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Comparative Evaluation of Anti-Fungal Efficacy of Phytochemicals and Probiotics against Candida Albicans-An In-Vitro Study

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ABSTRACT

Introduction: In the light of advancements made in the scientific field, the phytotherapy has received an incredible interest because of their low toxicity, pharmacological activities, and economic viability. Among compounds of natural origin, biological activities have been shown by essential oils from aromatic and medicinal plants and have received specific consideration because of their radical-scavenging properties. Therefore, numerous EOs and Probiotics should be considered as a potential anti-Candida agent, for the treatment of Candida species.

Materials and Methods: Swab samples from 50 subjects with Early childhood caries, oral candidiasis and from removable dentures or orthodontic appliances were aseptically collected andsent for microbiological analysis. C. albicans was identified using standard methods. Eight groups were included in the study viz Group I: Ajwain oil group, Group II: Cinnamon oil group, Group III: Lemon grass oil group, Group IV: Oregano oil group, Group V: Oil blend group, Group VI: Probiotic group, Group VII: Clotrimazole group and Group VIII: CHX (Chlorhexidine) group. The constituents responsible for the activity of test groups were identified using Gas-Chromatography Mass Spectrometry analysis. The effectiveness of test agents against C. albicans was tested by agar well diffusion method followed by Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC).

Satistical Analysis and Results: The obtained data was calculated using relevant statistical tools such as Students T test and ANOVA. Group II Cinnamon Oil Group was found to be highly effective with mean Zone of Inhibition (ZOI) being 19.83+0.29 mm. The mean MIC for cinnamon oil was 0.625mg/ml and MFC was .33+1.44 mg/ ml that was least concentration effective against C. albicans among all the test groups. There was mean Percentage reduction of colony forming units in all the oils at MIC and MFC with cinnamon being the highest at the lowest concentration.

Conclusion: The results of the present study provided conclusive evidence that Essential oils specifically Cinnamon Oil could be used against Candida present as various source of infections. It could be used for prevention of progression of Early Childhood Caries, as a root canal Irrigant and as a treatment for oral candidiasis and for prevention of the same.

Key Words: *Candida albicans, Essential oils, probiotic, phytochemicals, Early Childhood Caries, Root canal Irrigant, Oral Candidiasis*

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INTRODUCTION

In the light of advancements made in the scientific field, the phytotherapy has received an incredible interest because of their low toxicity, pharmacological activities, and economic viability. As the inhibition of many pathogenic microbes by using synthetic drugs failed and due to the carcinogenic effects, acute toxicity and environmental hazard potential, there was need for the development of novel anticandidal agents. Among compounds of natural origin, biological activities have been shown by essential oils from aromatic and medicinal plants and have received specific consideration because of their radical-scavenging properties [1]. Probiotics have also been evaluated as a novel anticandidal agent [2].

Invasive Candida infections pose major health concerns, especially in infants, hospitalised, immunocompromised, or critically ill patients [3, 4]. However, there are only four major classes of antifungals in clinical use, which include azoles, polyenes, echinocandins, and pyrimidine analogs [5,6]. For this reason, an intense search for new alternative antifungal compounds is very urgent and necessary. Previous research has explored the effect of 21 plant essential oils

against multidrug resistant Candida spp., where it was discovered that Cymbopogon martini (lemongrass, LEO), citral, and cinnamaldehyde exhibited great inhibitory activities with MIC ranging from 90–100 µg/mL [7].

For some decades, systemic and local antifungal agents such as clotrimazole, nystatin, and amphotericin B have been successfully used as therapeutic and prophylactic agents to obviate colonization of *Candida* (Ericson and Benjamin; Pappas). Also, Chlorhexidine (CHX) is the gold standard for chemical removal of microbial plaque, which is often prescribed as an adjunct to mechanical plaque removal methods. However, long-term use of chemical agents is not recommended [8,9]. Theefficacy of these is compromised due to an alarming increase in the emergence of drug-resistant Candida strains worldwide (Sanguinetti et al.). Hence, alternative, or adjunctive therapies have been explored for Candidal infections including the use of natural products such as peptides, oils, and phytochemicals (Coleman et al.; Sardi et al.; Sherry et al.).

Essential oils also called volatile oils, are aromatic oily liquids obtained from plant materials such as flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits,and roots. Many essential oils have both in vitro and in vivo biological activity, which has justified research on traditional medicine focused on the characterization of their antimicrobial activity [10]. The antimicrobial activity shown by plant oils is mainly due to several phenolic and terpenoid compounds, which have antibacterial or antifungal activity [11]. There is literature which has been published on antimicrobial activity of essential oils recognizing them as safe [12]. Essential oils prepared in ethanol, in general, are interesting antiseptics which can be used as alternative irrigation solutions for endodontic treatments [13,14]. In addition, essential oils have long been used in many commercialized mouthwashes as safe solutions for daily oral health [15].

Probiotics as defined by WHO are "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host". The prevailing antibiotic resistance and search for a better alternative, led to discovery of probiotics and its potential health benefits. Pioneer studies for the effect of Probiotics on caries, periodontitis, halitosis and later oral candidiasis were conducted [16,17].

Oral candidiasis is one of the common fungal infections caused by *Candida*, affecting oral mucosa and is most common in infants, toddlers, and immunocompromised cases. The main *Candida* species that colonize the human oral cavity is*C. albicans*. Wetzel et al. and Moalic et al [18,19]. determined the presence of *Candida* in saliva, dental plaque,and infected dentin of children with early childhood caries more common than caries-free children. It is generally recognized that the mutans-group streptococci are the prime movers of the caries process due to their acidogenic and aciduric nature [20].However, several studies have now shown that the aciduric oral yeasts, mainly belonging to Candida species, frequently co-inhabit these lesions with mutans- streptococci, and significantly contribute to the caries process [21,22]. Candida colonisation is also seen in patients with orthodontic appliances. Although it has been proven that bacteria cause pulpal disease, numerous studies have revealed the incidence of fungi to be 7%–55% within the infected root canals, *Candida albicans* being the most common [23,24]. Their presence was first reported by Grossman in the range of 1%–17% [25].

