Research article

Evaluation of antimycotic activity of grapefruit leaf extract on Candida species - An *in vitro* study

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ABSTRACT

Introduction and Aim: Candidiasis is one of the most common pathological conditions affecting the oral mucosa. Synthetic antifungals are being eliminated from the market due to the burden of environmental residues and carcinogenesis. Bioactive phytochemicals such as alkaloids, terpenoids, polyacetylenes, unsaturated isobutylamides, and phenolics are considered safer than synthetic products. The medical field is continuously exploring plant products against the increasing number of antibiotic-resistant organisms. Leaves of grapefruits were selected for the study because citrus species are well known for their antibacterial and antifungal properties.

Materials and Methods: Volatile oils extracted from grapefruit leaves by hydro distillation were tested for antifungal activity by agar diffusion method against various ATCC strains of Candida. MIC/MFC and ZOI were recorded, and a cytotoxicity test was carried out on human gingival fibroblasts.

Results: Leaf extract not only produced a larger zone of inhibition against test pathogens but was also less cytotoxic than Amphotericin B and Fluconazole.

Conclusion: Grapefruit leaf volatile oil extract had an antifungal effect on selected strains of candida and was less toxic than Amphotericin B and Fluconazole to the human gingival fibroblasts *in vitro.*

Keywords: Grapefruit; leaf extract; antifungals; cytotoxicity; healthcare.

INTRODUCTION

Candida, species of fungi constitute a common oral commensal and a potential opportunistic pathogen behind recurrent oral thrush and oropharyngeal candidiasis (1,2). Treating candida infections involve expensive antifungal drugs (3) and is fraught with the dangers of pathogen resistance and toxicity of therapeutic agents (4). Diversity of Indian flora offers abundant biochemicals of medicinal value and could be safer than synthetic antifungals (5).

Citrus products are well recognized for the presence of flavonoids and limonoids with anti-inflammatory and anti-cancer properties. Grapefruit belongs to the citrus family and has simple sugars, vitamin C, carotenoids, flavonoids, limonoids, fibers, folic acid, and potassium, with significant health benefits (6). The study aims to evaluate the antifungal activity of Grapefruit leaf volatile oil extract against candida species and cytotoxic effect on human gingival fibroblasts.

MATERIALS AND METHODS

Preparation of oil extract

The leaves of grapefruit trees were collected between November to January, from the South Coastal India region. Clevenger's apparatus was used to extract the volatile oil in the leaves mixed with water and glycerin. A graduated cylinder was used to collect the volatile distillate after returning the aqueous portion to the distilling flask. The volume of the oil obtained was measured and expressed in percentage v/w. Pale white light volatile oil was extracted from the grapefruit leaves by hydro distillation. The yield percentage was 0.066% v/w.

Fungal strains used and their source

Commercially available Amphotericin B and Fluconazole were used as positive controls. Commercially available ATCC (American Type Candidal Culture) strains of Candida *albicans* (ATCC 90028), Candida *krusei* (ATCC 14243), *Candida tropicalis* (ATCC 750), Candida *parapsilosis* (ATCC 22019) were procured from Himedia. The Zone of Inhibition (ZOI), Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of Citrus *paradisi* leaf essential oil were determined.

Antifungal assay

Determination of zone of inhibition

ZOI of leaf extract was determined by broth dilution technique. The Sabouraud dextrose broth (SDB) contains 160 µl of the oil that is 100% extract and 20 µl of the candida isolates without any extract.

Subsequent wells consisted of extracts with 50% reduction in concentrations to each well into which 20 µl isolate of Candida species was added respectively. The final concentrations were 50%, 25%, 12.5%, 6.25% and 3.75% of *Citrus paradisi* extract inoculated with actively dividing Candida. Sensitivity was evaluated from MIC (Minimum Inhibitory Concentration) and MFC (Minimum fungicidal Concentration) performed in triplicate.

The antifungal properties of herbal extracts were done according to the modified Kirby –Bauer method. Candida strains were cultured on Sabouraud Dextrose Agar (SDA). A single colony from the new culture was transferred with a sterile loop into SDB and incubated overnight at 37°C on a shaker. The density of the organism suspensions was adjusted to the 0.5 McFarland standard, 6mm wells were punched on SDA medium.

The cultures were plated on SDA. Commercially available antifungal discs of Amphotericin B and Fluconazole were transferred into the punched wells. The seeded plates were incubated aerobically for 18 hours at 37°C. The extract obtained was tested for anti-fungal activity without dilution. The study was repeated three times. The zones of inhibition were recorded for each species of Candida.

Determination of antifungal activity

To obtain the MIC, the positive control contained 100% concentration of the volatile oil extract of Citrus *paradisi* leaves and negative control consisted of 20 µl of the Candida isolates without any extract. Subsequent wells consisted of extracts with 50% reduction in concentrations to each well into which 20 µl isolate of Candida species was added respectively. The final concentrations were 50%, 25%, 12.5%, 6.25% and 3.75% incubated for 24 hours at 37°C overnight. After 24 hours, 20 µl of the solution was taken from each well and plated on SDA with the help of sterile glass spreader and incubated for 24 hours at 37°C overnight. The colonies formed on each plate were counted.

