therefore, MIC and MFC values were not determined for these groups.

In contrast, the 90% essential oil produced measurable inhibition zones, with mean diameters of 6.5 $\pm$ 0.5 mm for *C. albicans*, 10.5 $\pm$ 0.5 mm for *C. glabrata*, and 10.0 $\pm$ 1.0 mm for *C. krusei*. These values were significantly

smaller than those observed for 0.2% chlorhexidine ($P < 0.05$; Table 1).

Table 2 presents the MIC and MFC values of *Citrus reticulata* essential oil at 90% concentration. The MIC values were 1/8 for *C. albicans*, 1/32 for *C. glabrata*, and 1/4 for *C. krusei*, relative to the initial 90% essential oil concentration.

The MFC for *C. albicans* was 1/2, indicating fungicidal activity at this concentration. However, the essential oil showed no fungicidal effect against *C. glabrata* and *C. krusei*, suggesting that it acted only as a fungistatic agent against these strains.

Discussion

The present study evaluated the antifungal activity of *Citrus reticulata* essential oil and extracts against *C. albicans*, *C. glabrata*, and *C. krusei*. The results showed that neither the 50% nor the 90% concentration of the aqueous and alcoholic extracts, nor the 50% essential oil, exhibited any antifungal effects against the tested *Candida* species. In contrast, the 90% essential oil demonstrated measurable antifungal activity against all three species. However, the inhibition zones produced by the 90% essential oil were significantly smaller than those observed for the 0.2% chlorhexidine solution.

Citrus essential oils are rich in monoterpenes (such as d-limonene and citral) which are key contributors to their antifungal activity. In addition, flavonoids

Table 2. MIC and MFC of *Citrus reticulata* essential oil at a 90% concentration against the *Candida* species

Fungal species	MIC	MFC
<i>Candida albicans</i>	$\frac{1}{8}$	$\frac{1}{2}$
<i>Candida glabrata</i>	$\frac{1}{32}$	-
<i>Candida krusei</i>	$\frac{1}{4}$	-

extracted from *Citrus reticulata* peels have shown strong antifungal effects against various pathogenic fungi, including *Candida* species. These bioactive compounds exert their effects by interfering with fungal cell wall synthesis, disrupting membrane integrity, and modulating fungal cell signaling pathways (18, 19). Wu et al. (15) reported that polymethoxyflavones from *Citrus reticulata* peels inhibited *Aspergillus niger* by increasing cell membrane permeability. Furthermore, these compounds were shown to reduce chitin production, an essential component of the fungal cell wall, in a dose-dependent manner (15). It is believed that the antifungal activity of *Citrus* essential oils may vary considerably across different fungal species (20).

Chlorhexidine is a synthetic antiseptic with broad-spectrum antimicrobial activity and is effective against a wide range of pathogens (21). Its primary mechanism involves binding to microbial cell walls, leading to cell lysis and death (22). This binding is more potent than the mechanisms attributed to *Citrus* essential oils. In addition, chlorhexidine increases intracellular levels of reactive oxygen species (ROS), resulting in oxidative stress and apoptosis in various cell types, including fungi. It also disrupts metal ion homeostasis within cells, further contributing to cellular damage (23).

Despite its effectiveness, chlorhexidine is associated with low patient compliance, often due to unpleasant taste or side effects. In contrast, *Citrus reticulata* essential oil may offer a natural alternative with better patient acceptability. While citrus oils have demonstrated antifungal effects against certain yeast species, they typically exhibit higher MIC values and lower overall potency compared to chlorhexidine (20, 24). The present study also found that the inhibition zones produced by 90% *Citrus reticulata* essential oil were smaller than those of 0.2% chlorhexidine for all three *Candida* species tested.

When MFC values were evaluated, the 90% *Citrus reticulata* essential oil showed a fungicidal effect against *C. albicans* at a concentration of 1/2 of the original 90% stock. However, it demonstrated only fungistatic activity against *C. glabrata* and *C. krusei*.