Many studies of essential oils and probiotics have been conducted against bacteria but against *Candida*, studies are sparse. For this reason, the present study is undertaken to assess the antifungal effectiveness of essential oils, probiotics, and their combinations against *C. albicans*, to provide fundamental data for oral health promotion by demonstrating the possibility of the use of essential oils and probiotics as preventive and therapeutic agents for oral diseases.

MATERIALS AND METHODS

Materials

Samples Used for the Study

Essential oils (Fig1): *Cymbopogon citratus* (lemon grass), *Cinnamomum zeylanicum*(cinnamon), *Origanum vulgare* (oregano),*Trachyspermum ammi* (ajwain)
Biocide: Chlorhexidine
Antifungal agent: Clotrimazole
Probiotics (Fig 2):*Lactobacillus rhamnosus*
Test organism:*Candida albicans*
Culture media: Sabouraud's Dextrose Agar (SDA) and Sabouraud's Dextrose Broth (SDB), Luria Bertani Agar (LBA) and Luria Bertani Broth (LBB)

Fig 1: Raw Material of Essential oils

Fig 2: Probiotic(Lactobacillus rhamnosus GG Sachet) and colonies on LBA media

REQUIREMENTS FOR THE STUDY (Equipments and Reagents)

70% alcohol, Cotton wool swab, Mouth mirror, Explorer, Eppendorf tubes, Petri dishes,10%DMSO, Tween 20, Incubator, Sterile syringe, Micropipette tip, Inoculation loop, Micropipette, Vortex apparatus, Distilled water, Flask, Beaker, Clevenger type apparatus, Measuring cylinder, Microtiter plate, Test tubes, Test tube stand, Autoclave, Hand gloves, Masking tape, Vernier Calliper, GC-MS Apparatus.

PROCUREMENT OF THE EXTRACT OF THE PLANTS

The dried and powdered plant material was used for hydrodistillation. The essential oil was extracted by hydrodistillation of 500 g of the plant material in 1.5 L of water for 4 h using a Clevenger-type apparatus to collect the oil (Fig 3) [26]. To remove moisture, it was dried over anhydrous sodium sulphate, mixed with hexane, and dried with rota vapour. The essential oil was left open at room temperature for some time for the evaporation of hexane and was suitably diluted in 10% DMSO with 0.02% Tween 20. The essential oil was stored at 4^0till use.

10% DMSO preparation: For 8ml =0.8ml DMSO + 7.2ml H_2O
Essential oil sample preparation: 10 µl oil + 490µl DMSO (10%) + 0.2µl Tween 20

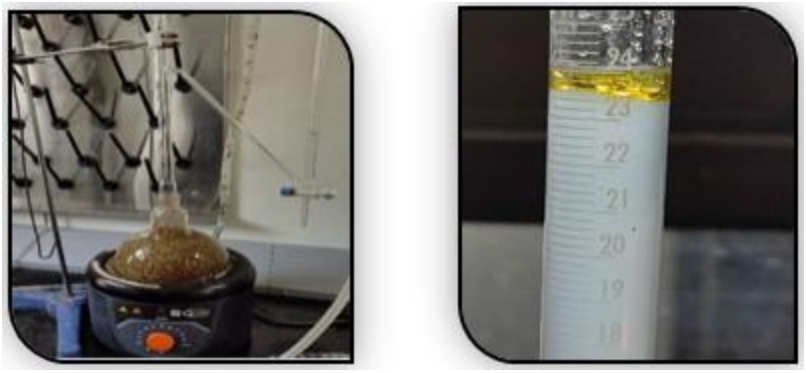


Fig3: Procurement of the extract of plants

PREPARATION AND DISPENSING MEDIA

Preparation of media was done according to the manufacturer's instruction used for culturing of microorganisms in the microbiology laboratory. Required amounts of media, SDA 65gm for 1L, SDB 35g for 1L, LBB 25g for 1L and LBA 40g for 1L i.e., SDA 9.75g for 150 ml, SDB 4.5g for 150 ml, LBB 3.75g for 150 ml and LBA 6g for 150ml were accurately weighed in a flask and distilled water was poured to make up to mark of 150 ml.It was tightly covered and autoclaved at 121^0C for 15 minutes according to manufacturer's instruction. Then the growth medium was dispensed into appropriate containers i.e., Petri-dishes for agar and falcons/flasks for broth

STERILITY TEST

Sterility test was carried out on samples to evaluate if they were free from microbial contamination and to ensure that the sample complies with the specifications for the test. Extracted oil was cultured on Sabouraud's dextrose agar (SDA) and incubated for seven days to ensure that the oil is completely sterile.

SAMPLE COLLECTION

The study included 50 subjects. The swab was collected from oral cavity of each subject having either candidiasis or Early Childhood Caries and from the removable dentures or orthodontic appliance surfaces by cotton bud sticks (2 swab each) and immediately transferred to the labfor microbiological analysis.*Candida albicans*was included in our study.

Isolation and identification of Candida species

Candida species was isolated and identified using standardmethods. The second swab from the subject was inoculated on Sabouraud'sdextrose agar (SDA). The inoculated agar plate was incubated at 37^0C for upto 14 days. The colony morphologyof the fungal growth on SDA was examined. Species identification and isolation was carried out according to the criteria established by Sidrim and Rocha [27], by the microscopic colony morphology, agar plate growth (Fig 4) and the tests performed in the microbiology lab [28]

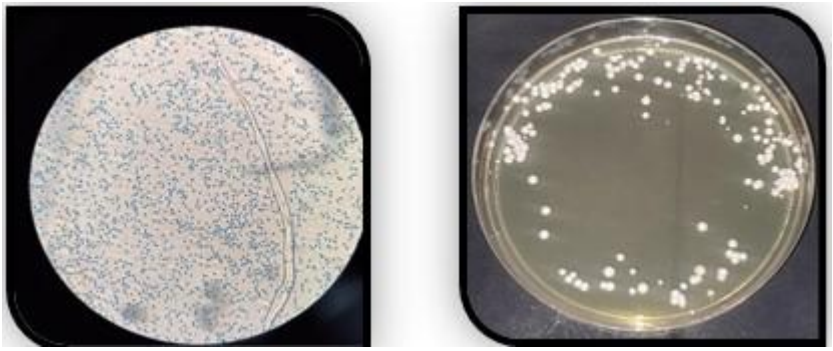


Fig 4: C. albicans microscopic picture and agar plate growth

INCLUSION CRITERIA

Age group 2 to 14 years, with orthodontic appliances, with oral candidiasis, with Early Childhood Caries and patients who give consent/assent

