Assiut University Journal of Multidisciplinary Scientific Research (AUNJMSR)
Faculty of Science, Assiut University, Assiut, Egypt.
Printed ISSN 2812-5029
Online ISSN 2812-5037
Vol. 52(2): 195- 231 (2023)
https://aunj.journals.ekb.eg

Mycobiota associated with teeth and oral cavity and their relevant to dental and periodontal diseases

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ARTICLE INFO

Article History:
Received: 2023-03-30
Accepted: 2023-04-27
Online: 2023-04-30

Keywords:
Dental caries, *Candida ciferrii* , *Cryptococcus laurentii*, Lipase, Protease, Hexitol

ABSTRACT

Teeth and oral cavity harbor diverse community of fungi and bacteria. Little is known about fungal community of oral cavity, teeth and gingiva. In this study, fungal diversity in various sites was evaluated using Sabouraud dextrose agar (SDA). Sixty swabs samples from patients suffering from gingivitis, periodontitis and dental caries in addition to healthy persons as control (15 each) were collected for fungal analyses. Thirteen fungal species related to 7 genera were isolated and identified. To the best of our knowledge, *Candida ciferrii* and *Cryptococcus laurentii* were recorded for the first time from the oral cavity in the current study. There is a highly significant variation in the frequency of *Aspergillus niger* and *Candida ciferrii* among isolated fungi. Screening of the yeast isolates for extra-cellular enzymes production resulted in 47.2% (34 out of 72 isolates) of the tested isolates showed positive lipolytic activities with varying capabilities; whereas 41 isolates (56.9%) were positive protease producers. Also, the antimicrobial activity of some toothpastes, mouth washes, natural oils and pharmaceutical preparations against yeast species were performed. Among the tested substances, Paradontax, Hexitol and Clove oil showed the best antimicrobial

activity against the tested yeast isolates. The minimum inhibition concentration (MICs) of Hexitol was high compared to Paradontax and Clove oil. This study provided clear image about fungal content in mouth, teeth and gingiva, the relation of these fungi in case of health or disease was also discussed.

INTRODUCTION

Oral microbiota is one of the most complex and diverse microbial ecosystems within the human body [1]. It has several appropriate niches for many microorganisms, as it is humid, warm, and nutrient-rich environment. These niches include the tongue, cheeks, palate, tonsils, gingival pockets, teeth, and saliva that enable the coexistence of numerous microorganisms within the complex and protecting structure forming a biofilm. The oral cavity is inhabited by more than 700 microbial species and many intrinsic and extrinsic factors affect the composition, metabolic activity and pathogenicity of the highly diverse microbiota [2]. The colonization of the oral cavity by fungi starts during childbirth [3] and progresses with age [4]. The majority of the oral adult mycobiome composition represents the phylum Ascomycota, Basidiomycota, Glomeromycota and Mucoromycota. These four groups include approximately 81 genera and 101 species of fungi [5, 6]. The most common representatives of the basal oral mycobiome originate from the phylum Ascomycota, namely the yeast-like genus *Candida* [5, 7-9].The less prevalent yeasts that have been reported are *Aureobasidium, Saccharomyces* and *Pichia* [7, 10, 11]. Some of the identified filamentous micromycetes are genera *Penicillium, Cladosporium, Aspergillus* and *Alternaria* [5, 7-9]. Some studies showed that airborne fungal genera such as *Phoma* and *Epicoccum,* whose spores could be inhaled by the study participants without the subsequent colonization of the oral cavity [7, 12]. Basidiomycota representatives by *Rhodotorula, Malassezia* and *Cryptococcus.* Minor relative abundances of other representatives are rarely reported [5, 7, 12].

Numerous studies have suggested a possible role of the genus *Candida*, the common isolated genus, in oral dysbiosis. This genus can participate in biofilm structure and function. It is able to form dentinal tubules and bind to denatured collagen and

secrete aspartyl proteases, resulting in the demineralization and dissolution of the dental hard tissues [13]. Teeth are composed of enamel, pulp–dentine complex, and cementum. Enamel has little or no collagen, and its organic matrix is made up of noncollagenous protein [14].The inorganic component of these hard tissues consists of biological apatite, $Ca_{10}(PO4)_6(OH)_2$ also known as calcium phosphate, and is naturally occurring mineral that's make up over 90% of our tooth enamel. Under normal conditions there is a stable equilibrium between Calcium and phosphate ions in saliva and the crystalline hydroxyapatite. When PH drops below a critical level (PH=5.5), it causes the dissolution of tooth mineral (hydroxyapatite)[15] due to the production of acid by oral microorganisms. The acidic environment may result from metabolic end products of dental plaque, usually organic acids produced through the fermentation of carbohydrates [16]. Dental plaque is the result of a large number of interactions between microorganisms that are present in the oral biofilm on the tooth surface [17]. *Candida* species are common colonizers of mucosal surfaces in oral cavity [18] and they are capable to adhere to tooth surfaces, participate in biofilm formation and proceede to carbohydrate fermentation. *Candida* possesses adhesive and proteolytic properties that are fundamental for dentinal caries progression. The adherence and dissociation of *Candida albicans* hydroxylapatite (HAP), which exhibit various hydrophobicity, was study by Nikawa et al. [19]. It was showed that the adherence between the yeast and HAP was extraordinary high [19, 20]. This association was effectively removed by high concentration of either phosphate or calcium ions, suggesting that either electrostatic interactions or ionic bonds between proteinaceous components of fungal cell wall and HAP may play an important role in tooth demineralization. *Candida* is dependent on high concentration of dietary sugars to produce acid [21], which leads to the dissolution of hydroxyapatite crystals in enamel and dentin. The C. albicans enzyme degraded both the native acid-soluble collagen and the insoluble dentinal collagen [22, 23] . It was though that the yeast may utilize the dentinal structure. which could help to promote the carious process in dentin [24]. The abundance of *C. albicans* and *C. dubliniensis* correlated with dental caries in early childhood, indicating that *C. dubliniensis* could be implicated in disease pathogenesis [25]. A higher abundance of *C. glabrata* resulted in tongue infections and the interactions between *C. glabrata* and hyphae of *C. albicans* established

oropharyngeal candidiasis [26]. Other etiological agents of oropharyngeal candidiasis are *C. parapsilosis*, *C. tropicalis* and *C. krusei* [26]. Despite the widespread use of oral hygiene products, the periodontal disease remains one of the most common diseases affecting adults and children [27]. Supragingival microbes are responsible for the mild course of gingivitis, and root caries disease. Subgingival microbial species accelerate the destruction of the tissues that support the teeth and might cause severe periodontitis [28]. Periodontitis is a chronic inflammatory disease that is triggered by bacteria in the oral cavity. As inflammation persists, periodontal tissues such as gingiva, periodontal ligament, cementum, and alveolar bone are destroyed. Periodontal tissues have an immune system against the invasion of these bacteria, however, due to the persistent infection by periodontopathogenic bacteria, the host innate and acquired immunity is impaired, and tissue destruction, including bone tissue destruction, occurs. Osteoclasts are essential for bone destruction. Osteoclast progenitor cells derived from hematopoietic stem cells differentiate into osteoclasts. In addition, bone loss occurs when bone resorption by osteoclasts exceeds bone formation by osteoblasts [29]. Gingivitis is a common and mild form of gum disease (periodontal disease) that causes irritation, redness and swelling (inflammation). Periodontitis is a severe gum infection that can lead to tooth loss and other serious health complications [30]. Yeasts can be expected to be present in periodontal pockets in one out of 6 periodontal patients independent of gender and age [31].

