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Evaluation of the Essential Oil of *Citrus paradisi* as an Alternative Treatment against *Candida albicans*

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Abstract

Introduction: The World Health Organization establishes that 80% of the world's population uses traditional medicine for their primary care, because they contain compounds responsible for their properties. Objective: To evaluate the antifungal effect of the essential oil of *Citrus paradisi* against *C. albicans* and the cytotoxic effect in three cell lines *in vitro*. Methods: The phytochemical characterization of the oil was carried out by chemical methods and Gas chromatography (GC-MS) and the antifungal effect against *C. albicans* (ATCC 90029) was evaluated by the Kirby-Bauer method, which evaluated concentrations of 0.75 - 20 µg/mL and compared with nystatin 100,000 Ul/mL as a positive control. The percentage of the relative inhibitory effect was calculated. The minimum inhibitory concentration (MIC) was determined at 24 hours. Moreover, the cytotoxic effect on *C. albicans* and cell lines was determined by the colorimetric MTT tetrazolium assay. Finally, the antifungal effect against *Candida* strains isolated from clinical samples was evaluated at a concentration of 20 µg/mL. Results: The essential oil showed an antifungal effect with a percentage of inhibition of 123%. The MIC was 2.5 µg/mL, and the cytotoxicity index was 5.44 µg/mL for *C. albicans*. The IC_{50} values were 21.060, 9.482 and 4.176 µg/mL for Vero E6, J774A.1 and MDBK cells respectively. Conclusion: These results show the use of *C. paradisi* essential oil as an alternative treatment in oral antifungal therapy, it is beneficial due to its antifungal effect and its low toxicity on cell cultures.

Keywords

Citrus, *Candida*, Candidiasis, Essential Oil, Herbal Medicine

1. Introduction

Candida albicans was first described more than 150 years ago [1]. It is known as a commensal microorganism and the most common fungal pathogen in humans [2] and is a member of the healthy microbiota that colonizes the gastrointestinal tract, reproductive tract, oral cavity and skin [3]. The different types of cells of *C. albicans* are yeasts, which are cells of round to oval morphology with a size of 2 to 4 microns; the hyphae, which are thin cells with a tubular shape that measure between 3 to 5 microns; and finally, the pseudohyphae, which are ellipsoidal shape [2]. The primary fungal disease manifested in children is oral candidiasis caused by *C. albicans*. Some reports indicate the presence of the disease in up to 95% of immunosuppressed patients, such as terminal patients, HIV positive patients, patients with leukemia, anemia, and those who receive head and neck radiotherapy [1] [4]. Studies have shown that 65% of patients who use dentures or orthodontic appliances [5] [6] and between 46% to 65% in susceptible children such as premature babies have the disease due to the lack of hygiene in bottles and pacifiers. Other causes of *C. albicans* infection include drug therapy of antibiotics, corticosteroids and immunosuppressant's long-term, malnutrition [1] [7] [8], xerostomia, poor oral hygiene and mouth breathing [9]. The use of plants for medicinal purposes is known as phytotherapy, and worldwide, there are thousands of plants that synthesize active substances that act as defense mechanisms against microorganisms [10] [11]. For its part, *Citrus paradisi* (grapefruit) belongs to the family *Rutaceae* and is located in tropical regions such as Asia, China, the Philippines, and New Guinea [12] [13]. The fruit measures approximately 15 cm in diameter and is a pale yellow color protected by a tough outer covering called the cortex, which is formed by an epicarp corresponding to the epidermis and hypodermis and a mesocarp and an endocarp surrounded by a membrane (septas) containing the seeds.

The essential oil of *C. paradisi* is a lipophilic liquid volatile substance with aromatic properties extracted from the fruit's rind [13] [14]. It contains active substances, such as terpenes, hydrocarbons, sesquiterpenes, alcohols, aldehydes and esters [12] [14], and it acts as an antibacterial, antiviral, antifungal, insecticide, antioxidant, astringent, cell regenerator and detoxifier [13] [14]. The cytotoxic evaluation of natural products in cell cultures allows for the determination of cell viability, that is, changes in morphology, growth alteration, and death or cell disintegration [15]. Taking into account the biological properties described, the main objective of this study was to evaluate the antifungal and cytotoxic action of the essential oil of *C. paradisi* against *C. albicans* ATCC and cell cultures.

