RESEARCH ARTICLE

Effectiveness of *Citrus paradisi* Extract, its Synergistic Effect and Cytotoxicity when Incorporated into Tissue Conditioners against *Candida* Species

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ABSTRACT

Purpose: One of the most common fungal infections seen in complete denture wearers is candidiasis. Unscrupulous use of tissue conditioners, in the long run, can lead to irreversible damage to the tissues if the underlying infection is not attended to in a timely manner. Carcinogenesis and environmental residues associated with synthetic antifungals are eliminating them from the market and herbal alternatives are replacing them due to lesser side effects. Since *Citrus* species are known for antifungal and antibacterial effects, the raw fruits of *Citrus paradisi* were selected for study.

Materials and Methods: The fresh fruits of *C. paradisi* were hydrodistilated to obtain their volatile oil. Antifungal impact studies were conducted using agar diffusion and colony count techniques on certain Candidal strains from the ATCC. The research was conducted on human gingival fibroblasts to determine cytotoxicity, biofilm inhibition, synergistic action, minimum inhibitory concentration, minimum fungicidal concentration, and zone of inhibition (ZOI).

Results: Volatile extract of *C. paradisi* showed higher efficiency compared to commercially available antifungals. Cytotoxic effects were less than the toxicity showed by commercial antifungals.

Conclusion: *C. paradisi* oil extract showed antifungal effect on tested strains of *Candida*. *In-vitro* cytotoxicity on human gingival fibroblast was less in comparison to flucanazole and amphotericin B.

Keywords: *Citrus paradisi*, Antifungal, *Candida*, Cytotoxicity, Biofilm inhibition.

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INTRODUCTION

Tissue conditioners are soft resilient and porous materials used in prosthodontics to increase the longevity and fit of complete removable dentures and heal the abused foundation tissues.[1] *Candida*, a commensal in oral cavity becomes virulent with compromised local and systemic conditions and are harbored in the tissue surface of complete dentures.[2] Increased cost of commercial antifungals, multidrug resistance of *Candida* strains and adverse reactions and toxic nature of antifungals have encouraged the research for herbal alternatives that are believed to be safer.[3]

Citrus products are exploited and explored for their known anti-cancer and anti-inflammatory properties. These properties are attributed to the presence of fibres, limonoids, flavonoids, pinenes and other components in them.[4] The aim of the study is to evaluate the antifungal and synergistic effect of citrus paradise oil extract incorporated into tissue conditioners with the commercial antifungals and to study the cytotoxic effect on human gingival fibroblast.

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MATERIAL AND METHODS

The proposed research work data and the proposed proforma for data collection were presented to the Central Ethical Committee and the ethical clearance was obtained (Ref No:NU/CEC/Ph.D-60/2012).

Preparation of Oil Extract

Raw fruits of *Citrus paradisi* were collected between the months of October to February from the region of South Coastal India. Fruit peels were mixed with water, and glycerine and volatile oil were extracted from Clevenger's apparatus. Volatile distillation was collected in a graduated cylinder while returning the aqueous portion back into the flask. Volatile oil extracted was expressed as percentage v/w. The percentage yield remained 0.12% v/w.

Fungal Strains and Source

We used the commercially available antifungals amphotericin B (AMB) and flucanazole (FCZ) as a control. The following *Candida* strains were obtained from Himedia: ATCC 22019, ATCC 14243, ATCC 90028, and ATCC 750, all of which are part of the American Type candidal culture (ATCC) collection (Figure 1). The essential oil of *C. paradisi* was tested for cytotoxicity, synergistic action, biofilm formation, minimum inhibitory concentration, minimum fungalicidal concentration, and zone of inhibition (ZOI).