The present findings are consistent with those of Roos et al. (20) who demonstrated that the essential oil from *Citrus deliciosa* inhibited biofilm formation in *C. albicans*, *C. glabrata*, *Candida parapsilosis*, and *Trichosporon asahii*. Similarly, Hernawan et al. (25) reported that the essential oil from *Citrus limon* peel effectively inhibited *C. albicans*, with a MIC of 80%, while lower concentrations showed no antifungal activity. The findings of this study also imply that the antifungal effect

of *Citrus reticulata* essential oil is dose-dependent, with no activity observed at the 50% concentration. Ayoola et al. (26) found that *Citrus reticulata* essential oil exhibited antimicrobial activity against gram-positive and gram-negative bacteria, as well as *C. albicans*, attributing this effect to active compounds such as d-limonene. Carvalhinho et al. (27) evaluated the susceptibility of various *C. albicans* strains to antifungal drugs and herbal essential oils. They found that all strains were sensitive to amphotericin B, nystatin, and fluconazole. However, sensitivity to essential oils varied, with rosemary showing the highest activity and tangerine (from *Citrus reticulata*) the lowest. The present findings also indicate that *Citrus reticulata* essential oil has limited antifungal activity, particularly against *C. albicans*. However, its fungicidal effect at higher concentrations suggests that it may be considered an adjunctive treatment in combination with standard antifungal agents against *C. albicans*.

In this study, the 90% essential oil of *Citrus reticulata* exhibited a larger inhibition zone and lower minimum inhibitory concentration (MIC) values against *C. glabrata* and *C. krusei* compared to *C. albicans*. Developing new antifungal agents is particularly important for combating *C. glabrata* and *C. krusei*, given the high prevalence of antifungal resistance in *C. glabrata* and the intrinsic resistance of *C. krusei* to commonly used antifungal drugs. It is assumed that early intervention is associated with reduced mortality in cancer patients with fungemia (28, 29). *C. krusei* is also emerging as a significant nosocomial pathogen, especially in immunocompromised individuals and patients with leukemia (30). Therefore, targeted management of these two pathogenic fungi is critical due to the serious clinical challenges they pose.

Few studies have investigated the antifungal effects of *Citrus* species against *C. glabrata* and *C. krusei*. Ruiz-Pérez et al. (24) evaluated essential oils from *Citrus sinensis* and *Citrus latifolia* and reported moderate antifungal activity against *C. glabrata*. Additionally, some studies have highlighted the antifungal potential of active components such as citral and linalool against *C. krusei* (31). The combination of these compounds with fluconazole demonstrated synergistic effects in certain isolates (31). The present study also showed moderate antifungal activity of 90% *Citrus reticulata* essential oil against both *C. glabrata* and *C. krusei*.

This study had several limitations. The cytotoxic effects of *Citrus reticulata* essential oil on human mucosal cells were not evaluated and require further investigation. In vivo studies are necessary to confirm

the present findings, as dilution by saliva may reduce the antifungal efficacy of *Citrus reticulata* essential oil. Future research should aim to identify and purify the active components of *Citrus reticulata* essential oil and examine their potential synergistic effects in combination with existing antifungal agents.

Conclusions

Within the limitations of the present study, the following conclusions can be drawn:

- The 90% *Citrus reticulata* essential oil demonstrated antifungal activity by producing inhibition zones against *Candida albicans*, *Candida glabrata*, and *Candida krusei*, although its effect was less than that of 0.2% chlorhexidine.
- The essential oil exhibited lower minimum inhibitory concentration (MIC) values against *C. glabrata* and *C. krusei* than against *C. albicans*, indicating greater sensitivity in these species.
- At half the 90% concentration, the essential oil showed a fungicidal effect only against *C. albicans*, whereas it exerted a fungistatic effect against *C. glabrata* and *C. krusei*.
- The aqueous and alcoholic extracts, as well as the 50% concentration of the essential oil, were ineffective against all tested *Candida* species.

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Conflicts of Interest

The authors declare no conflict of interest.

Ethical Considerations

The protocol of the present in-vitro study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.DRC.REC.1398.070).

Author Contributions

Z.N. and Z.M.S. contributed to the study design and data interpretation; Z.Y. and J.B.T. contributed to the conceptualization of the study, data analysis, and manuscript editing; M.Ha., M.Ho. and H.M. contributed to data collection and manuscript preparation. All authors approved the final manuscript.

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