Cytotoxicity test

Human Gingival fibroblasts (HGF) collected from freshly extracted teeth from the department of oral and maxillofacial surgery (with approval from ethical review board and prior consent from patient) were used for the study.

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-Glutamine, 100 IU/ml Penicillin, 100 µg/ml Streptomycin and 5 µg/ml Amphotericin B at 37°C in a humidified atmosphere of 95% air and 5% CO2. In a 24-well plate, cells were seeded with 5×10^4 cells per well and treated with Citrus *paradisi* leaf oil extract at 2 × MFC in serum-free medium (SFM) for 240 and 480 min. The cytotoxicity of Grapefruit leaf oil was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.

The ELISA reader with the absorbance of 540 nm was used to measure the absorbance of extract. The viable cell number was calculated from the standard curve of cell number by plotting a scattergram of the absorbance value against the known number of cells. Optical density of the Formazan product in solution is measured as the outcome. Spectrophotometer of 570 nm wavelength measures living cells in the form of blue color reaction indicating the MTT product (7).

RESULTS

Table 1 compares the MIC, MFC and the Zone of Inhibition of *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* when treated with 50% concentration of essential oil of *Citrus paradisi* leaves, 50mcg/disc concentration of Amphotericin B, 25mcg/disc concentration of Fluconazole. MIC and MFC of *Citrus paradisi* leaf essential oil for *Candida albicans* was 250 µl/mL, and the ZOI was 18mm (Fig. 1).

Table 1: Comparison of MIC, MFC and ZOI between *Citrus paradisi* leaf extract and control drugs on various Candida strains.

		MIC	**MFC**	**ZOI**
***Candida albicans* ATCC 90028**	*Citrus paradisi* leaf essential oil	250 µl/mL	250.0 µl/mL	18.00 mm
	Amphoterin B	25 mg/mL	25.0 mg/mL	17.00 mm
	Fluconazole	25.2 mg/mL	25.0 mg/mL	18.67 mm
***Candida parapsilosis* ATCC 22019**	Citrus paradisi leaf essential oil	250.0 µl/mL	500.0 µl/mL	27.33 mm
	Amphoterin B	19.3 mg/mL	19.3 mg/mL	43.33 mm
	Fluconazole	12.8 mg/mL	12.8 mg/mL	47.33 mm
***Candida tropicalis* ATCC 750**	Citrus paradisi leaf essential oil	500.0 µl/mL	500.0 µl/mL	16.67 mm
	Amphoterin B	25.0 mg/mL	25.0 mg/mL	23.33 mm
	Fluconazole	12.8 mg/mL	12.8 mg/mL	25.33 mm

***Candida krusei* ATCC 14243**	Citrus paradisi leaf essential oil	250.0 µl/mL	500.0 µl/mL	16.00 mm
	Amphoterin B	41.6 mg/mL	50.0 mg/mL	12.00 mm
	Fluconazole	25.0 mg/mL	50.0 mg/mL	34.33 mm

ATCC – American Type Culture Collection; MIC-Minimum Inhibitory Concentration; MFC-Minimum Fungicidal Concentration; ZOI (Zone of Inhibition)

Fig.1: ZOI of Candida albicans when treated with Citrus paradisi leaf essential oil

MIC and MFC of Amphotericin B and Fluconazole on *Candida albicans* were 25 mg/mL. ZOI of Amphoterin B was 17 mm, and that of Fluconazole was 18.67mm on average. MIC and MFC of *Citrus paradisi* leaf essential oil for *Candida parapsilosis* were 250 µl/mL and 500 µl/mL respectively, and the ZOI was 27.33mm. MIC and MFC for Amphotericin B was 19.3 mg/mL with a ZOI 43.33mm.MIC and MFC for Fluconazole were 12.8 mg/mL and ZOI 47.33mm. MIC and MFC of *Citrus paradisi* leaf essential oil for *Candida tropicalis* was 500 µl/mL and ZOI 16.67mm. MIC and MFC for Amphotericin B were 25 mg/mL with the ZOI being 23.33mm. MIC and MFC for Fluconazole was 12.8 mg/mL, and the ZOI recorded was 25.33mm. MIC and MFC of *Citrus paradisi* leaf essential oil for *Candida krusei* 250 µl/mL and 500 µl/mL respectively, and the ZOI was 16mm. MIC and MFC for Amphotericin B was 41.6 mg/mL and 50.0 mg/mL and ZOI 12mm. MIC and MFC for Fluconazole was 25 mg/mL and 50mg/mL respectively and ZOI 34.33 mm on average.