Preparation of Tissue Conditioner Pellets

We used metal molds that were 5 mm in diameter and 2 mm thick. Filling the mold voids with a tissue conditioner separator was the next step. After preparing the pellets, the tissue conditioner was combined in accordance with the manufacturer's directions. About 140 such plain tissue conditioner discs were made to be used as control. About 120 discs each of AMB, tissue conditioner combination and FCZ, tissue conditioner combination were made by this method. In 60 discs of tissue conditioners had 1:1 wt/wt essential oils incorporated (MIC concentration). To compare synergistic activity, the AMB and FCZ incorporated tissue conditioner pellets were dipped in the MIC concentration of essential oil for 5 minutes before use. A total number of 420 tissue conditioner pellets were made. The tissue conditioner pellets with and without antifungals were stored separately in amber-colored glass containers in a cool, dry environment until used for the study.

Figure 1: ATCC strains of *Candida*

GCMS Analysis

Gas chromatography–mass spectrometry (GC-MS) analysis (Figure 2) was carried out using a Shimadzu gas chromatograph with a SE-30 10% Chromosorb-W packed stainless steel column (2 x 2 mm).

Oven program: 60°C (5 minutes), 60 to 260°C (5°C/min), 260°C (10 minutes); carrier gas –nitrogen, flow rate 40 mL/min injector temperature 240°C; detector temperature 240°C. Literature available in the libraries like NIST and WILEY were used to identify.

Matching and comparing the mass spectra of individual components using a database is performed. One way to measure a compound's LRI is to compare its retention time on a gas chromatographic column to that of homologous series n-alkanes. Adams RP *et al*., provided the retention time, and the following equation is used to derive the linear retention index (LRI) from that.[5]

Antifungal Assay

Determination of zone of inhibition

Well diffusion method was used to test for the antimicrobial properties. Figure 3 (a-f) shows that the *Candida* species were cultivated on sabouraud dextrose agar (SDA). Using a sterile loop, one colony was removed from the new culture and placed into sabouraud dextrose broth (SDB). Shaken and kept at 37°C overnight, the broth was then tested. The density was set to match the 0.5 McFarland standards for the organism suspensions. Using a sterile cork borer, 6 mm wells were punched into a dry SDA sample." Cells were grown in a petri dish that contained the SDA. After that, 50 µL of the extracts with known concentrations was put into the wells that

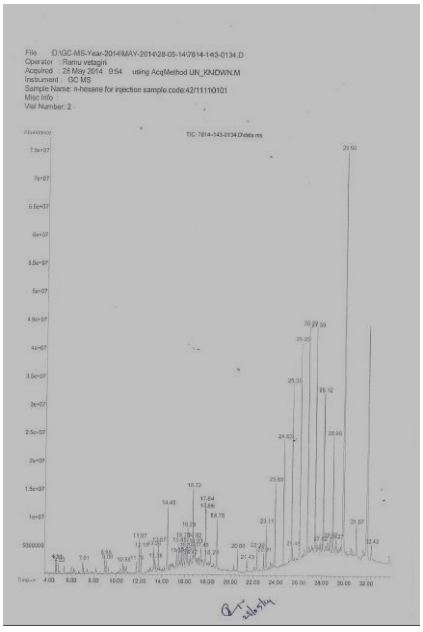
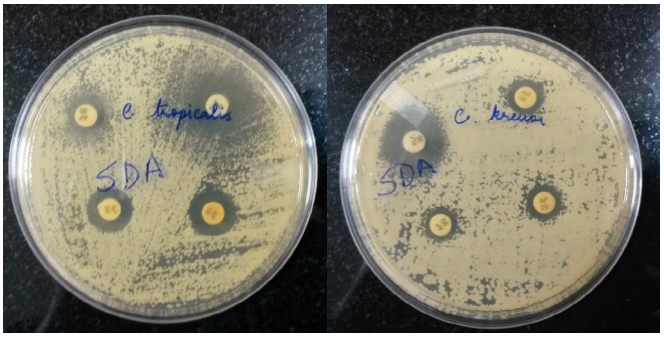
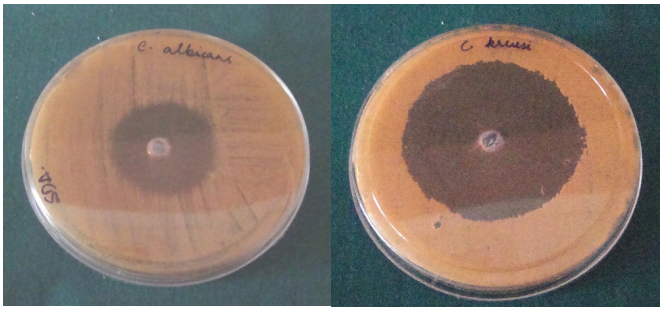