EXCLUSION CRITERIA

Not on any topical or systemic antifungal or antibiotic drugs

PREPARATION OF THE INOCULUM

Inoculum for fungal isolate was prepared by taking four to five colonies with the help of the loop from 24-hour old culture and dissolving them into respective falcon containing 15 mL of sterile saline solution. The suspensions were shaken for 2 min with the aid of a vortex apparatus. After shaking, the turbidity of each suspension was assessed and

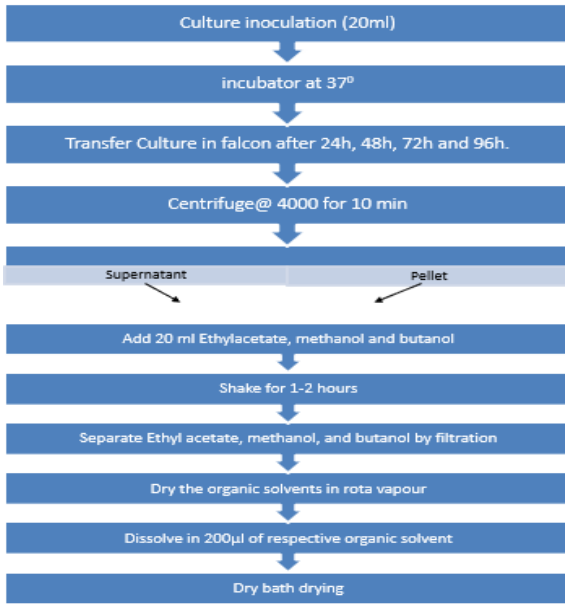
adjusted to produce a turbidity of 0.5 on McFarland scale. This corresponds to a concentration of 10^6colony forming units per ml [29,30]. Cell suspensions were with the fungal isolate were evenly spread on to the freshly prepared respective agar plates using the lawn technique to ensure even distribution of the inoculum. The inoculated agar plates were kept overnight at 37°C in an incubator.

Method for 0.5 McFarland:

0.5 mL of a 1.175% (wt./vol) anhydrous barium chloride solution + 99.5 mL of 1% (vol/vol) sulfuric

PREPARATION OF LACTOBACILLUS CULTURES FOR ANTIFUNGAL ACTIVITY ASSAYS:

Flask containing 100 ml LB broth was used for inoculation of *Lactobacillus* strain. The culture was aerobically grown at 37°C in a shaker incubator for 24 h, 48h, 72h and 96h. At zero time and every 24 h, the samples of culture were harvested under aseptic conditions and transferred in falcon. The samples were centrifuged at 4000 rpm for 15 min at 18 degrees. The broth was used for extraction of secondary metabolites by addition of organic solvent ethyl acetate and butanol. The extractwasthen assayed for antifungal activity against *C. Albicans*(Flowchart 1).



Flowchart 1: Procedure for Probiotic Sample Preparation

METHODOLOGY

GROUPS INCLUDED:

Group I: Ajwain oil group	Group VI: Probiotic group
Group II: Cinnamon oil group	Group VII: Clotrimazole group
Group III: Lemon grass oil group	Group VIII: CHX group
Group IV: Oregano oil group	
Group V: Oil blend group	

PHASE I

- **Gas Chromatography-Mass Spectrometry Analysis:**

The essential oils and probiotic were analysed by GC-MS. The chromatographic analyses were carried out with a Trace GC ultra-gas chromatograph, coupled with a DSQ mass spectrometer. The identification of the components was based on a comparison of their mass spectra with those of a computer library (NIST17R.lib MS Search library). Further confirmation was done by referring to retention index (RI) data generated from a series of alkanes (C10– C23) and comparison with literature data (Aiemsaard et al., Babushok and Zenkevich).

- **Evaluation of Essential Oils (Eo) and Combinations for Antifungal Activity:**

Microbiological studies were performed on strains of *Candida albicans*. The colonies were stored in -4°C until the experiment is performed.

Agar well diffusion method:

Agar well diffusion method is widely used to evaluate the antifungal activity of plants or microbial extracts [31,32]. The SDA plate surface was inoculated by spreading a volume of the microbial inoculum over the entire agar surface with the spreader. Then, a hole with a diameter of 6 mm was punched aseptically with a micropipette tip, and a volume of the 50µl antifungal agent (standard) and 50µl test agent was introduced into the well. The DMSO was used as the negative control and Clotrimazole and Chlorhexidine were used as the positive control. To allow a good diffusion of the agents throughout the agar, the inoculated plates with the tested agents were maintained for 2 hours at room temperature.

INCUBATION

The SDA agar plates inoculated with *C. Albicans*was incubated in aerobic chamber for 48 hours at 37°C —in aerobic conditions.After removing from the chamber, diameter of the zone of inhibition around the wells was measured with the vernier calliper towards the centre and compared. The assays were performed in triplicate.

Assay for Antifungal Activity of Lactobacillus:

Hole-plate diffusion method [33]:

The supernatants and the pellets of *Lactobacillus* were sterilized by filtration through a 0.22µm Milliporefilter paper. Sabouraud's dextrose agar (SDA) plates were inoculated with *C. albicans* andused to test anti-*Candida* effects of the collected supernatants from each culture. 6 mm diameter holes were punched aseptically in each plate with micropipette and filled with 100µl of the cell-free supernatants. The plates were incubated aerobically for 48 h at 37°C and the diameters of the inhibition zones (mm) were measured by vernier calliper and compared. The assay was performed in triplicate.