Most previous studies on periodontal diseases have limited their focus to the oral bacteriome and few have indicated a possible role of the fungal microbiome. Thus, this work was aimed to study oral Mycobiota in healthy and diseased (gingivitis, periodontitis and dental caries) individuals and to test the sensitivity of some isolated oral mycobiota to some pharmaceutical preparations and natural oils. The capability of the isolated fungi for lipase and protease enzymes was also assessed in addition to discuss the relationship of these microorganisms in health and disease.

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cheeks, palate, tonsils, gingival pockets, teeth, and saliva that enable the coexistence of numerous microorganisms within the complex and protecting structure forming a biofilm. The oral cavity is inhabited by more than 700 microbial species and many intrinsic and extrinsic factors affect the composition, metabolic activity and pathogenicity of the highly diverse microbiota [2]. The colonization of the oral cavity by fungi starts during childbirth [3] and progresses with age [4]. The majority of the oral adult mycobiome composition represents the phylum Ascomycota, Basidiomycota, Glomeromycota and Mucoromycota. These four groups include approximately 81 genera and 101 species of fungi [5, 6]. The most common representatives of the basal oral mycobiome originate from the phylum Ascomycota, namely the yeast-like genus *Candida* [5, 7-9].The less prevalent yeasts that have been reported are *Aureobasidium, Saccharomyces* and *Pichia* [7, 10, 11]. Some of the identified filamentous micromycetes are genera *Penicillium, Cladosporium, Aspergillus* and *Alternaria* [5, 7-9]. Some studies showed that airborne fungal genera such as *Phoma* and *Epicoccum,* whose spores could be inhaled by the study participants without the subsequent colonization of the oral cavity [7, 12]. Basidiomycota representatives by *Rhodotorula, Malassezia* and *Cryptococcus.* Minor relative abundances of other representatives are rarely reported [5, 7, 12].

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impaired, and tissue destruction, including bone tissue destruction, occurs. Osteoclasts are essential for bone destruction. Osteoclast progenitor cells derived from hematopoietic stem cells differentiate into osteoclasts. In addition, bone loss occurs when bone resorption by osteoclasts exceeds bone formation by osteoblasts [29]. Gingivitis is a common and mild form of gum disease (periodontal disease) that causes irritation, redness and swelling (inflammation). Periodontitis is a severe gum infection that can lead to tooth loss and other serious health complications [30]. Yeasts can be expected to be present in periodontal pockets in one out of 6 periodontal patients independent of gender and age [31].

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MATERIALS AND METHODS

1. Collection of samples

This study was carried out on 60 cases of both sexes from the out-patient clinic, Oral Medicine and Periodontology Department, Faculty of Dentistry, Al-Azhar University, Assiut, Egypt. These cases were classified into 4 groups, healthy, gingivitis, periodontitis and dental caries (15 samples each). The samples were collected by sterilized swabs from tooth, gingiva, tongue, saliva for each case and transferred immediately to the Mycological lab for fungal analysis. A medical history was taken for the cases if they are suffering from chronic diseases or smokers

2. Mycological analysis of the samples

The collected swabs were cultured by streaking on the surface of Sabouraud dextrose agar (SDA) only to allow fungal growth. All cultures were incubated under

aerobic conditions at 30°C for 7 day until the colonies appear. Microscopic examination was done to ensure the presence of fungal propagules.

2.1. Identification of filamentous fungi

Fungal isolates recovered were identified based on their macro- and microscopic features using the following keys of Raper and Fennell [32], Pitt [33], Leslie and Summerell [34]. Ellis [35].

2.2. Identification of yeast isolates

The developing yeast colonies were purified and characterized using traditional methods such as germ tube test, chlamydospore formation, and plating on CHROMagar *Candida* [36].Some isolates were confirmed by the carbohydrate assimilation and fermentation patterns which evaluated at the Department of Clinical Pathology, Assiut University Hospital, Assiut University, Assiut, Egypt, using the VITEK 2 identification system (Model: Compact 15; BioMérieux; France), according to the Yarrow [37] and Barnett *et al*. [38] protocols using pure colonies streaked in SDA medium, incubated at 37 °C for 24 h.

3. Morphological identification of yeast isolates

3.1. Growth on CHROM agar *Candida* Plus medium

CHROM agar (CHROM agar Company, Paris, France) is a good differential medium for some *Candida* species [39]. The growing isolates of *Candida* were inoculated on the surface of CHROM agar plates and incubated at 30 °C for 24 – 48 h.

3.2. Germ tube test (GT)

Candida albicans and *C. dubliniensis* are two closely related species, Germ tube test was used for differentiate both species from other *Candida* species. Yeast isolates were inoculated in a small sterile tube containing 0.5 ml of human serum and incubated at 37 °C for 2 – 4 h. Then, a loop -full of the culture was transferred to a glass slide and examined microscopically for formation of GT [40].

3.3. Chlamydospore production (Dalmau plate technique)

Corn Meal Agar (CMA) containing 1% Tween 80 was prepared and poured in 90 mm sterile Petri dish. The plates were then inoculated with yeast isolates using a sterile straight wire. A flame-sterilized glass cover 22 mm square was placed over the inoculum to provide partially an anaerobic environment at the margins of the cover slip. The plates were incubated at 25 °C for 3-5 days. Examination was carried out by removing the lid of the Petri dish and placing the plates on the microscope stage to observe the edge of the glass cover to detect morphological features of the yeast strain, such as hyphae, pseudohyphae, blastospores and chlamydospores [41].

4. Molecular identification of fungal isolates:

Seven-day old culture on SDA was sent to the Molecular Biology Research Unit, Assiut University for DNA extraction using Patho-gene-spin DNA/RNA extraction kit provided by Intron Biotechnology Company, Korea. The fungal DNA was then sent to SolGent Company, Daejeon South Korea for polymerase chain reaction (PCR) and gene sequencing. PCR was performed using forward (ITS1) and reverse *ITS4) primer set which were incorporated in the reaction mixture. The purified PCR product (amplicons) was sequenced with the same primers [42]. The obtained sequences were analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website. The sequences of the identified strains were submitted to NCBI giving accession numbers and phylogenetic trees were constructed using MUSCLE (Phylogeny.fr).