Figure 2: GC-MS analysis



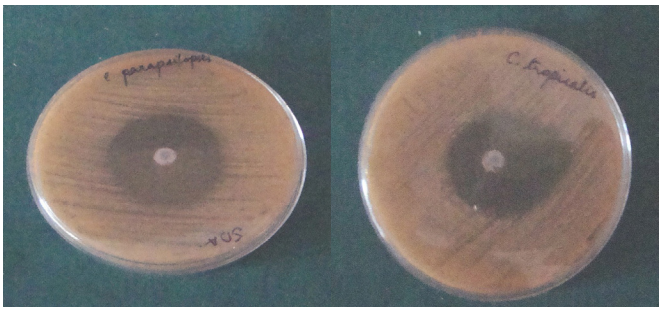
(a) ZOI for *C. tropicalis* with commercially available antifungals amphotericin B and flucanazole

(b) ZOI for *C. krusei* with commercially available antifungals amphotericin B and flucanazole



(c) ZOI for *C. albicans* with essential oil of *C. paradisi*

(d) ZOI for *C. krusei* with essential oil of *C. paradisi*



(e) ZOI for C. *parapsilosis* with essential oil of *C. paradisi*

(f) ZOI for C. *tropicalis* with essential oil of *C. paradisi*

Figure 3: The *Candida* species were cultured on sabouraud dextrose agar.

had been punched. For 18 hours at 37°C, the seeded plates were incubated aerobically. At 24, 48, and 72-hour intervals, the millimeter-wide clean zone around the punched well was measured. The sensitivity was tested in triplicate using the minimum fungicidal concentration (MFC) and the minimum inhibitory concentration (MIC) as shown Figure 4 in Table 1.

Biofilm assay

With minor adjustments, Merritt JH *et al.*[6] described the colony biofilm test. After incubating at 37°C for 18 hours, six isolates of new agar plates were added to SDB and diluted with fresh medium to a 1 in 100 concentration. A 0.2 mL portion of diluted cultures containing tissue conditioner pellets submerged in the culture was added to each well of sterile polystyrene 96-well flat-bottom tissue culture plates. The tissue conditioner discs were placed in the tissue culture plates and let to incubate at 37°C for 24 hours. Floating organisms and broth were