PHASE II

DETERMINATION OF MIC AND MFC:

Determination of MIC:

The Minimal Inhibitory Concentration (MIC) was determined by microdilution technique [34,35]in 96-well plates divided into eight columns (A to H) and 12 lines. Each column from A-H, line 1, 2, 3, 6, 7 and 8 corresponds to a test agent, C12, D12 corresponds to chlorhexidine,A11, A12was represented by clotrimazole and F12, G12 corresponds to DMSO. The range of concentration of plant products from 10mg/ml to 0.078mg/ml was used from column A to H. Each well contained 100uL Sabouraud's-Dextrose broth, 100uL of test agent and 100uL of the inoculum. Plates were placed in incubator at 37°C for 24 hours. MIC was determined by the visual method using Alamar Blue (AB) dye. The lowest concentration of the test product capable of producing visible inhibition on the growth of *Candida albicans* used in microbiological assays was considered as MIC [36]. Aftervisually seeing the inhibition without the dye, 10µl of Alamar Blue was added, and the plates were shaken and incubated at 37°C for 1 hour. After 1 h in the presence of Alamar Blue, the colour of each well was recorded. The Alamar Blue MIC was defined as the lowest drug concentration resulting in a purplish well 60 min after the addition of Alamar Blue (that accounts for ≤50% reduction of Alamar Blue) [37].The assays were performed in triplicate.

Determination of MFC:

Aliquots of 50 µl corresponding to MIC and the two previous concentrations (MIC, MICx2, MICx4) were sowninPetri dishes containing SDAand then incubated for 24 hours at 37°C. Then the concentrations at which no growth of the pathogen or less than four colony forming units (CFU) was considered as fungicidal concentrations and the lowest considered as MFC. MFC/MIC $\geq$ 4: Fungistatic; MFC/MIC $\leq$ 4: Fungicidal. The Assay was performed in triplicate.

STATISTICAL ANALYSIS

The obtained data was calculated using relevant statistical tools such as Students T test and ANOVA. All the data was analysed thrice. Mean results were evaluated statistically using IBM SPSS VERSION 21. Continuous data was expressed in terms of mean and standard deviation and was compared by using student t test for independent samples and ANOVA for all group comparison. Qualitative data was expressed in terms of proportion and percentages and compared by using chi square test and fisher exact test and the normality of the data was checked by box and whisker plot. P-value < 0.05 was considered as statistically significant and other wise non-significant.

RESULTS

Data presented in this research work were obtained by a step-by step approach based on different techniques to evaluate the biological effect of four essential oils, their blend and probiotic (*Lactobacillus rhamnosus)* against *Candida albicans.*The first step was the identification of the components of the essential oils and probiotic. This was carried out by Analysis using GC-MS. According to the chemical components of the tested essential oils, they are characterized by a few major active components at practically high concentrations. The major constituents of the various Test agents are listed in the table (Table 1)

Table 1: Major Constituents of Test agents by GC-MS Analysis

Test Agent	Major Components	Retention time	Area%
Cinnamon	Cinnamaldehyde, (E)-	8.840	74.48
	cis-Calamenene	12.008	5.46
	.alpha.-Muurolene	11.794	5.46
Oregano	Phenol, 2,3,5,6-tetramethyl-/ Thymol	9.263	83.74
	Linalool	5.991	4.58
	Terpin-4-ol/ endo-Borneol	7.012	1.90
Ajwain	2,3,5-Trimethylanizole/ Thymol	9.092	50.76
	1,3,8-p-Menthatriene/ Mono terpine	5.157	24.49
	Gamma- Terpine	5.550	19.64
Lemon Grass	2,6-Octadienal, 3,7-dimethyl-, (E)-	9.061	44.55
	Neral	8.599	35.10
	D-Limonene	4.999	5.68
Probiotic	Dibutyl phthalate	15.006	30.62
	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	14.885	12.00
	cyclooctasiloxane-hexadecamethyl	13.005	11.37

The second step was evaluation of the antifungal activity of the essential oils against *C. albicans* by using the agar well diffusion method. All essential oils showed significant antifungal activity against *C. albicans* at the concentration of 20mg/ml. Cinnamon showed stronger antifungal activity against *C. albicans*with mean halo of zone of inhibition 19.83±0.29 mm than other essential oils and comparable to clotrimazole 19.17±0.76 mm. While ajwain showed the least zone of inhibition 4.5±0.5. The positive control clotrimazole against *C. albicans* however allowed the formation of regrowth inside the halo (Fig 5 - Fig 8). The Inter group Comparison of mean zone of inhibition of all the groups against *Candida albicans*was found to be highly significant with p value = <0.001 (Table 2, Chart 1). DMSO was used as the negative control and did not show any inhibition of the growth. *Lactobacillus rhamnosus* showed no antifungal effect i.e., no zone of inhibition halo was observed against *C. Albicans*

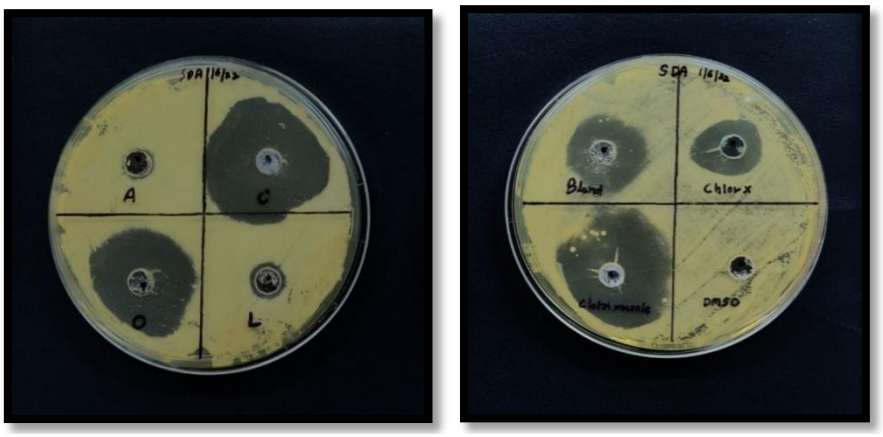


Fig. 5: Zone of Inhibition of Essential oils, oil blend and controls against C. albicans