5. Screening of yeast isolates for extracellular enzyme production

5.1. Protease enzyme

Casein hydrolysis medium was prepared in tubes, inoculated with the tested isolates and incubated at 35 ± 2 ºC for 7 days. After incubation, protease producing fungi resulted in complete degradation of milk protein that was seen as a clear depth in the tube. The clear depth below the colony was measured in mm [43].

5.2. Lipase enzyme

Detection of lipase production by isolated yeasts was carried out on the medium of Ullman and Blasins [44]. The medium containing tubes were inoculated with the tested isolates and incubated at 35 ± 2 ºC for 7 days. Then, the lipolytic ability of the isolate was expressed as a visible precipitate due to the formation of crystals of calcium salt of the oleic acid liberated by the enzyme. The depth of each visible precipitate was measured (in mm).

6. Antimicrobial susceptibility test

Agar well diffusion technique was employed to evaluate the antimicrobial activity of 9 compounds including 4 pharmaceutical preparation toothpastes (Close-up, Signal, Sensodyne and Paradontax) and 3 mouth washing solutions (Hexitol, Betadine and Platina) and 2 natural oils (Clove oil and Ginger oil) against isolated yeast. Cultures were prepared using yeast extract -malt extract (YM) agar medium. After solidification 50 µ l from each substance tested which dissolved in DMSO (dimethylsulphoxide) were put in the agar well (0.5 cm diam). The plates were incubated for 24 – 48 h at 30 °C. Antimicrobial activities were expressed as the diameters of inhibition zone (in mm).

7. Determination of minimum inhibition concentration (MIC):

The double fold micro-dilution method was used according to the Clinical and Laboratory Standards Institute. A hundred microliters were prepared starting with the original concentration used in the screening experiment, with various concentrations of two-fold serial dilution of each of the most effective drugs (Clove oil, Signal and Hexitol). One ml of the spore suspension of each of 16 isolates yeast was dispersed with 20 ml Yeast extract and Malt extract (YM) medium in the agar plate. After solidification, 50 µl of each concentration were put in the agar well (0.5 cm diam). The plates were incubated at 30°C for 24 – 48 h, the minimum inhibitory concentration (MIC) was expressed as the lowest concentration that inhibits the visible growth of each strain [45].

Statistical analysis

Frequency of occurrence fungi isolated from each group was subjected to chi-squared analysis using IBM "SPSS" statistics Version 25**.** To determine the differences in antimicrobial activity of various pharmaceutical preparation and natural oils tested, one-way analysis of variance (ANOVA) and Duncan Multiple range tests were performed between different yeast isolates using SPSS statistics software. $P < 0.05$, 0.01 or 000 was determined as significant, highly significant or very high significant, respectively.

RESULTS AND DISCUSSION

During this study, a total of sixty cases of healthy, gingivitis, periodontitis; gum disease starts with mild inflammation called gingivitis. If it's left untreated, it progresses to periodontitis and dental caries (15 each) were sampled form 4 different, sites one swab had taken from 4 sites (tooth, gingiva, tongue and saliva) for each case for fungal biodiversity including 73.3% females and 26.6% males. This may be because females are more interesting with their health and high dental hygiene. The cases located in age group ranged between 19-62 (healthy), 15–75 (gingivitis), 20–60 (periodontitis) and 15–65 years (dental caries). From the studied cases, 20 usually use brushing (healthy, 10; gingivitis, 5, periodontitis, 2 and dental caries, 2 cases), 4 cases use mouth washes. Healthy status of dental caries patients were irregular teeth brushing, and increase soda pop consumption. There are no chronic diseases except one case having high blood pressure and another one have breast cancer. Two of gingivitis patients are smokers and one of them have high blood pressure. Periodontitis patients also, have no chronic disease except one that has respiratory disease Pneumonia.

1. Fungal analysis

Mycobiota analysis associated with 4 different sites in mouth (tooth, gingiva, tongue and saliva) resulted in a total of 210 isolates representing 13 identified species belonging to 7 fungal genera (Table 1). These fungi were isolated from healthy (4 species and 2 genera); gingivitis (9, 6); periodontitis (10, 5); and dental caries (8, 5) (Table 2). The results revealed that *Aspergillus niger* and *A. flavus* were isolated from gingivitis, periodontitis and dental caries patients with 8.09%, 9.04% of the total isolates

respectively. In this study *Aspergillus niger* was isolated from periodontitis and dental caries cases only and couldn't isolate from gingivitis cases or healthy persons. *Aspergillus niger* was previously isolated from the oral cavity of a young woman with underlying disease, and could be isolated from 75% of the individuals, followed by *Cladosporium*, Saccharomycetales, *Fusarium* and *Cryptococcus* [46].

Penicillium (2 species) was occupied the second place behind *Aspergillus*. It was isolated from gingivitis represented 5.71% of the total fungal isolates. From the genus *P. citrinum* (3.8%) and *P. chrysogenum* (1.9%) were identified. *Fusarium* (represented by 2 species) were isolated from gingivitis and periodontitis patients with percent (1.42 %). Two species from the genus namely *F. solan* (0.95%) and *F. oxysporum* (47%) were isolated. *Fusarium* species are the second-most common mould causing a broad spectrum of infections in humans, including superficial, locally invasive, and disseminated infections. invasive fungal infections in immunocompromised individuals [47].

Although, in the present work *Cladosporium cladosporioides* was isolated rarely represented by 2 isolates comprising 0.95 % of the total isolates from dental caries and periodontitis cases, Ghannoum *et al*. [5] concluded that the oral cavity contained 74 culturable and 11 non-culturable fungal genera. They identified 101 species belonging to *Cladosporium* (65%), Saccharomycetales (50%), *Aspergillus* (35)%, *Fusarim* (30%) and *Cryptococcus* (20%) *Cladosporium cladosporoides* was present in 53% of total dental plaque samples of Australian children [13].

Bibi Khadija[10] investigated salivary mycobiome of three postpartum females along with one healthy non-pregnant female omit by targeting ITS region. They detected a total of 55 genera and 92 species as follow: *Penicillium* (6.85%), *Aspergillus* (6.56%), *Olpidium* (5.15%), *Cochliobolus* (4.78 %), *Malassezia* (4.61%), *Neurospora* (4.3%), and *Cristinia* (3.04%) in all samples. The filamentous fungi identified in oral samples were mostly ubiquitous moulds. The most prevalent were *Penicillium* spp., *Aspergillus* spp., and *Cladosporium* spp., which are among the most frequent airborne moulds found indoors and outdoors[48].