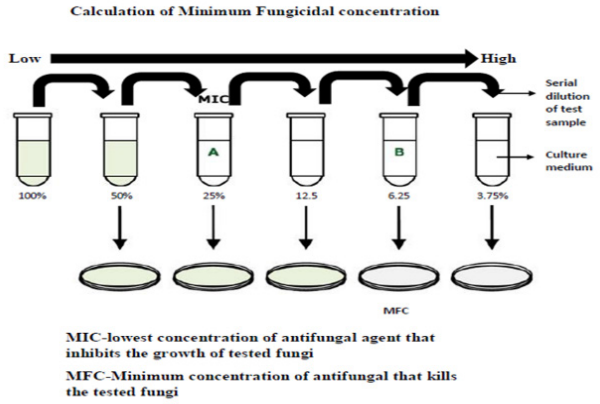


Figure 4: Determination of MIC and MFC

delicately removed from the wells after incubation by inverting and tapping them. To eliminate the free-floating "planktonic" bacteria, the wells and tissue conditioner discs were rinsed four times with 0.2 mL of phosphate-buffered saline. After incubating the biofilm for 24 hours at 37°C, 0.1 mL of the oil extract was added to it. Following incubation, the test material was carefully removed by tipping and flipping the wells. The wells and tissue conditioner discs were rinsed four times with 0.2 mL of phosphate buffer saline to eliminate any evidence of the test substance. New 0.1 mL of SDB was added to the microtitre wells that had been rinsed. In the test tubes with the new broth, the pellets of tissue conditioner were mixed by vortexing. After transferring the broth from each well or tube to the SDA, it was incubated at 37°C for 24 hours. The density of the organisms was determined by counting the colonies growing on the agar. The positive control included the wells with the organisms devoid of oil extract which were streaked on the plates. The colonies grown were counted and compared with the control.

Synergistic activity

Fungicidal concentrations of MIC of AMB and FCZ at which 90% of the isolates were inhibited, were incorporated into tissue conditioner powder which was mixed according to the manufacturer's instructions. Tissue conditioner discs without antifungal incorporation were used as controls. Tissue conditioner discs with AMB and tissue conditioner discs with FCZ were immersed in oil extract of *C. paradisi* separately for 5 minutes and were further used to test for synergistic activity by colony biofilm assay method.[7] The colonies were counted at intervals of 24, 48, and 72 hours.

Cytotoxicity test

For the purpose of assessing cytotoxicity, human gingival fibroblasts (HGF) were procured from gingival tissue explants connected to non-carious, recently removed third molars. Before tissue was collected, individuals who had healthy third molars extracted gave their informed permission. Under a humidified environment consisting of 95% air and 5% CO_2, cells were grown in DMEM that was supplemented with 10% fetal bovine serum (FBS), 2 mML glutamine, 100 IU/mL

Table 1: GC-MS analysis table

Peak	*RT*	*Name*	*%Area*	*Quality%*
1.	4.490	alpha-Pinene	0.41	96
2.	4.605	Butanedioic acid	0.24	47
3.	4.783	Hexane,3-bromo-3octene-2,5-dione	0.25	43
4.	5.184	beta-Phellandrene	0.43	91
5.	5.298	beta-Pinene	2.25	97
6.	5.438	beta-Myrcene	2.06	81
7.	6.265	D-Limonene	89.04	90
8.	6.494	beta-Ocimene	0.33	98
9.	6.946	Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl) propan-2-yl carbonate	0.09	87
10.	7.226	2-Furanmethanol,5-ethenyltetrahydro-alpha,5-trimethyl-cis-Linaloloxide	0.08	64
11.	7.430	1,6-octadien-3-ol,3,7-dimethyl	0.49	90
12.	7.856	2,6-octadienal,3,7-dimethyl,(Z) Limonene oxide	0.11	46
13.	8.098	cis-p-Mentha-2,8-dien-1-ol	0.11	97
14.	8.842	Terpinen-4-ol	0.11	94
15.	9.058	L-alpha-Terpineol	0.39	87
16.	9.141	Furan,2,3-dihydro-4-methyl-2-Butenal	0.24	46
17.	9.733	2,6-Octadienal,3,7-dimethyl-,(Z) Citral	0.18	74
18.	10.140	2,6-Octadienal,3,7-dimethyl-,(E) Citral	0.13	93
19.	11.100	Cyclohexane,1-ethyl-1-methyl	0.06	68
20.	11.139	2-Carene	0.06	95
21.	11.896	Cyclohexane	0.06	64
22.	11.972	Heptadecane 2,6,10,14-tetramethyl	0.05	52
23.	12.316	beta-ylangene	0.10	96
24.	12.347	Caryophyllene	0.20	99
25.	12.475	Bicyclo[4,4,0]dec-1-ene,2-isopropyl-5-methyl-9-methylene	0.10	93
26.	12.659	gamma-Muurolene	0.03	87
27.	12.761	Arachidonic acid	0.03	38
28.	13.143	beta copaene	1.76	95
29.	13.327	gamma-Elemene	0.24	64
30.	13.384	gamma-Muurolene	0.05	94
31.	14.479	Hexadecane	0.09	98
32.	16.718	Octadecane	0.08	90
33.	17.838	7,9,Di-tert-butyl-1-oxaspiro(4,5)eca-6,9-diene-2,8-dione	0.11	99
34	18.741	Eicosane	0.05	93