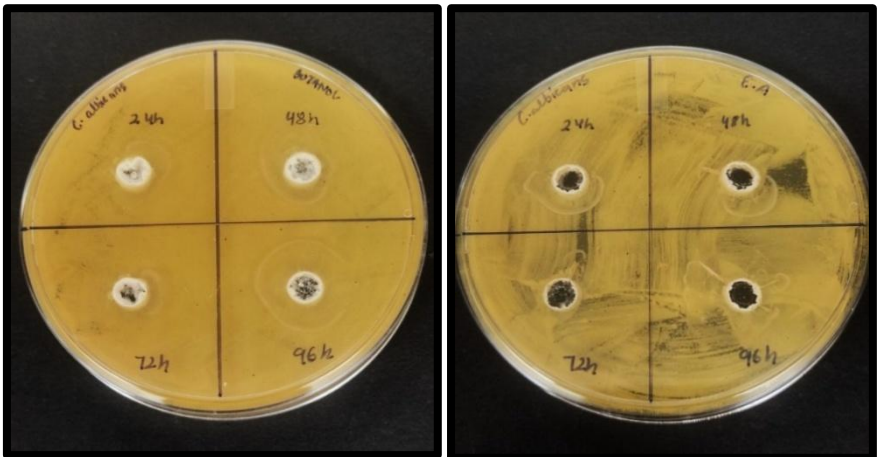


Fig. 6: ZOI of Probiotic with Butanoland Ethyl acetate, against C. albicans

Table2: Inter group Comparison of mean ZOI of all the groups against C. Albicans

Test Agent	Concentration of test agent (mg/ml)	Mean mm	SD	Min	max
Ajwain	20	4.5	0.5	4	5
Cinnamon	20	19.83	0.29	19.5	20
Lemon grass	20	5.83	0.76	5	6.5
Oregano	20	15	0.5	14.15	15.5
Oil Blends	20	11	0.5	10.5	11.5
Chlorhexidine	2%	12	0.5	11.5	12.5
Clotrimazole	20	19.17	0.76	18.41	19.93
DMSO	10%	0	0	0	0
Probiotic (*Lactobacillus rhamnosus*)	1	0	0	0	0
P-VALUE		0.0001*			

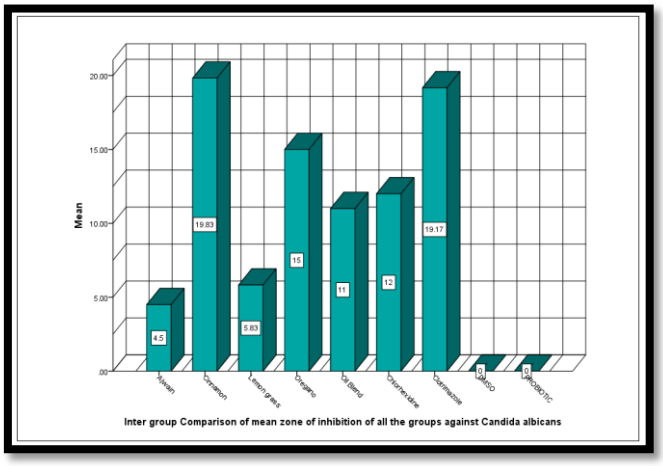


Chart 1: Intergroup Comparison of mean ZOI of all the groups against C. albicans

The next step was determination of MIC of four essential oils viz Ajwain oil, Cinnamon oil, Lemon grass oil, Oregano oil and Oil blend against *C. albicans* with 10% DMSO as negative control and Clotrimazole and Chlorhexidine as positive controls. The oils exhibited concentration dependent inhibition of growth. The Minimum inhibitory Concentration was the concentration at which more than 80% inhibition was there.0.625mg/ml concentration of Cinnamon oil was enough to completely inhibit the growth. Ajwain oil, Lemongrass oil, Oregano oil and Oil blend exhibited inhibition at the concentration of 1.25mg/ml, 5mg/ml, 2.5mg/ml and 5mg/ml respectively (Fig 7). The Inter group Comparison of mean Minimum Inhibitory Concentration (MIC) of all the groups against*Candida albicans*was found to be statistically significant with p value = <0.001 (Table 3, Chart 2). There was mean Percentage reduction of colony forming units by all the oils at MIC with cinnamon being the highest at the lowest concentration (Table 4, Chart 3).

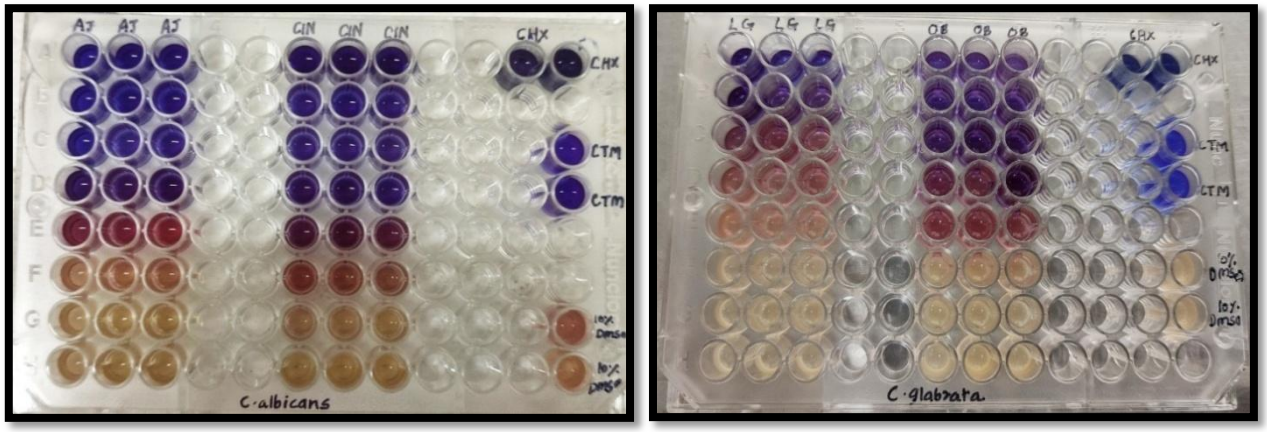


Fig. 7: Plates with alamar blue dye after incubation

Table 3: Inter group Comparison of mean MIC of all the groups against Candida albicans