2. Materials and Methods

2.1. Essential Oil

The essential oil of *C. paradisi* was obtained from the grapefruit peel by cold pressing extraction method from the company Frutech International Corpora-

tion de México S.A. by C.V. The fresh air-dried *C. paradisi* peels were subjected to water-distillation boiling (>100˚C) for 3 h by using a Clevenger apparatus. The obtained essential oil was dried over anhydrous sodium sulfate and after filtration stored at 4˚C inside amber containers [16].

2.2. Basic Phytochemical Analysis

Phytochemical screening of the essential oil by conventional chemical tests was performed to determine the main chemical groups present in the essential oil using the following tests: Liebermann Burchard (sterols and triterpenes), Shinoda (flavonoids, flavanones and flavanonols), Baljet (sesquiterpene lactones), sulfuric acid (quinones), ferric chloride (tannins), Molisch (carbohydrates), sodium hydroxide (coumarins) and Dragendorff (alkaloids) [17] [18] [19]. Lastly, GC-MS chromatography was performed on a sample of the essential oil obtained. Gas chromatography (GC) was conducted in a HP-5 MS (30 m × 0.25 mm to 0.25 m) capillary column. The GC conditions were as follows: injection temperature, 250˚C; and oven temperature controlled at 70˚C for 1 min with a heating rate of 10˚C/min, at 200˚C for 2 min, with a heating rate of 10˚C/min, and at 300˚C for 5 min. The following parameters were used for the EM 5973N analysis: ion source, EI; electronic energy, 70 e; quadrupole temperature, 150˚C; interface temperature, 230˚C; and m/z, 30 - 400 amu [20].

2.3. *Candida albicans* ATCC

For the activation of the strain of *C. albicans* (ATCC 90029), 100 µL of *C. albicans* was inoculated in 1 mL of Sabouraud broth and incubated at 37˚C for 24 hours. The morphological identification of the strains was performed using the Gram technique [21]. The number of colony forming units per milliliter (CFU) was carried out using the turbidity technique equivalent to 0.5 of the McFarland scale (1×10^6 CFU/mL).

2.4. Antifungal Effect of *Citrus paradisi* and Minimum Inhibitory Concentration

Sowing of *C. albicans* on Sabouraud agar was performed by dissemination until confluent growth was obtained. Then, disc diffusion was performed (Kirby-Bauer method) using serial dilutions of the essential oil of *C. paradisi* at concentrations from 0.75 to 20 µg/mL, compared with nystatin 100,000 Ul/mL as a positive control and distilled water as a negative control, and incubated at 37˚C for 24 hours [13] [22] [23]. After incubation, the measurement of the inhibition zones was performed, and the mean and standard deviation were calculated, as well as the percentage of the relative inhibitory effect is interpreted as a high antifungal activity when its relative inhibition percentage is >70%, intermediate between 50% - 70% and low when it is <50% [24] [25], using Equation (1):

$$\%\ \text{inhibition} = \frac{\bar{x}\ \text{halo diameter of oil}}{\bar{x}\ \text{halo diameter of positive control}} \times 100 \quad (1)$$

2.5. Index of Cytotoxicity of *Citrus paradisi* against *Candida albicans*

One hundred microliters of *C. albicans* equivalent to 1×10^6 CFU/mL was placed in a sterile 96-well microplate with negative controls (Sabouraud medium), positive controls (nystatin 100,000 Ul/mL) and serial concentrations of the essential oil of *C. paradisi* from 0.75 to 20 µg/mL. The cells were incubated for 24 hours at 37˚C. Subsequently, the spores were concentrated by centrifugation at 1700 rpm for three minutes. Then, the supernatant was removed, and 100 µL of the MTT reagent was added and incubated for 2 hours under the conditions described above. Finally, the supernatant was removed, and 100 µL of dimethylsulfoxide (DMSO) was added and incubated for 20 minutes. The absorbance was quantified in an Epoch spectrophotometer (BioTek) at 540 nm with the Gen5 program [26] [27]. All experiments were performed in triplicate with three independent repeats.