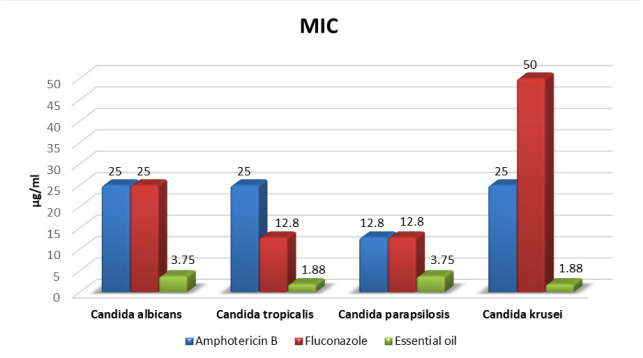
penicillin, 100 µg/mL streptomycin, and 5 µg/mL amphotericin B. The temperature was maintained at 37°C. Cells were placed in 24-well plates with 5×10^3 cells per well. After that, the cells were exposed to *C. paradisi* oil extract at a concentration of 2 × MFC in serum-free medium (SFM) for 240 and 480 minutes, respectively, in order to measure the extract's cytotoxic impact. A 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) test was used to measure the cytotoxicity. Metabolically active cells release dehydrogenase enzymes, which are the basis of the MTT experiment. These enzymes reduce the yellow tetrazolium salt to purple formazan crystals. In order to determine the solution's absorbance at 540 nm, an ELISA reader was used. Scatter gram analysis of absorbance value *vs* known cell number allowed for the calculation of viable cell number from the standard curve of cell number.[6]

RESULT AND DISCUSSION

When analyzing gas chromatography-mass spectrometry (GC-MS) data, a total of 34 components were identified in the essential oil derived from the fruit rind. The mass fragmentation revealed a similarity below 70%, making 11 out of 34 unidentifiable. A whopping 89.04% of the total was limonene. According to Table 1, three other active chemicals were β-Pinene (2.25%), β-Myrcene (2.06%), and β-Copaene (1.76%) (Table 1).

The antifungals' minimum inhibitory concentrations on different *Candida* species (Graph 1).

For *Candida albicans*, the MIC and MFC values for Flucanazole and amphotericin B were 25 mg/ML and 18.2 and 17.5 mm, respectively. The minimum inhibitory concentration (MIC) of the oil extract was 3.75 µL/mL, followed by 6.25 µl/mL and so on. A depth of 29.4 mm was measured during the zoom-in interval. *Candida parapsilosis* was effectively treated with amphotericin B and flucanazole, with MICs of 12.8 mg/mL and 25 mg/mL, respectively. Zeta potential of amphotericin B was 14 mm and flucanazole was 16.2 mm. The oil extract had a minimum inhibitory concentration (MIC) of 3.75 µl/mL and an MFC of 6.25µl/mL. The Z-score implanted was 43.4 mm. The MIC and maximum concentration (MFC) of amphotericin B against *Candida tropicalis* was 25 mg/mL, whereas the MIC and MFC of flucanazole were 12.8 in the same volume. When comparing amphotericin B with flucanazole, the ZOI for the former was 14



Graph 1: Minimum inhibitory concentrations (mic) of the antifungals on different species of *Candida*.

mm while for the latter it was 16.2 mm. MIC and MFC values for the oil extract were 1.88 µl/mL and 42.6mm, respectively. The MIC and MFC of Amphotericin B against *Candida krusei* were 50 mg/mL, whereas the MIC and MFC of flucanazole were 50 mg/mL." The zone of inhibition (ZOI) for amphotericin B was 14.6 mm and for flucanazole it was 15.8 mm. Based on Table 2, the oil extract had MIC of 1.88 µl/mL, MFC of 3.75 µl/mL, and ZOI of 41.6 mm (Table 2).