Essential oil	Concentration of Essential oil (mg/ml)	Mean MIC (mg/ml)	SD	Min	Max
Ajwain	20	1.25	0	1.25	1.25
Cinnamon	20	0.625	0	0.625	0.625
Lemon grass	20	5	0	5	5
Oregano	20	2.5	0	2.5	2.5
Oil Blends	20	5	0	5	5
P-VALUE		0.0001*			

* p value is highly significant

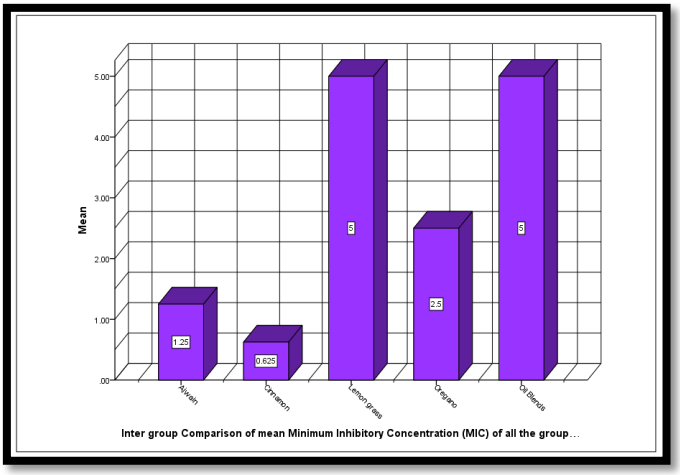


Chart 2: Intergroup Comparison of mean MIC of all the groups against C. albicans

Table 4: Comparison of percentage reduction of CFU/ml (log 10 values) of all the groups at MIC against C. albicans

Essential oil	Concentration of the test agent (mg/ml)	Percentage reduction at MIC
Ajwain	1.25	82
Cinnamon	0.625	99.20
Lemon grass	5	83
Oregano	2.5	87
Oil Blends	5	87
Chlorhexidine	2%	86
Clotrimazole	20mg/ml	99
DMSO	10%	0

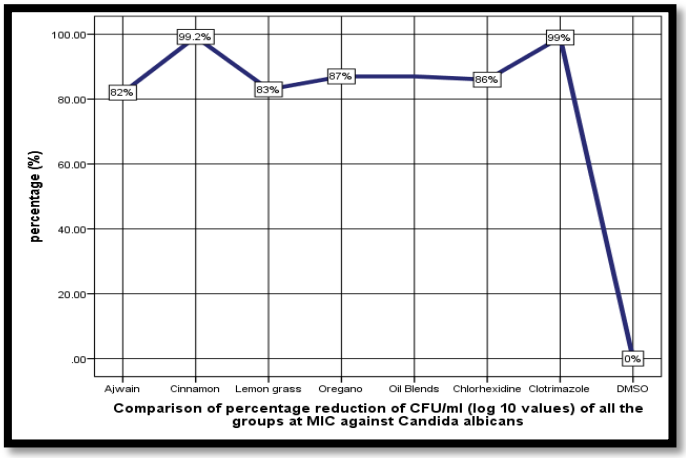


Chart 3: Comparison of percentage reduction of CFU/ml (log 10 values) of all the groups at MIC against C. albicans

After MIC, MFC was determined. The MFC was the lowest concentration of the drug resulting in 99.9% death of the inoculum. All the oils inhibiting growth showed fungicidal activity. In general, the fungicidal concentration was found to be higher than the MIC for all the oils except for the oil blends for which MFC was equal to MIC. Cinnamon oil was the best, having fungicidal activity against *C. Albicans*with mean MFC of 3.33±1.44 mg/ ml (Fig8). Against *C. albicans*, lemon grass and oil blend had the fungicidal activity at higher concentrations of 8.33±2.89 mg/ml and 10mg/ml for both the oils. Ajwain oil and oregano oil had MFC 5mg/ml and 4.17±1.44 mg/ml respectively. The Inter group Comparison of mean Minimum Fungicidal Concentration (MFC) of all the groups against*Candida albicans*was found to be statistically significant with p value < 0.001 (Table5 and Chart 4). There was mean Percentage reduction of colony forming units by all the oils at MFC with cinnamon being the highest at the lowest concentration (Table 6, Chart 5).

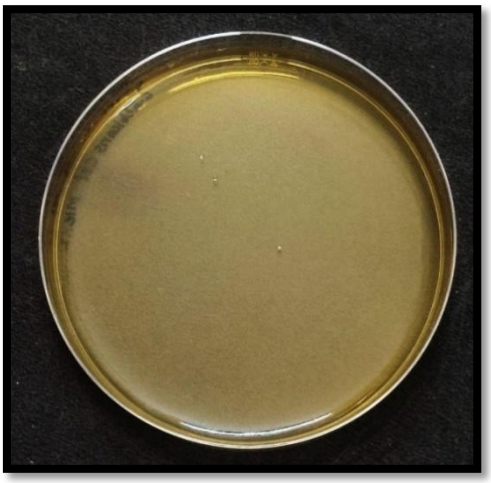


Fig. 8: Colonies on SDA at MICX2/MFC (CEO)

Table 5: Inter group Comparison of mean Minimum Fungicidal Concentration (MFC) of all the groups against C.Albicans

Essential oil	Concentration of oil (mg/ml)	Mean MFC (mg/ml)	SD	p value
Ajwain	20	5	0	0.001*
Cinnamon	20	3.33	1.44	0.001*
Lemon grass	20	8.33	2.89	0.001*
Oregano	20	4.17	1.44	0.001*
Oil Blends	20	10	0	0.001*