2.6. Index of Cytotoxicity of the Essential Oil on Cell Cultures

To evaluate the cytotoxic capacity of the essential oil of *C. paradisi*, three cell lines of the different lineages were used: phagocytic cells (mouse macrophages J774. A1) and kidney cells of monkeys and cows (Vero E6 and MDBK respectively). The cell strains were incubated in CRPMI culture medium with 10% fetal bovine serum at 37˚C, with 5% CO_2 and a relative humidity and confluence of the cell monolayer of 80%. Subsequently, 5×10^4 cells/well from each of the cell lines were placed in 100 µl of CRPMI medium, and the different serial concentrations of the essential oil of *C. paradisi* were added, ranging from 5 to 50 µg/mL, and the medium was allowed to cool to a minimum temperature. The final volume of 200 µL was incubated for 24 hours. A positive control (nystatin 100,000 Ul/mL) and a negative control (cells without treatment) were also included. Then, the medium was removed, and 100 µL of the MTT reagent was added and incubated for 3 hours. Finally, the supernatant was removed, and 180 µL of DMSO + 20 µL of glycine buffer was added and incubated for 30 minutes, and the absorbances were quantified in an Epoch spectrophotometer (BioTek) at 540 nm with the Gen5 program [28] [29]. All experiments were performed in triplicate with three independent repeats. Percent cytotoxicity calculated according to Equation (2):

$$\%\ \text{cytotoxicity} = 100 - \left[\left(\frac{\text{Abs of the sample}}{\text{Abs control}}\right)\right] \times 100 \tag{2}$$

In addition, the IC_{50} is defined according to each of the cell lines and *C. albicans*, according to Equation (3):

$$Y = \text{Min} + \frac{\text{Max} - \text{Min}}{1 + \left(\frac{x}{IC_{50}}\right)\text{Hill Coefficient}} \tag{3}$$

2.7. Antifungal Effect of *Citrus paradisi* against *Candida* Strains Isolated from Patients

The essential oil of *Citrus paradisi* was evaluated with *Candida* strains isolated from patients with oral candidiasis, it was provided by the Laboratorio de Microbiología, Facultad de Odontología, Universidad Autónoma de Nuevo León. The *Candida albicans* and *Candida krusei* strains were taken from the clinical isolates from *Candida,* seeding was carried out by dissemination on Saboraud agar, then disk diffusion was performed (Kirby-Bauer method) using essential oil of *Citrus paradisi* for a concentration of 20 µg/ml, compared to the positive control of nystatin 100,000 Ul/ml, and distilled water as a negative control, then were incubated at 37˚C for 24 hours. The procedure was performed in triplicate [13] [22] [23].

2.8. Statistical Analysis

All results are expressed as the mean ± standard deviation. To establish the differences between the essential oil of *C. paradisi* and the positive control (nystatin 100,000 Ul/mL), the data were analyzed in the IBM SPSS Statistics 24 program. The t-test was performed to evaluate the statistical significance, with a value of $p \leq 0.05$ considered significant with a confidence level of 95% [29].

3. Results and Discussion

The *Citrus paradisi* essential oil was positive for sterols, triterpenes, sesquiterpenectones, quinones and coumarins (**Table 1**). The principally identified components were limonene (94.427%[a]), myrcene (1.852%[a]), *α*-pinene (0.544%[a]), sabinene (0.340%[a]), decanal (0.211%[a]), citral (0.104%[a]), linalool (0.085%[a]), *β*-pinene (0.065%[a]), *γ*-terpinene (0.049%[a]), 1-terponen-4-ol (0.012%[a]) y nootkatone (0.009%[a]), to which the antifungal activity of the *Citrus paradisi* essential oil were attributed (**Table 2**) (**Figure 1**). The presence of compounds in the essential oil of *Citrus paradisi* has been reported, identifying monoterpenic hydrocarbons as limonene (94.8%) was the most abundant, *α*-terpinene (1.8%), *α*-pinene (0.5%) and sabinene (0.4%),*β*-pinene, *γ*-terpinene and myrcene (<0.05%), sesquiterpenic hydrocarbons (0.3%), aliphatic aldehydes such as octanal (0.4%), decanal (0.3%), dodecanal (0.1%) and tetradecenal, alcohols such as linalol, (*E*)-p-menthadien-1-ol and *α*-terpineol (0.1%), esters (0.4%) and nootkatone (0.1%) [30]; the results of this study agree with the major compounds identified in the oil. Other authors have referred to the presence of flavonoids, amino acids, phenolic and vitamins, alkaloids, flavonoids, steroids, terpenoids, saponins, cardiac glycosides and sugars in *C. paradisi* oil [31] [32]. Terpenes, *α*-pinene, vinyl sesquiterpene [16], sabinene, ocimene, linalool, *β*-pinene, limonene, *β*-myrcene, and terpinen-4-ol have also been reported in the essential oil by gas chromatogram [20]. In this study, the presence of previously reported compounds was also identified in the essential oil of *Citrus paradisi* by chemical tests and GC/MS, highlighting limonene and myrcene; therefore, it could be suggested that these agents are responsible for its biological activity and their