Mean zones of inhibition of commercially available antifungals (control) and the test materials (extracts). Mean zones of inhibition exhibited by essential oil extract of raw fruit peel of *C. paradisi* was 29.40 ± 1.95, 42.60 ± 3.97, 43.40 ± 3.78, 41.60 ± 3.05 mm, respectively, on *C. albicans, C. tropicalis, C. parapsilosis* and *C. krusei* which was much higher than the inhibition zones exhibited by amphotericin B which was 17.40 ± 1.34, 23.20 ± 1.10, 14.00 ± 1.41, 14.60 ± 0.89 mm when treated on *C. albicans, C. tropicalis, C. parapsilosis, C. krusei* respectively and Flucanazole which was respectively 18.20 ± 1.30, 25.60 ± 0.55, 16.20 ± 1.10, 15.80 ± 0.84 mm on *C. albicans, C. tropicalis, C. parapsilosis* and *C. krusei* (Table 3 and Graph 2). MFC values of the antifungals on different species of *Candida* (Graph 3)."

For each of the four time points 24, 48, 72, and 96 hours Graph 1 shows the biofilm development potential of several *Candida* species in the presence of different antifungals and extracts. At a statistically significant level ($p \leq 0.001$), essential oil extract inhibited the formation of *Candida* biofilms more effectively than amphotericin B and fluconazole. The biofilm growth, however, showed not much difference in relation to time interval. The number of colonies remained almost same up to 96 hours only the density increased.

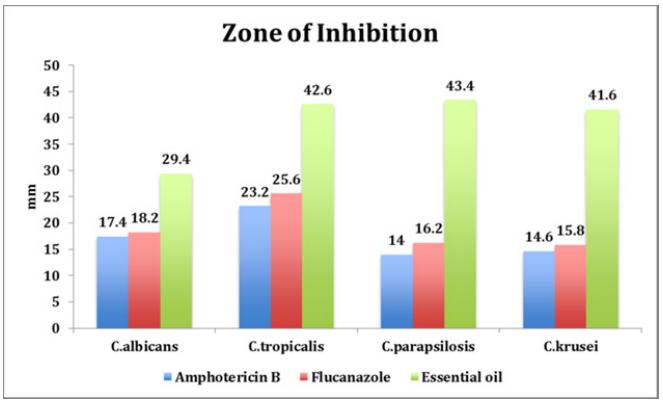
The synergistic activity of various medicaments on the different tested strains of *Candida*. As compared to the control group, amphotericin B and fluconazole showed decrease in the growth capacities of *Candida* strains when tested by colony count method. Tissue conditioners with essential oil incorporated showed absence of any colonies. When used with amphotericin B and fluconazole, essential oil extract showed complete absence of *Candida* colonies, indicating that the essential oils had excellent synergistic activity when combined with commercially available antifungals (Graph 4).

Table 2: Comparison of MIC, MFC and ZOI between *C. paradisi* oil extract and control drugs on various *Candida* strains

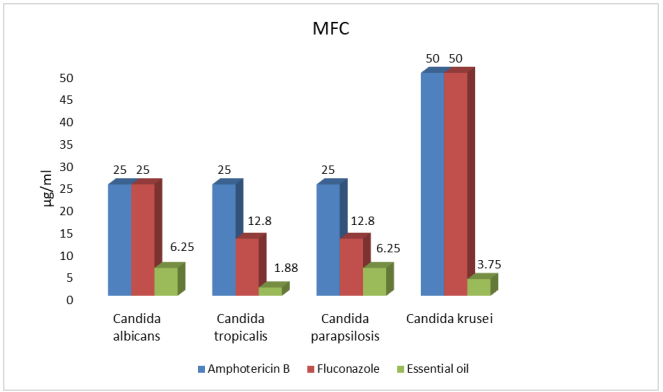
VAriable		*MIC*	*MFC*	*ZOI (mm)*
C. albicans ATCC 90028	*C. paradisi* oil extract (µL/mL)	3.75	6.25	29.4
	Amphoterin B (mg/mL)	25	25.0	17.4
	Flucanazole (mg/mL)	25	25.0	18.2
C. parapsilosis ATCC 22019	*C. paradisi* oil extract (µL/mL)	3.75	6.25	43.4
	Amphoterin B (mg/mL)	12.8	25	14
	Flucanazole (mg/mL)	12.8	12.8	16.2
C. tropicalis ATCC 750	*C. paradisi* oil extract	1.88	1.88	42.6
	Amphoterin B (mg/mL)	25.0	25.0	23.2
	Flucanazole (mg/mL)	12.8	12.8	25.6
C. krusei ATCC 14243	*C. paradisi* oil extract (µL/mL)	1.88	3.75	41.6
	Amphoterin B (mg/mL)	25	50.0	14.6
	Flucanazole (mg/mL)	50	50.0	15.8