* P value is highly significan

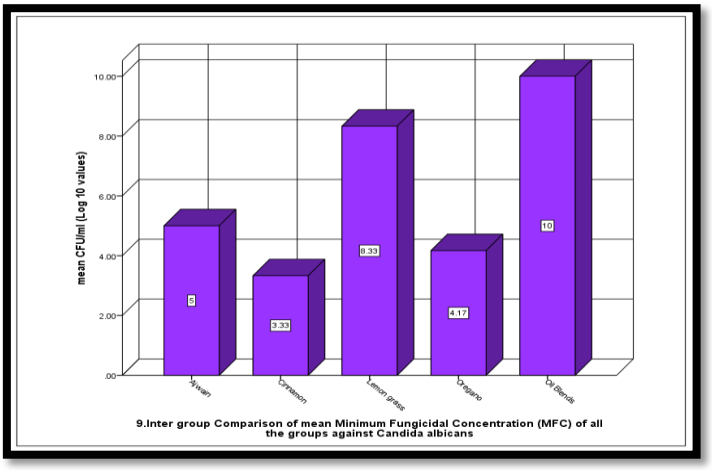


Chart 4: Inter group Comparison of mean Minimum Fungicidal Concentration (MFC) of all the groups against C. albicans

Table 6: Comparison of percentage reduction of CFU/ml (log 10 values) of all the groups at MFC against C. Albicans

Essential oil	Concentration of the test agent (mg/ml)	Percentage reduction at MFC
Ajwain	5	96.90
Cinnamon	3.33	99
Lemon grass	8.33	94.50
Oregano	4.17	95
Oil Blends	10	95
Chlorhexidine	2%	86
Clotrimazole	20mg/ml	99
DMSO	10%	0

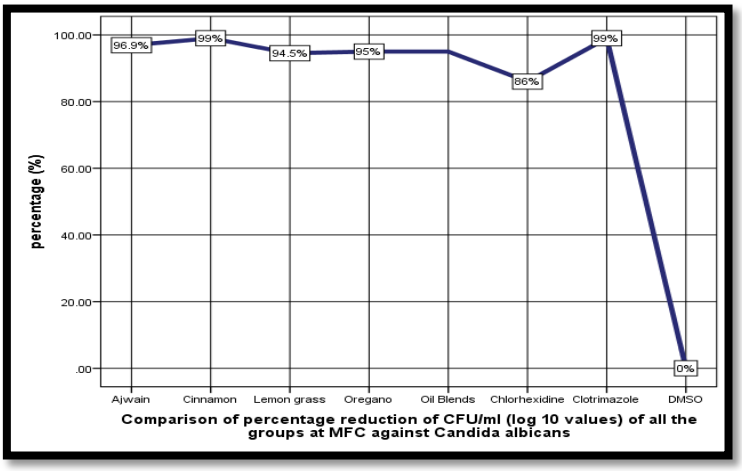


Chart 5: Comparison of percentage reduction of CFU/ml (log 10 values) of all the groups at MFC against C. albicans

DISCUSSION

Candida albicans, the typical fungi causing various oral infections, are the most common fungal pathogens present in the oral cavity [38].To eliminate *Candida* from the human oral cavity, substances that can replace antibiotics are currently being developed [39].Studies on the antimicrobial effect of plant extracts or essential oils have been actively conducted of late, and natural antimicrobial substances that can be used for preventing or inhibiting the progress of these diseases have been reported [40].Essential oils have been steadily studied in relation to causative organisms of dental caries, oral infections, and periodontal diseases [41]but the studies as such on *Candida* are sparse. This study demonstrated the effect of four essential oils viz; ajwain oil, cinnamon oil, oregano oil, lemon grass oil and their combination against*Candida albicans* compared to Clotrimazole and Chlorhexidine.

Azole drugs have fungistatic activity via interference with the synthesis of fungal ergosterol. These broad-spectrum drugs are frequently used for the clinical treatment of microbial and fungal infections. It stimulates phagocytosis and inhibits the filamentous growth of *C. Albicans* [42]. Hence, in the present study, Clotrimazole is taken as the standard

antifungal agent, against which other antimicrobial agents are tested. In this study, the mean zone of inhibition of clotrimazole against *Candida albicans* was 19.17±0.76mm. The values of percentage reduction of clotrimazole for *Candida albicans* was 99%.

The mechanism of action of these drugs involves interference with certain human functional pathways; therefore, they have important side effects on the human body [43].The development of drug resistance among Candida species in clinical infections is problem physicians commonly encounter. Resistance of *C. albicans* to clotrimazole is well documented. Holt and Azmi reported one case of *C. albicans* resistance to clotrimazole, miconazole, and econazole emerging in a pediatric patient previously treated for 2 months with miconazole [44].

Chlorhexidine digluconate is used as a broad-spectrum antiseptic often incorporated in mouth rinses. It has a broad spectrum of activity against a variety of organisms, including *C. albicans*. Chlorhexidine is capable of inhibiting candidal adhesion to biological and inert surfaces [45].It acts as a fungicide and has a fungistatic function, leading to the coagulation of nucleoproteins and changes in cell walls allowing the possible escape of cytoplasmic components through the plasmalemma.The ability of CHX to inhibit Candida growth is well documented [46,47]. Giuliana et al [48]. found that chlorhexidine is an effective means to inhibit candidal growth due to its ability to break the permeability barrier and leakage of cytoplasmic contents. Hence it was used as a positive control in this study along with Clotrimazole. Further in 2007, Torres et al [49]revealed that even though chlorhexidine reduces *C.albicans* counts, when patient discontinues treatment, intensity of colonization rises again. In the present study chlorhexidine showed significant antimicrobial activity against *C.albicans* with a mean zone of inhibition of 12±0.5 mm and the difference with that of clotrimazole was statistically significant (*P* value 0.001), with clotrimazole being more effective.In this study, the percentage reduction of chlorhexidine against *C. albicans* was 86%.