The results in Tables (1, 2) showed that *Candida* was the most prevalent fungi. It was isolated from 70% of the total isolates. From the genus, 5 species were identified of which *Candida albicans* species was the most common fungal species, being represented by 63 isolates comprising 30% of total yeast isolates followed by *C. glabrata* and *C. tropicalis* (20.95% and 12.85 % of total isolates, respectively). *Rodotorula* spp and *Cryptococcus laurentii* were less encountered isolated from 2.85% and 1.9% of the total yeasts, respectively. The remaining yeast species (*C. ciferrii* and *C. dubliniensis*) were less frequent encounted in 6.13% of the total yeast (Table 1). In this study *Candida ciferrii* and *Cryptococcus laurentii* were recorded for the first time from the oral cavity, to the best of our knowledge. Identification was performed by automated Vitek2 system (BioMerieux ®).*C. ciferrii* was isolated rarely from periodontitis cases, while it was reported previously from clinical specimens of onychomycosis [49]. Also it has been reported to cause various human infections such as endophthalmitis, intraorbital abscess, systemic mycosis, and otitis media [50-52]. *Cryptococcus laurentii* was isolated rarely from gingivitis cases while it was reported previously from the blood of a cancer patient [53] This fungus was previously considered saprophytic and non-pathogenic to humans, but it has been isolated as the etiologic agent of infections such as skin infection, keratitis, endophthalmitis, lung abscess, peritonitis, meningitis, and fungemia [54, 55]. The highest frequent species in periodontitis and gingivitis cases was *C. albicans* but *C. glabrata* was considered a relatively nonpathogenic commensal fungus of human mucosal tissues, this may explain their predominant in healthy cases (Table 2). In patients with chronic periodontitis, *C. albicans* has been the most isolated species because it has been typically found on the ou.ter layers of the plaque and deep in periodontal tissues [56]. This fungus has a range of virulence factors that can be potentially relevant to the pathology of periodontal disease, such as the ability to adhere and invade gingival connective tissue [57]. One important virulence characteristic of *C. albicans* is its cell surface hydrophobicity due to the mannosylated surface proteins that cover the fungal cells [58]. Some of these proteins allow the yeast to adhere to the host cells through the increase in hyphal forms and resistance against macrophages. When yeasts gain access to underlying periodontal tissues , more damage may result from the metabolites produced by them [59]. *C. albicans* can secrete proteinases enzymes which are able to

degrade extracellular matrices and membrane components [22, 60], it has the capacity to interact with periodontal pathogens and influence their behaviors. Specific bacteria frequently co-isolated with *C. albicans* in periodontal pockets include the anaerobes. *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. Remarkably, it has been shown that fungi are able to rapidly deplete oxygen within mixed species environments, which may explain why obligate anaerobes and yeasts are observed together [61]. For instance, *P. gingivalis* modulates and enhances the germ tube formation of *C. albicans* [62].

From all studied cases, 8, 1 and 2 healthy, gingivitis and dental caries cases were free from fungal contamination. Kleinegger *et al.* [63] concluded that number of natural barriers are established in the mucosal surfaces and body fluids, preventing the colonization in healthy individuals. These barriers depend on factors related to age, gender, smoking, diet, drugs, and the host immune status. Furthermore, lactoferrin and lysozyme, in addition to, other important proteins are the main proteins in the innate immune response present in saliva and exert an antifungal and antibacterial modulating effect on the implantation of fungal and bacterial species in the oral cavity [64]. Furthermore, saliva has antifungal substances that control fungi in oral cavity and consequently keep balance between normal bacteria and fungi in the mouth. Reduction of these antifungal components is one of the most factors predisposing oral fungal infection [65].

Table 1: Number and percentage of the appearance of fungal isolates from all cases

Genera and Species	No. of isolates	%
Aspergillus	36	17.14
A. flavus Link	19	9.04
A. niger Van Tieghum	17	8.09
Penicillium	12	5.71

P. chrysogenum Thom	4	1.9
P. citrinum Thom	8	3.8
Fusarium	3	1.42
F. oxysporum Schltdl	1	0.47
F. solani Martius	2	0.95
Cladosporium cladosporioides *(Fresen.) G.A. de Vries*	2	0.95
Candida	147	70
C. albicans (C.P. Robin) Berkhout	63	30
C. ciferrii Kreger-van Rij	3	1.42
C. dubliniensis D.J. Sullivan	10	4.76
C. glabrata H.W. Anderson	44	20.95
C. tropicalis (Castell.) Berkhout	27	12.85
*Cryptococcus laurentii (*Kuff.) C.E. Skinner	4	1.9
Rhodotorula spp F.C. Harrison	6	2.85
Total isolates		
No. of genera = 7		
No. of species = 13	210	100

Table 2: Comparisons of frequencies of fungi collected from healthy, gingivitis, periodontitis and dental caries cases using Chi-square test

Fungal Taxa	Healthy		Gingivitis		Periodontitis		Dental Caries		Phi
	P	A	P	A	P	A	P	A	
Aspergillus									
Aspergillus niger	0	60	0	60	9	51	8	52	0.277***
Aspergillus flavus	0	60	4	56	10	50	5	55	0.22
Penicillium									
penicillium citrinum	0	60	4	56	2	58	2	58	0.131
Penicillium chrysogenum	0	60	4	56	0	60	0	60	0.225
Fusarium									
F. solani	0	60	4	56	0	60	0	60	0.225
F. oxysporum	0	60	0	60	3	57	0	60	0.195*
Cladosporium cladosporioides	0	60	0	60	4	56	4	56	0.131
Candida spp.	25	35	22	38	28	32	29	31	0.092
C. albicans	12	48	16	44	18	42	17	43	0.086
Candida ciferrii	0	60	0	60	3	57	0	60	0.195*
C. dubliniensis	3	57	2	58	3	57	2	58	0.042
C. glabrata	15	45	7	53	12	48	10	50	0.126
C. tropicalis	2	58	8	52	7	53	10	50	0.155

Cryptococcus laurentii	0	60	4	56	0	60	0	60	0.225
Rhodotorula spp	3	57	2	58	0	60	1	59	0.119
No. of identified genera=7	2		6		5		5		
No. of identified species=13	4		9		10		8		

P: present A: absent * and *** express significant and very high significant differences at levels of 0.05 and < 0.0001, respectively

2. Phenotypic and genotypic characteristics of yeast isolates

2.1 Macroscopic characteristics

Some medically important *Candida* species showed specific color of colonies when culturing on chromogeneic medium (CHROM agar *Candida* Plus medium), due to differential uptake of the chromogenic compounds. After incubation at 37˚C for 2-3 days, preliminary identification of the yeast isolate tested was done according to certain colony colors comparing with standard sheet of colors recommended by CHROM agar Company, Paris, France (Table 3 & Fig. 1).