application against *Candida albicans*.

The essential oil of *C. paradisi* presented the best antifungal activity at a concentration of 20 μg/mL with average inhibition halos of 25.6 mm, followed by the concentration of the essential oil at 10 μg/mL, which presented halos of average inhibition of 14.6 mm, compared with the positive control (nystatin), which showed average inhibition halos of 20.8 mm ($p \leq 0.05$). The percentage of relative inhibition of the essential oil was considered high at the concentrations of 20 μg/mL (123%) and 10 μg/mL (70.1%), intermediate at 5 μg/mL (51.9%) and low at 2.5 μg/mL (41.3%) (Table 3). In this study, the percentage of inhibition was dependent on the concentration of the essential oil, similar to those reported by other authors who indicated halos from 8 to 12.63 mm [32] [33], on the other hand, studies have reported the antifungal activity of *C. paradisi* essential oil to reduce the growth of *Penicillium chrysogenum* and *Penicillium verrucosum* [34], however, few studies have examined the antifungal activity of essential oils of citrus fruits.

Table 1. Partial phytochemical characterization of *Citrus paradisi*.

Chemical methods	Chemical groups	Result
Liebermann Burchard	Sterols, triterpenes	+
Shinoda	Flavonoids	−
Baljet	Sesquiterpenectones	+
Sulfuric acid	Quinones	+
Ferric chloride	Tannins	−
Molisch	Carbohydrates	−
Sodium hydroxide	Coumarins	+
Dragendorff	Alkaloids	−

−: negative; +: positive.

Table 2. Compounds obtained in the chromatogram obtained from the GC/MS analysis of the *Citrus paradisi* essential oil.

Compounds	RT	%[a]
Limonene	6.644	94.427
Myrcene	5.123	1.852
α-pinene	3.923	0.544
Sabinene	4.732	0.340
Decanal	12.815	0.211
Citral	15.145	0.104
Linalool	8.850	0.085
β-pinene	4.836	0.065
γ-terpinene	7.384	0.049
1-terpinen-4-ol	11.709	0.012
Nootkatone	30.911	0.009

Retention time (RT), percentage of area (% a).

Table 3. Antifungal activity of *C. paradisi* against *C. albicans* ATCC.

Inhibitor effect (mm)	Concentrations of essential oil (μg/mL)							
	20	10	5	2.5	1.25	0.75	C(+)	C(−)
R1	26	16	12	10	0	0	22	0
R2	27	14	12	8	0	0	20	0
R3	27	16	10	8	0	0	21	0
R4	24	14	10	9	0	0	20	0
R5	25	14	10	8	0	0	21	0
R6	25	14	11	9	0	0	21	0
$\bar{x}$ ± s	25.7 ± 1.21	14.7 ± 1.03	10.8 ± 0.98	8.7 ± 0.82	0	0	20.8 ± 0.75	0
%	123	70.1	51.9	41.3	0	0	-	-

R: repetitions; $\bar{x}$: mean of the sample; s: standard deviation; %: percentage of relative inhibitory effect; C(+): nystatine 100,000 Ul/mL; C(−):distilled water; $p < 0.05$. $F = 811.6$, $df = 6$.