Table 3: Variation in zone of inhibition by *Candida* when treated with various medications

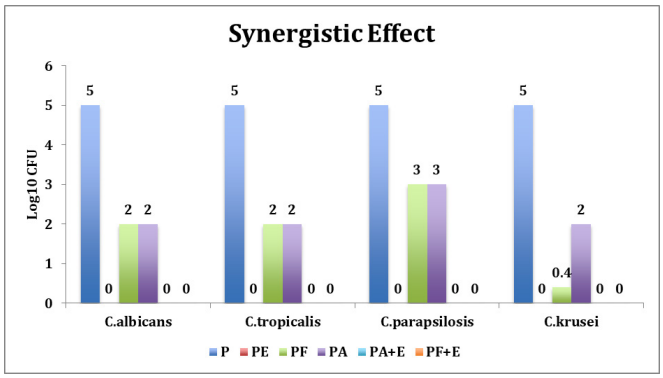
Microorganism		*Amp B vs Flucanazole*	*Amp B vs Essential oil*	*Flucanazole vs Essential oil*
C. *albicans*	U statistic	8.50	0	0
	Z	-0.85	-2.64	-2.62
	p-value	0.39(NS)	0.008*	0.009*
C. *tropicalis*	U statistic	0	0	0
	Z	-2.69	-2.65	-2.65
	p-value	0.007*	0.008*	0.008*
C. *parapsilosis*	U statistic	2.50	0	0
	Z	-2.18	-2.65	-2.65
	p-value	0.03*	0.008*	0.008*
C. *krusei*	U statistic	4.00	0	0
	Z	-1.84	-2.64	-2.63
	p-value	0.07(NS)	0.008*	0.009*



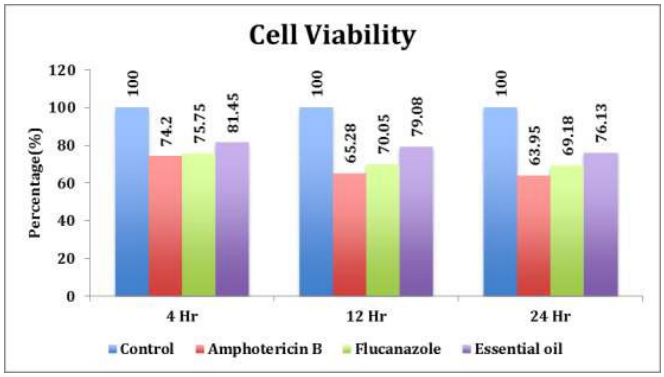
Graph 2: Zone of inhibition (ZOI) shown by antifungals and essential oil



Graph 3: MFC of various medicaments on *Candida* spp.



Graph 4: Synergistic activity of essential oil when mixed with commercial antifungals



Graph 5: Percentage viability of HGF at different time intervals on exposure to antifungals and essential oil

The percentage viability of HGF after treating with amphotericin B was 74.2 ± 0.91, 65.28 ± 0.50 and 63.95 ± 0.13. Viability of cells after treatment with fluconazole was 75.75 ± 0.65, 70.05 ± 0.21 and 69.18 ± 0.31 and viability after treatment with essential oil extract of *C. paradisi* was 81.45 ± 0.60, 79.08 ± 0.17 and 76.13 ± 0.22 at 4, 12 and 24 hours respectively. This shows that the oil extract of *C. paradisi* was superior and had less cytotoxic effects (Graph 5).