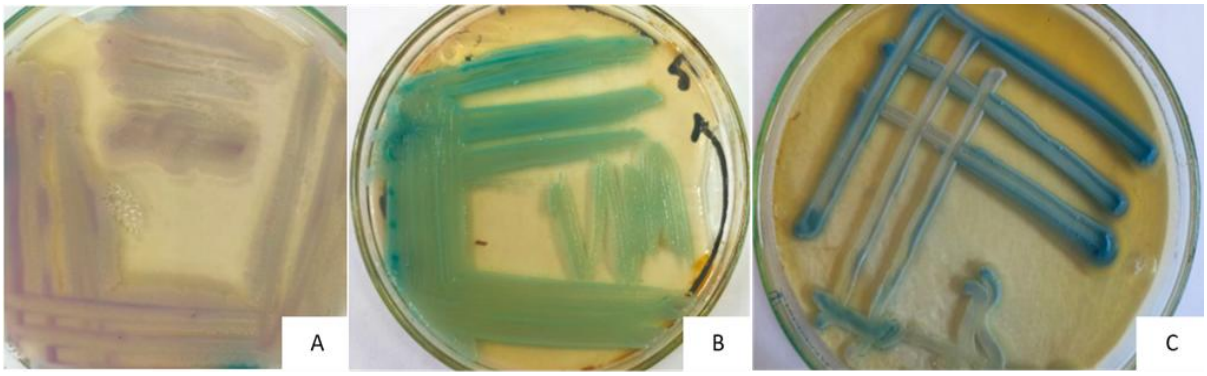

Fig. 1: Colony color of different *Candida* species on CHROMagar *Candida* plus medium showing:

(A) Dark pink to Mauve (*C. glabrata*), (B) Green (*C. albicans*) and (C) Dark blue (*C. tropicalis*).

Morphological features of *Candida* species colonies on Sabouraud agar such as *C.albicans* has white to cream colour glistening soft and smooth growth, *C. glabrata* has white, smooth, butyrous and entire colonies but *C. tropicalis* has white, smooth, butyrous and soft with ringed border. *Rhodotorula* spp colonies are moist, glistering, smooth to mucoid, and salmon pink to red in color.

Table 3: Growth and colony color of *Candida* spp on different media, germ tube test and chlamydospores formation

	Colony color			
***Candida* spp**	**SDA**	**CHROMagar**	**CMA**	**GT**
C. albicans	white to cream	Green	+	+
C. ciferrii	milky white	Blue green	-	-
C. dubliniensis	White	Green	+	-
C. glabrata	White	Mauve	-	-
C. tropicalis	White	Metallic blue	-	-

SDA: Sabouraud Dextrose agar, GT: Germ tube test, CMA: Corn meal agar, (+): Positive, (-): Negative

2.2 Microscopic features:

Some *Candida* species, such as *C.albicans* produced globose to ovoid Shaped budding cells, single, in pairs and clusters 3.5 - 6.0 µm diam in size, but *C. glabrata* has subglobose to ovoid shaped cells, 2.5-4.0 µm diam single and in pairs. *Candida albicans* and *C. dubliniensis* can be distinguished from other *Candida* species by the formation of germ tubes within 2 hours after inoculation in human serum. A total of 20 isolates of *Candida* showed positive for germ tube test indicating their relevant to either *Candida albicans* or *C. dubliniensis* as shown in Fig. (2).

2.2.1 Microscopic appearance of *Candida* species on Corn meal/Tween 80 agar

C.albicans has abundant pseudohyphae and true hyphae, bearing chlamydospores terminally. Twenty strains of *C. albicans* were examined and reported to produce chlamydospores. On contrast, *C. glabrata* didn't produce pseudohyphae or hyphae (Fig. 2). All morphological diagnostic features (macr- and microscopic) of different strains of *Candida* spp. isolated in the current work are illustrated in table (3).

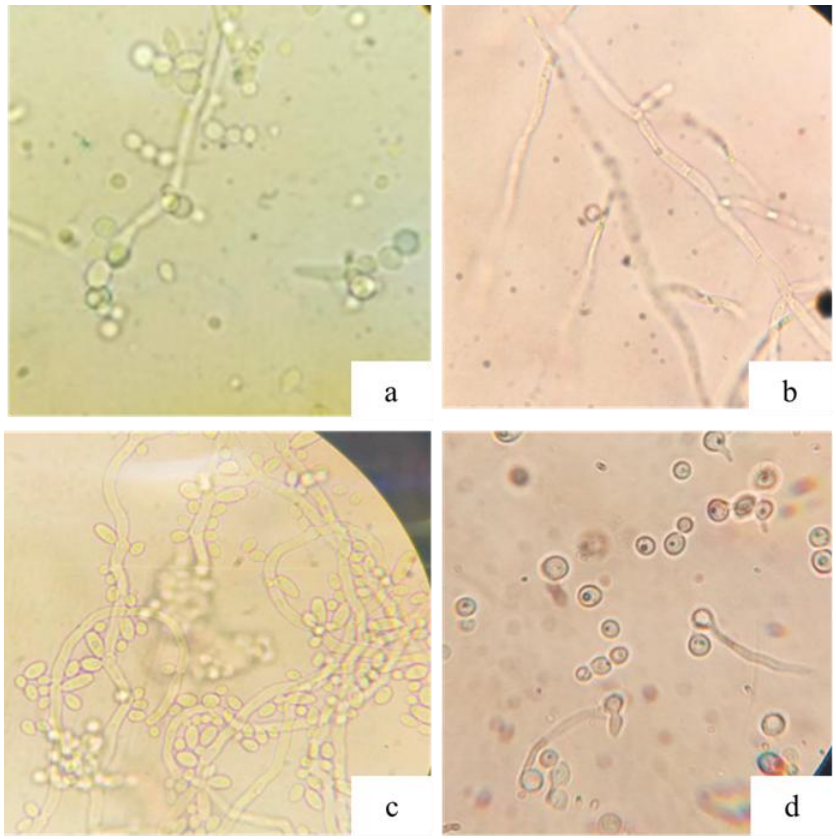

Fig.2: (a) Chlamydospores production on CMA, (b) true hyphae of *C. albicans* (c) pseudohyphe with chains of elongate cells of *C. tropicalis* (d) Positive germ tube test for *C. albicans*

2.3 Molecular identification of yeast isolates:

The two most common yeast strains that isolated, respectively from healthy and dental caries were subjected to molecular identification by ITS sequencing analyses. According to high similarities with isolates from GeneBank database, both strains were identified as *Candida albicans* AUMC 16031 (=OQ256207) and AUMC 16029 (=OQ256189) (Fig. 3).