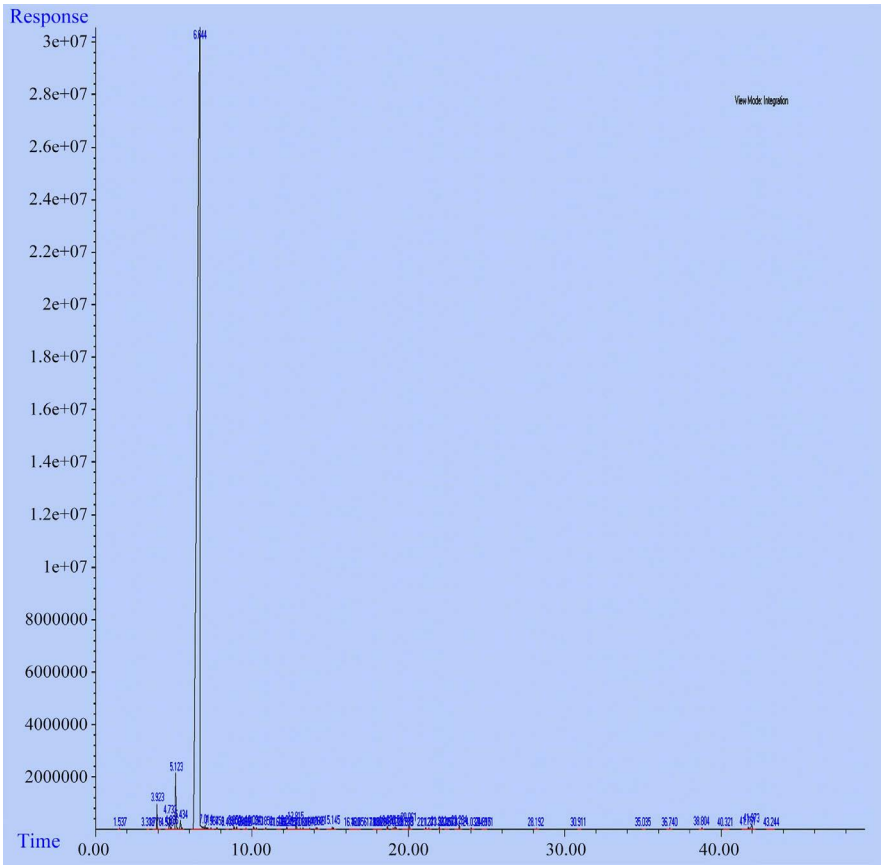

Figure 1. Chromatogram obtained from the GC/MS analysis of the *Citrus paradisi* essential oil.

Concerning the cytotoxic effect of the essential oil of *C. paradisi* on the viability of *C. albicans* evaluated by the MTT assay, a cytotoxicity index (IC_{50}) of 5.44 μg/mL was obtained as the concentration of the essential oil increased, without statistical differences between the concentrations, but with a statistical difference

of $p \leq 0.01$ with respect to the positive cotrol and $p \leq 0.001$ with respect to the vehicle control (**Figure 2**). It is important to note that there are no studies that used a procedure similar to this study. However, some studies mention the use of XTT salts (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide [26] and MTT to evaluate the mitochondrial metabolism of *C. albicans* using the same method in this study [35].

Regarding the cytotoxic effect of the essential oil of *C. paradisi* on different cell cultures by the MTT assay, a cytotoxicity index 50 (IC_{50}) of 21.060 µg/mL was identified for Vero E6 cells, 9.482 µg/mL for J774A.1 cells, and 4.176 µg/mL for MDBK cells (**Figure 3**). This suggests its possible use as a tool in the control of yeast growth on body surfaces with therapeutic doses of 5.44 µg/mL lower than the doses with cytotoxic effect in Vero E6 and J774A.1 cells, which makes it a natural alternative for the control of mycoses. Other studies reinforce these postulates, where they show that the essential oil of *C. paradisi* inhibits the proliferation of leukemia cells through apoptosis mechanisms at a concentration of 250 µg/mL when employing the MTT assay [36]. With a concentration higher than that of this study, the use of the MTT assay has been reported to measure the antitumoral potential of citrus fruits such as *C. maxima, C. lemon* and *C. reticulata* against Dalton's lymphoma ascites cells [37]. It has also been used to measure the growth and viability of lung cancer cells using tangerine peel [38]; both studies support cell viability assays through the use of MTT. It should be mentioned that there are not enough studies about the cytotoxic effects of the essential oil on cell lines; the results from this study increase the knowledge of the therapeutic use of citrus fruits based on their cytotoxic effects, this was due to the fact that a positive viability-concentration relationship is observed in non-phagocytic MDBK and Vero E6 cell strains however, in J774A.1 cells, no differences associated with concentration were observed, which attributed cell viability to apoptotic processes.