This study evaluated the anti-candidal activity of four plant essential oils, their combination, and probiotics, with their antimicrobial properties recognized and well documented [50,51].The test agents viz Cinnamon essential oil, Ajwain essential oil, Lemon grass essential oil, Oregano essential oil, Oil blends and Probiotic were screened using agar well diffusion assay. The antifungal activity was assessed by MIC and MFC using broth dilution method. The mean ZOI order against *Candida albicans* is CIN = CTM > ORG > CHX > OB > LG > AJ > Probiotic. The mean MIC order against *Candida albicans* is CIN > AJ > ORG > OB = LG. The mean MFC order against *Candida albicans* is CIN > AJ = ORG > LG = OB. The plant oils were screened using this very convenient agar well diffusion assay method. This study indicates that caution is needed since different oils may have different diffusion rates on agar plates and this may contribute to variation in the inhibitory zones, leading to erroneous conclusions regarding their antifungal activity. For example, some of the oils which exhibited smaller inhibition zones compared to others like Ajwain oil were very effective against *Candida* strains in the microbroth dilution assay which does not involve diffusion. For example, against *Candida albicans*, Ajwain oil exhibited a ZOI of 4.5±0.5 mm in the disc diffusion assay, whereas Oregano oil showed 15±0.5 mm. But in the MIC assay Ajwain oil (1.25ug/ml and 1.25ug/ml) was found better than Oregano oil (2.5ug/ml). It is encouraging to note that most of the oils used in this study were fungicidal at low concentrations. In this present study, Cinnamon had the maximum mean zone of inhibition of 19.83±0.29 mm against *C. albicans* which was more than all the other oils and antifungal controls and the results were statistically significant with p value =0.001. These results were in consistent with the studies by Carvalhinho et al. and Condò et al., that showed significant activity of cinnamon against *Candida albicans* [52,53]. Gucwa et al., exhibited both fungistatic and fungicidal activity of some plant oils towards *Candida albicans* isolates and the highest activity was demonstrated for cinnamon oil [54]. Against *C. albicans*, 0.625mg/ml was the Minimum Inhibitory Concentration (MIC) of Cinnamon oil enough to completely inhibit the more than 80% of growth. The results were consistent with the study of Aneja et al[55].Depicting that Cinnamon extract exhibits even much higher antifungal activity than the standard antifungal drugs against Candida. The Comparison of Baseline and Post mean Colony Forming Unit/ml (CFU/ml) (Log10 values) of Cinnamon at MIC *against Candida albicans*was statistically significant with p value<0.05 and the intergroup comparison of the CFU/ml of cinnamon with the rest of test agents was also highly significant with p value=0.001. Cinnamon oil was the best, having fungicidal activity against *C. albicans* with mean MFC of 3.33±1.44 mg/ml. These results were similar as found in the study by Carvalho et al [56], Brnawi et al.[57]and Ranasinghe et al. [58] who found cinnamon essential oil (CEO) to be a more effective antifungal agent. The results of Comparison of Baseline and Post mean Colony Forming Unit/ml (CFU/ml) (Log10 values) of all the groups at MFC and intergroup comparison of all the groups against*Candida albicans*was statistically significant with p value= <0.05. The percentage reduction of planktonic *Candida albicans* at MIC and MFC was 99% and 99.2% respectively. In *C. albicans*, virulence gene expression is linked to its ability to transition from yeast form to hyphal form [59].In the presence of CEOs, hyphal formation was inhibited in the *C. albicans*, suggesting a potential mechanism of action of the EO as hyphae formation is associated with release of virulence factors.

According to a WHO/FAO report (2002), probiotics are "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host." Use of probiotics and molecular genetics to replace and displace cariogenic micro-organisms with noncariogenic micro-organisms has shown promising results. Lactobacilli are bacteria that naturally colonize the oral cavity and gastrointestinal tract of healthy humans. In the oral cavity, certain strains of lactobacilli can cause caries through their acidogenic and aciduric characteristics and are frequently detected in lesions of deep cavities. However, some studies suggest an additional beneficial role for oral lactobacilli. Hatakka et al [60]showed

a reduced prevalence of *C. albicans* after taking probiotics.The clinical studies and animal model experiments reported that probiotics such as *L. rhamnosus* have the effect of inhibiting the excessive growth of Candida [60-62].The Results of our study were in accordance with that obtained and data published recently by Strahinic et al. [63]where it was reported that the growth of *C. albicans* was not inhibited by the probiotics. The probiotic in the present study was *Lactobacillus rhamnosus*, the extracts used were ethyl acetateand butanol but it didn't inhibit the growth of *C. albicans*. These conflicting results regarding antimicrobial activity could be the result of differences in methodology, but could also be related to microbial factors, like the strain-specific antimicrobial activity of lactobacilli as well as variability in the sensitivity of different target strains.

SUMMARY AND CONCLUSION

The present study was carried out to compare the antifungal efficacy of phytochemicals, probiotic and their combinations against *Candida albicans*.The test agents viz Cinnamon essential oil, Ajwain essential oil, Lemon grass essential oil, Oregano essential oil, Oil blends and Probiotic were screened using agar well diffusion assay. The antifungal activity was assessed by MIC and MFC using broth dilution method.The results of the present study provided conclusive evidence that Essential oils specifically Cinnamon Oil could be used against Candida present as various source of infections. It could be used for prevention of progression of Early Childhood Caries, as a root canal Irrigant and as a treatment for oral candidiasis and for prevention of the same.However, further studies should be undertaken to evaluate the significance of these essential oils and more of in vivo studies should be carried out to make it a boon for the dentistry.

The following conclusions could be drawn from the present study:

1) Cinnamon showed the maximum antifungal activity compared to the rest of the essential oils and the positive controls followed by oil blends and lemongrass.
2) *Lactobacillus rhamnosus* showed no antifungal effect i.e., no zone of inhibition halo was observed against *C. albicans*.
3) The mean ZOI order against *Candida albicans* is CIN=CTM>ORG>CHX>OB>LG>AJ>Probiotic
4) The mean MIC order against *Candida albicans* is CIN>AJ>ORG>OB=LG
5) The mean MFC order against *Candida albicans* is CIN>AJ=ORG>LG=OB

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