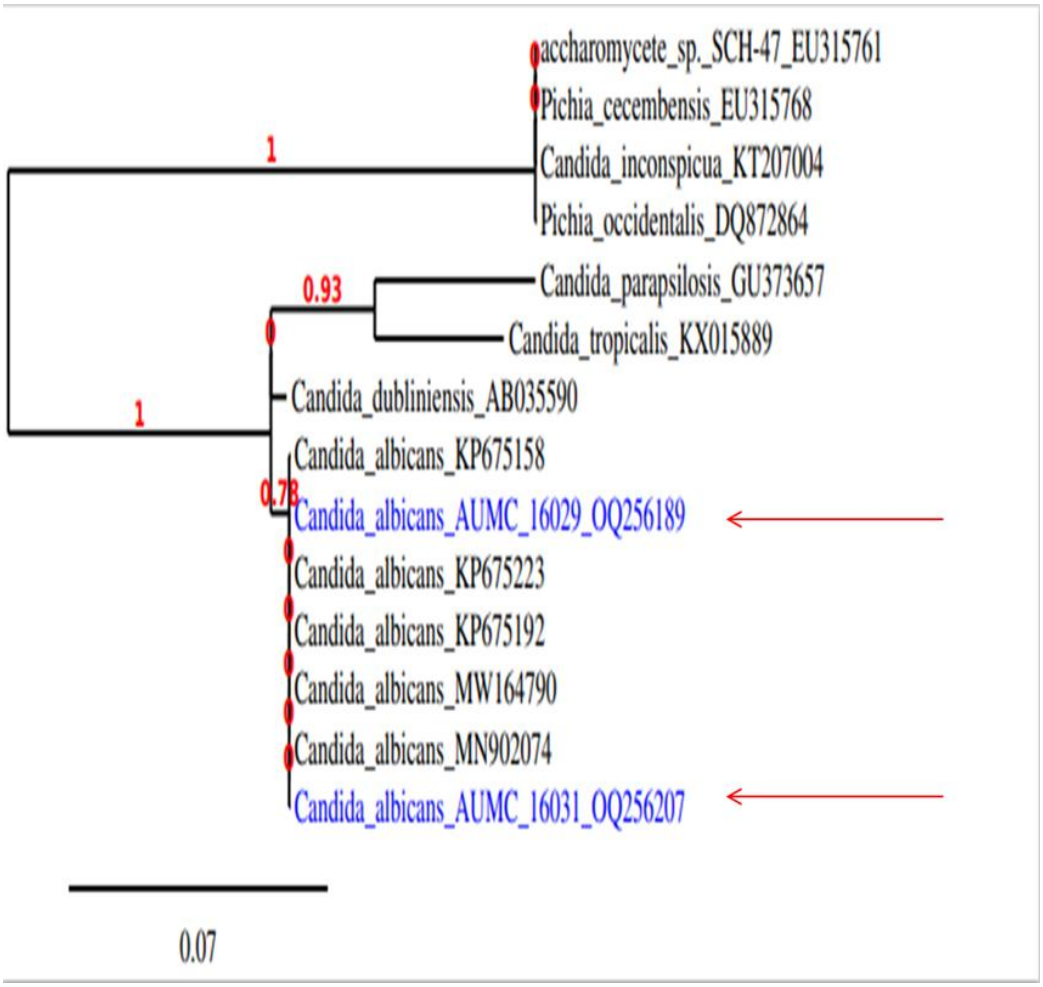

Fig. 3: Phylogenetic tree constructed after analyses of ITS sequence data of two *Candida albicans* strains, AUMC 16031, 16029 (=OQ256207, OQ256189 respectively) in blue color matching with other relevant strains obtained from genebank database.

A total of147 isolates of yeasts were identified from 60 patients after culturing on chromogenic media as well as after performing germ tube test and production of chlamydospores on corn meal agar. From an etiological standpoint, *Candida albicans* was the most frequent species (63 isolates out of 147) representing 30.0% of total cultures. *C. glabrata* came second sharing with 44 isolates matching 20.95% of cultures. The remaining species; *C. tropicalis* and *C. dubliniensis* accounted for 12.8% and 4.75% of total *Candida* isolates. The two most common yeast strains that isolated, respectively from healthy and dental caries were subjected to molecular identification to confirm identification of this species. They identified as *Candida albicans* strains, AUMC 16031,

16029 (=OQ256207, OQ256189 respectively) Our findings were supported by the study of Patel et al. [66] *Candida albicans* is a prominent member of the human mycobiota in the oral cavity.

Table 4: The total count percentage of different fungi associated with each of the different four sites of healthy, gingivitis, periodontitis, and dental caries

Site	Healthy			Dental caries			Gingivitis			Periodontitis		
	%TC	NG	NS	%TC	NG	NS	%TC	NG	NS	%TC	NG	NS
Tooth	55.5	2	5	51.16	3	5	59.15	4	6	45.82	3	6
Gingiva	34.36	1	4	3.3	1	2	28.93	3	5	17.68	2	4
Tongue	4.62	2	3	44.74	3	6	10.25	2	4	0.27	2	4
Saliva	5.5	1	3	0.77	2	2	1.64	2	3	8.84	3	4
Total %TC	19.59			22.18			23.56			34.65		

(%TC): Total count percentage, (NG): No. of genera, (NS): No. of species

The results in Table (4) showed that healthy cases have lowest percentage of total count (19.59 %), comparing with the other cases. Low numbers of the fungi are naturally found in the mouth and digestive system of most people. The highest incidence of fungi in healthy oral cavity occurred at tooth and saliva 55.5 % and 34.36 %, respectively. Dental caries and gingivitis have close ratio in percentage of total count 22.18 % and 23.56 %, respectively. Periodontitis cases had the highest percentage of total count (34.65 %) comparing with the other cases as in periodontitis the *Candida* spp. have virulence factors that facilitate colonization and proliferation in the oral mucosa and, possibly, in periodontal pockets. These fungal organisms can co-aggregate with bacteria in dental biofilm and adhere to epithelial cells. These interactions, which are associated with their capacity to invade gingival conjunctive tissue, may be important in microbial

colonization that contributes to progression of oral diseases [67]. In addition to these properties, *Candida* spp. also produces enzymes, such as the collagenases and proteinases that degrade extracellular matrix proteins, and immunoglobulin [56]. In gingivitis and periodontitis gingiva is the second richest site with fungi 28.93 % and 17.68%, respectively. Tongue and saliva have low fungal population due to mechanical movements during speaking, chewing and swallowing make tongue unstable site. Saliva contains multiple antifungal components such as histatins, lysozyme, lactoferrin and lactoperoxidase [56] which are active against *C. albicans* when evaluated individually *in vitro*.

3. Extra-cellular enzymes production by yeast

The results in Table (5) and Fig (4) showed that 47.2% (34 out of 72 isolates) of the yeast isolates under investigation had positive lipolytic activities with varying capabilities. From the positive isolates, 7 (27.5%) exhibited high lipolytic activity. These included *Candida albicans* (4 isolates), *C. glabrata* (2) and *C. tropicalis* (1). Moderate lipase activity was achieved by *C. albicans* (11 isolates), *C. glabrata* (3) and *C. tropicalis* (2 isolates). The remaining positive isolates (*C. albicans*, 5; *C. glabrata*, 1 and *C. tropicalis*, 5 isolates) exhibited low lipase enzyme production (Table 4). Some species of *Candida* and other yeasts or yeast- like fungi were reported as lipase producers like *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* [68-72] .