DISCUSSION

Candida is commonly occur in the oral cavity. The tissue surface of complete and partial removable dentures and tissue conditioners used for lining loose dentures are mainly reasons for *Candida*.[6,7] In compromised conditions of oral cavity due to local factors like poor oral hygiene and tissue injury or systemic conditions that decrease the salivary flow, it encourages the commensals to attain virulence.[8-10] Compromised immunity of elderly denture wearers and multidrug resistance developing in *Candida* species demands the discovery of drugs with minimum side effects

The variables that determine an extract's composition include the extraction method, extraction time, temperature, solvent type, concentration, and polarity. How effective and safe natural medications are depends on a lot of things. These included things like using fresh plants, the part and age of the plants harvested, the time, the length, the season, the techniques of collecting, the temperature of processing, the nutrients, the light, the availability of water, the drying, packaging, storing, and transportation of the raw materials.[11,12]

In this investigation, the oil extract was most effective against *C. parapsilosis,* then *C. tropicalalis, C. krusei,* and *C. albicans*, in that order (Table 1). Comparing oil of *C. paradisi* to commercially available antifungals, it was shown to completely prevent biofilm development of all *Candida* strains. Essential oils' antimicrobial properties stem from their ability to suppress prostaglandins, autocoids, and membranes. In addition to complexing with cell walls and binding to adhesins, quinones and flavonoids also inactivate enzymes. Along with these effects, tannins and polyphenols damage membranes and deprive them of their substrates.[10]

Antifungal incorporation into tissue conditioners has several advantages wherein healing of abused tissues and treatment of candidiasis occur simultaneously, there is no need for patient compliance, a reduced frequency of antifungal application by patient is appreciated, decreased dependency on caretakers and cost-effectiveness. Antifungals' effectiveness depends on the antifungal's concentration, dissolution capacity, molecular weight, permeability of tissue conditioner, molecular size of the drug and drug release rates.[13]

In the present study, percentage viability of human gingival fibroblasts in the presence of essential oil extract ranged 81.45 ± 0.60% (4 hours.), 79.08 ± 0.17% (12 hours.), 76.13 ± 0.22 (24 hours.) which was less than the percentage viability of cells treated with commercially available antifungals amphotericin B and fluconazole. The increase in cytotoxic effect in commercial antifungals may be due to the presence of preservatives. The decreased cell destruction by oil extract proves that herbal product is an excellent replacement for commercial antifungals in terms of antifungal effect and biocompatibility.

Using *in-vitro* tests at various concentrations, Delgado *et al*. demonstrated that the essential oil of *C. paradisi* inhibited the growth of *Candida albicans* and oral candidiasis clinical isolates. This effect was attributed to the chemical groups of *C. paradisi* oil, which included sterols, triterpenes, coumarins, quinones, and sesquiterpenectones, and it did not harm Vero E6 cells or J774.A1 macrophages.[14]

The peel of *C. paradisi*, which is an ethanolic extract (96% concentration), exhibited inhibitory action against a range of bacterial and fungal species, according to Ayat *et al*.[15]

CONCLUSION

C. paradisi oil extract outperformed commercial antifungals Amphotericin B and Flucanazole in terms of antifungal action, within the constraints of the research. It effectively inhibited *Candida* species that are linked with biofilms and planktonic environments. The combination of oil extract, amphoterin B, and flucanazole showed a substantial synergy of activity. The antifungal effect was seen when mixed with tissue conditioners as well. Cytotoxic effect was substantially less as compared to commercial antifungals. The oil extract can be a promising alternative to commercial antifungals and beneficial to geriatric patients in terms of ease of use.

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