The essential oil of *Citrus paradisi* also demonstrated antifungal activity against *Candida* strains isolated from patients with oral candidiasis, at the concentration of 20 µg/ml it had an inhibitory effect against *Candida krusei* with average inhibition of 25.3 mm compared to the positive control which was 20.6 mm, against *Candida albicans* the inhibitory effect was 14.3 mm, unlike the positive control of 13.6 mm. The essential oil demonstrated a high relative inhibition percentage against both strains (**Table 4**), similar to that reported where the antifungal activity of the essential oil of *C. paradisi* was evaluated on *C. albicans* strains isolated from patients with subprosthetic stomatitis with an average inhibition of 12.6 mm [14], likewise, studies have evaluated the minimum fungicidal concentration of *C. paradisi* against *C. albicans* isolated from patients with prosthetic stomatitis, reporting a broad spectrum of fungicidal activity at concentrations of 0.1 - 16 µg/ml [39], however, these results coincide with the studies where it is demonstrated that the essential oil presents an inhibitory effect against clinical isolates of *Candida*.

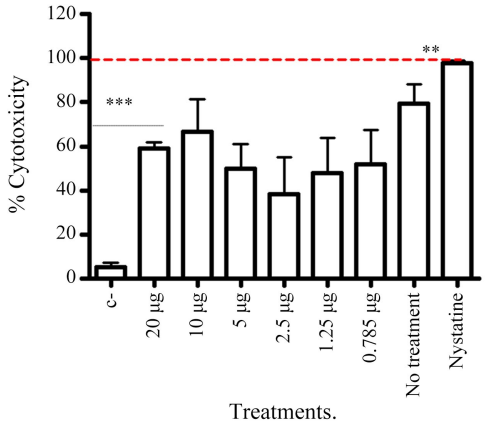

Figure 2. Cytotoxic effect of grapefruit essential oil on the viability *Candida albicans.*

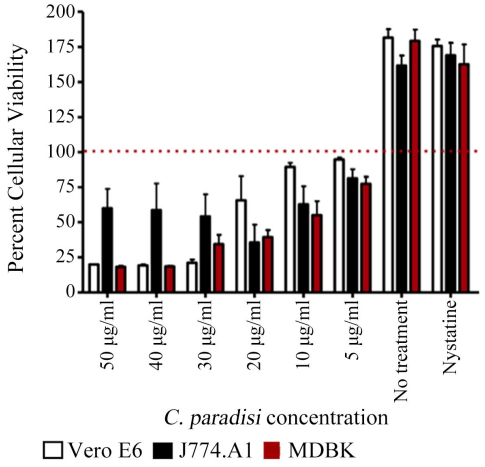

Figure 3. Effect of *C. paradisi* essential oil on cell viability.

Table 4. Antifungal activity of *C. paradisi* against *Candida* strains isolated from patients with oral candidiasis.

Inhibitor effect (mm)	*Candida albicans*		*Candida krusei*		C(−)
	C. paradisi 20 µg/mL	C(+)	*C. paradisi* 20 µg/mL	C(+)	
R1	13	13	24	21	0
R2	15	14	27	21	0
R3	15	14	25	20	0
$\bar{x}$ ± s	14.3 ± 1.15	13.6 ± 0.57	25.3 ± 1.52	20.6 ± 0.57	0
%	105	-	123	-	0
Student's t-test p value	0.183	-	0.034	-	-

R: repetitions; $\bar{x}$: mean of the sample; s: standard deviation; %: percentage of relative inhibitory effect; C(+): nystatine 100,000 Ul/mL; C(−): distilled water; $p \leq 0.05$.

4. Conclusion

The essential oil of *C. paradisi* showed an inhibitory effect against *C. albicans*

and samples from clinical isolates of oral candidiasis at different concentrations evaluated in the *in vitro* tests and was associated with the activity of its chemical groups identified as sterols, triterpenes, coumarins, quinones, and sesquiterpenectones, without toxic effect on Vero E6 cells and J774.A1 macrophages. The results of this study set a precedent for future research on this plant material and its possible applications in the dental field and related areas as an alternative antifungal treatment based on natural products.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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