Table 5: Number (N) and percentage (%) of yeast isolates showing lipolytic activities

Yeast species	**No. of tested strains**	**High**		**Moderate**		**Low**		**Total Positive**	
		N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Candida albicans	30	4	13.3	11	36.6	5	16.6	20	66.5

C. glabrata	21	2	9.5	3	14.2	1	4.7	6	28.4
C. tropicalis	21	1	4.7	2	9.5	5	23.8	8	38.0
Total	72	7	27.5	16	60.3	11	45.1	34	47.2

High: Depth of visible precipitate ≥ 15 mm, Moderate: Depth of visible precipitate 6-14 mm and Low: Depth of visible precipitate ≤5 mm.

Data in Table (6) and figure (4) indicated that 41 isolates out of 72, (56.9%) were positive protease producers. From these isolates, only 11 had high proteolytic activity (*C. albicans*, 5; *C. glabrata*, 4 and *C. tropicalis*, 2 isolates) while, 12 isolates (6 *C. albicans*, 1 *C. glabrata* and 5 *C. tropicalis*) and 18 isolates (10 *C. albicans*, 2 *C. glabrata* and 6 isolates of *C. tropicalis*) had moderate and low proteolytic activity, respectively. These fungi were also recorded previously as protease producers. Hydrolysis of protein was observed by *Candida albicans*[73], *C. tropicalis*[74], C. glabrata[75-77].

Table 6: Number (N) and percentage (%) of yeast strains showing proteolytic activities

Yeast species	**No. of tested strains**	**High**		**Moderate**		**Low**		**Total Positive**	
		N	%	N	%	N	%	N	%
C. albicans	30	5	16.6	6	20	10	16.6	21	70
C. glabrata	21	4	19.04	1	4.7	2	9.5	7	33.3
C. tropicalis	21	2	9.5	5	23.8	6	28.5	13	61.9
Total	72	11	15.3	12	16.6	18	25	41	56.9

High: Depth of clear media ≥ 18 mm, Moderate: Depth of clear 15-17 mm and Low: Depth of Depth of clear ≤ 14 mm.

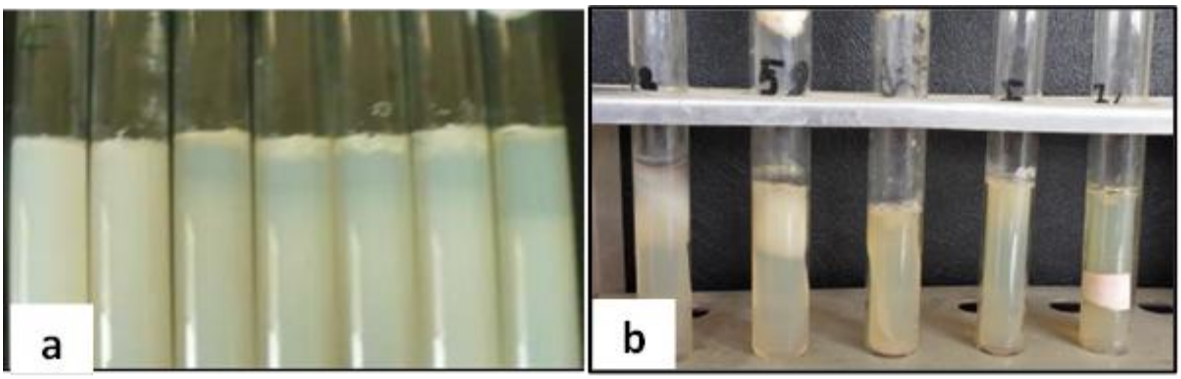

Fig.4:Proteolytic(a) and liopolytic (b) activities of *Candida* species.

Candida species employ a repertoire of virulence factors, including phenotypic switching, dimorphism, and production of hydrolytic enzymes, to invade the oral cavity. These hydrolytic enzymes facilitate adherence of *Candida* spp. to the host tissue, rupture of cell membranes, invasion of mucosal surfaces and blood vessels, and evasion of the host's immune response [78]. Secreted proteinases are principal among such enzymes and degrade proteins related to structural and immunologic defenses, such as collagen, keratin, mucin, antibodies, complement, and cytokines, during tissue invasion. The putative roles of microbial extracellular lipases include digestion of lipids for nutrient acquisition, adhesion to host cells and host tissues, initiation of inflammatory processes by affecting immune cells, and self-defense mediated by lysing competing microflora [79].

3.4. Susceptibility of yeast isolates to pharmaceutical preparations and natural oils

Four toothpastes (Close-up, Signal, Sensodyne and Paradontax), 3 mouth washes (Hexitol, Betadine and Platina) and 2 natural oils (clove oil and Ginger oil) were screened against 60 *Candida* isolates belongs to *C. albicans*, *C. glabrata C. tropicalis* (20 isolate from each) the most predominant isolated species related to gingivitis, periodontitis and dental caries patients Interestingly, all isolates tested were inhibited by Paradontax, Hexitol and Clove oil (Table 7). Other natural oils almost had no inhibitory effect against all tested microbial strains. These oils may have either weak active substances or have some additives which inactivate the inhibitory action of the oil [80]. Paradontax was proved previously to be used as a good antimicrobial agent against yeasts

(*Candida albicans* and *C. tropicalis*) obtained from saliva, teeth, root canals or radiation caries [81]. Furthermore, it could decrease the rate of both plaque and gingivitis [82]. Statistical analysis using one way ANOVA and Duncan multiple range tests showed mostly high significant differences among the tested compounds (Table 7). Clove oil was reported to have antimicrobial activity against food borne pathogens, when used in a combination with cinnamon oil [83]. The antimicrobial action of natural plant oils is suggested to be attributed to the most active phytochemical components, such as: phenolic compounds, aldehydes, ketones, alcohols, and hydrocarbons. These components have the hydrophobicity behavior, leading to their action with lipids on the microbial membranes, that disturb the permeability, and break homeostasis [84]. In the current study, it was observed that Signal and Close-up have a good antimicrobial effect against yeasts (*Candida albicans, C. glabrata* and *C. tropicalis*) compared to Sensodyene which has no effect on those microorganisms. Platina mouth wash and Betadine have either weak or no antimicrobial effect against yeasts, especially oral *candida*

Table 7: Antimicrobial activity of toothpastes, mouth washes and natural oil against yeast strains isolated from gingivitis, periodontitis and dental caries (Inhibition zone in mm: Mean[a] ± SE).