The following equation was used for determining the percentage inhibition from the ZOI (8).

$$PIDG\ (\%) = \frac{\text{Diameter of sample} - \text{Diameter of control}}{\text{Diameter of control}} \times 100$$

IC_{50} was calculated using linear regression y=mx+b, Where y is the percentage of inhibition, m is the constant, x is the concentration of compound tested in µg/mL, and b is the y-intercept of the line of standard curve. The IC 50 values are given in (Table 2) for various Candida species tested.

Upon investigation of the cytotoxic effects of grapefruit leaf oil extract, the cells were treated for 24 hours with the extract to determine the viability of the cells. The cells treated with the extract were compared with the normal cells, and it was found that 99% cells were viable for essential oil extract.

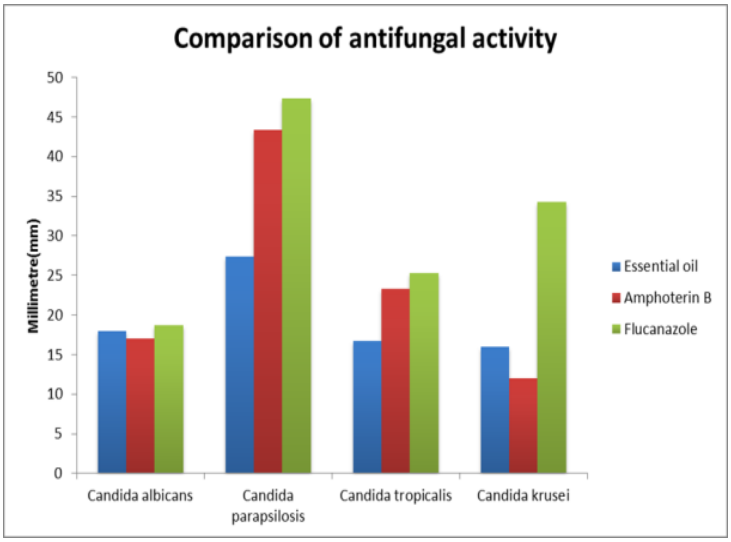
Table 2: IC_{50} value of the grapefruit leaf volatile extract for various Candida species

Organism	**IC_{50} (mg/mL)**
Candida tropicalis	214.5
Candida krusei	359.2
Candida albicans	73.6
Candida parapsilosis	297.07

DISCUSSION

Candida is a yeast-like fungus seen as commensals in the oral cavity. In complete and partial removable denture wearers, the tissue surface of the denture provides habitat to these commensals. In consort with other microorganisms, candida can become infectious causing denture stomatitis and oropharyngeal candidiasis. (6,9) Development of adaptive mechanism by candida against commercial antifungals and decreased immunity in geriatric populations calls for identifying antimicrobials from herbal products (10).

In the present study (Fig. 2), the effectiveness of essential oil extract of grapefruit leaves on candida species was in the following order *Candida parapsilosis* > *Candida krusei* > *Candida tropicalis* > *Candida albicans*. Essential oil extract was more active on *Candida albicans* and *Candida krusei* compared to the commercially available Amphotericin B.



Compares the antifungal activities of essential oils with that of the control

Fig. 2: Comparison of antifungal activity of essential oil with commercial antifungals

Disruption of the bacterial membrane and liberation of the cytoplasmic contents were the mechanism of action of grapefruit extracts according to Cvetni *et al.,* (11) According to Han (10) grape seed extract had a synergistic effect against Candida when used with commercial antifungals. Citrus flavonoids and naringenin present in grapefruits were responsible for the antifungal activity (11,13).

In the present study the yield percentage of essential oil extract was 0.066% v/w. The yield percentages reported by previous studies for grapefruit, malta, mandarin, mosambi and tangerine were 0.45%, 0.37%, 0.33%, 0.30%, and 0.28%. measured in terms of v/w, orange, lemon, mandarin and bigaradier(bitter orange) yielded 0.96%,1.02%,0.51% and 0.73% v/w respectively (14,15).

Major constituents detected were 3,7-Dimethyl-,(n)-6-octen-1-ol (31.41%) and 1,1,3,3,5,5,7,7,9,9,11,11,13,13- tetradecamethyl-heptasiloxane (62.03%), Caryophyllene (18.77u%), (-)-Spathulenol (10.36%), Caryophyllene oxide (10.95%) and 1-Bromo-4-bromomethyldecane (12.89%)whereas from studies on various Citrus peels,(11,14-16) the compounds detected were β-pinene, γ-terpinene, limonene, spathulenol, citral, β -myrcene, trans-β-ocimene, α-pinene. Further studies are required to extract the active components and know which specific content is responsible for the antifungal effect of the essential oil.

CONCLUSION

Within the limitations of the present study, we concluded that, the essential oil extract of grapefruit leaves possessed a strong anti -fungal activity against the tested ATCC strains of candida, then the commercial antifungals Fluconazole and Amphotericin B. Effective concentration of essential oil was nontoxic on human gingival fibroblasts. Essential oil extract of the grapefruit leaves can prove to be promising as an antifungal agent in oral candidiasis. It can be used as an adjunct in health care products for geriatric patients. Animal studies with essential oil extract can prove to be beneficial.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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