Taxa (n=20)	Clove oil	Ginger oil	Hexitol	Betadine	Platina	Signal	Close up	Pardontex	Sensodyene	F
C. albicans	27.73 ± 0.94^{d}	4.60 ± 1.30^{b}	22.10± 0.64^{c}	0^{a}	5.35 ± 1.12^{b}	21.47 ± 0.53^{c}	21.75 ± 0.52^{c}	22.85 ± 0.62^{c}	0^{a}	215.69***
C. trobicalis	30.63 ± 1.58^{e}	0^{a}	21.94 ± 0.64^{d}	0^{a}	5.70 ± 1.20^{b}	20.84 ± 0.45^{cd}	21.55 ± 0.47^{cd}	19.60 ± 0.55^{c}	0^{a}	250.46***
C. glabrata	30.9 ± 1.53^{e}	0^{a}	22.65 ± 0.59^{d}	0^{a}	6.95 ± 1.22^{b}	21.50 ± 0.50^{cd}	21.95 ± 0.53^{cd}	20.30 ± 0.40^{c}	0^{a}	263.0***

*** express very high significant differences at levels of 0.05 and < 0.0001, respectively

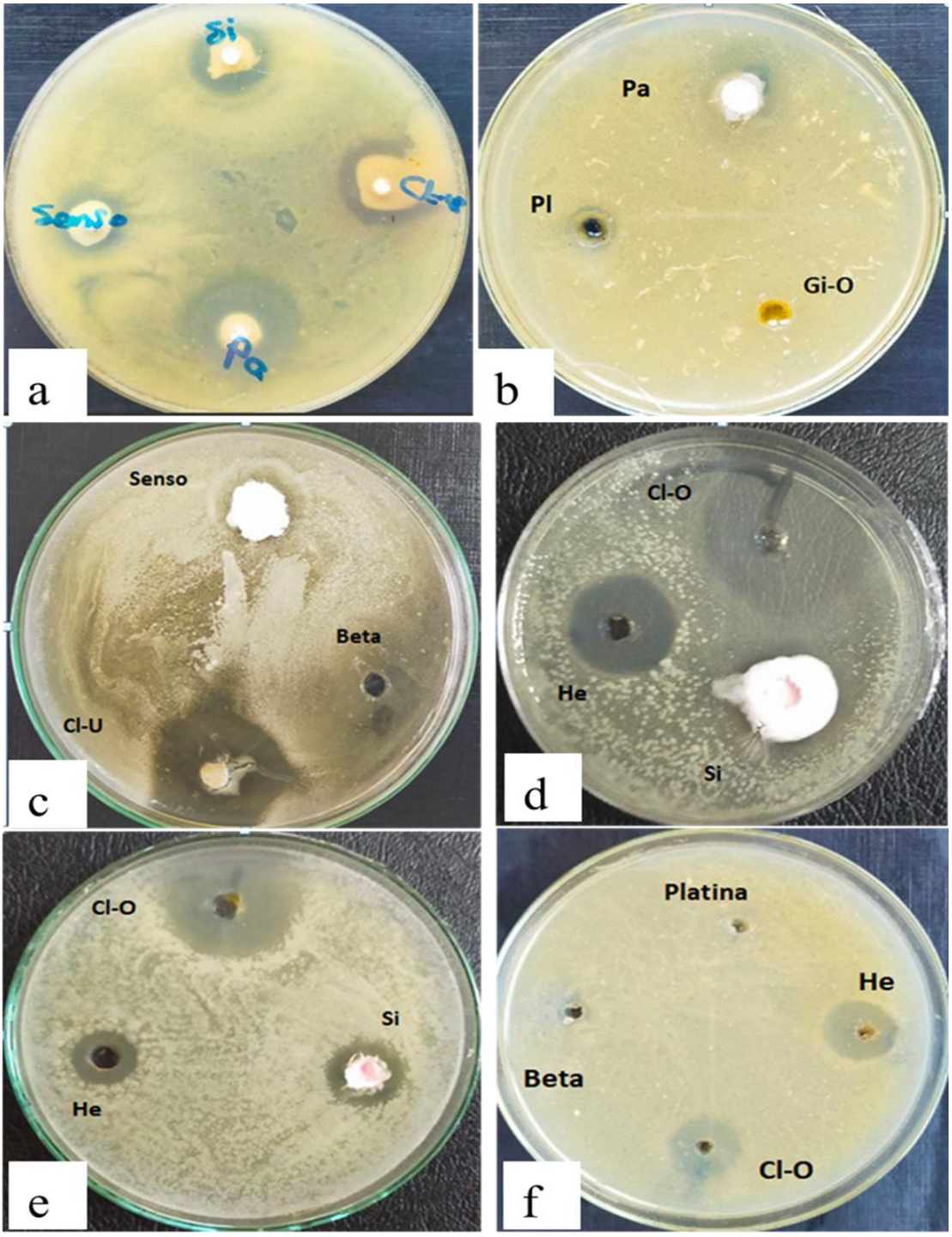

Fig.5: Growth of *Candida* spp. a-b *C. trobicalis*, c-d *C. albicans*, e-f *C. glabrata* in the presence of some pharmaceutical preparations (teeth pastes and mouth washes) and natural oils; Close-up (Cl-U), Sensodyen (Senso), Ginger oil (Gi-O), Platina (Pl), Paradontax (Pa), Clove oil (Cl-O), Signal (Si).

4. Minimum inhibition concentration (MIC) for yeast isolates

Hexitol had the highest inhibitory effect against the tested strains in very small concentrations (MIC =0.078 – 0.039 mg/ml), followed by clove oil (0.25 – 0.125 ml) and paradontax (0.7 – 0.35 mg/ml). The inhibitory effect of Hexitol may be due to the lethal effect of its active ingredients, sodium fluoride or other substances that have antimicrobial activity through membrane disruption causing growth inhibition and cell death [85]. Also, it could strongly inhibit plaque growth and prevent the binding to the tooth structure thus reducing pellicle formation [86]. Another suggested mechanism is that the component of Hexitol drug is Chlorhexidine which is a positively-charged molecule that binds to the negatively-charged sites on the cell wall; it destabilizes the cell wall and interferes with osmosis. The fungus uptakes chlorhexidine in a short amount of time and impairs the integrity of the cell wall and the plasma membrane entering the cytoplasm resulting in leakage of cell contents and cell death [62, 87].

CONCLUSION

Although, highly resistant pathogenic strains increase continuously, the current work recommend Paradontax toothpaste and Clove oil as antifungals, but the most amazing is Hexitol mouth washing solution. Clinical studies are urgent to evaluate the activity of clove oil and other natural oils against dental and periodontal pathogenic microorganisms and highlight their mechanism of action. Oral microorganisms could be considered as risk factors aid in predisposing serious infections in immunocompromised patients. There is a need to additional studies to that shed light on the pathogenicity of these microorganisms and creating novel and safe antimicrobial agents. Fungal population of various niches in oral cavity is not the same .Tooth is the highest site in oral cavity rich with fungi in all types of cases. Culturing of these fungi is not enough technique to provide a complete picture on oral microbial community, because several strains are unculturable. For this reason, advanced molecular analyses using modern metagenomic techniques are indispensable to provide the actual diversity of microbial community. Finally, regular oral examinations, prophylaxis and oral hygiene, in addition to periodical use of appropriate toothpaste and mouth wash are valuable in protection against oral infection by microbes.

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