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The antifungal effect of *Salvadora persica* and *Euclea natalensis* on *Candida* isolates from Libyan patients with type 2 Diabetes mellitus.

By

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DECLARATION

I declare that that this work "The antifungal effect of *Salvadora persica* and *Euclea natalensis* on *Candida* isolates from Libyan patients with type 2 Diabetes mellitus." is my original work and that all the sources that I have used or cited have been indicated and acknowledged by means of complete references, and that this document has not been submitted for degree purposes at any other academic institution.

Mustafa Hasan Mustafa Esmaio
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Signed:…… Date:……

DEDICATION

This study is dedicated to my parents and my family members.

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I would like to extend a word of thanks to everyone who has contributed positively to my PhD journey and to my personal development.

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ABSTRACT

Improved oral hygiene plays a vital role on quality of health and well-being of diabetic patients. Poor oral health leads to an increased incidence of oral diseases, particularly oral candidiasis. The emergence and global spread of azole- resistant *Candida* species has necessitated the need for novel, cost effective antifungals to stop further spread of resistant *Candida* infections.

This project is the first documented investigation of *Candida* species prevalence in Libyan type 2 diabetes mellitus (T2DM) patients and investigated the antifungal effect of Salvadora persica and Euclea natalensis on azole-resistant *Candida* isolates. In this study, 182 *Candida* isolates from the oral mucosa of T2DM patients were identified using presumptive species identification by chromogenic media followed by confirmation using API ID 32 C, YST Vitek 2 and phenotype microarrays. Their drug susceptibility profiles were tested using the disc diffusion and the AST Vitek 2 compact system.

High-pressure liquid chromatography and nuclear magnetic resonance were employed to separate, isolate and purify the bioactive compounds and fractions of the plant extracts which were then tested for their antifungal activity. The results showed that both *Salvadora persica* and *Euclea natalensis* promise to provide beneficial alternatives to conventional drugs in treating oral candidiasis in diabetic patients.

Keywords

Candida species

Oral Candidiasis

Type 2 Diabetes Mellitus candidiasis

Oral “Health”

API 32 ID C identification

Fluconazole resistance

Antifungal activity of plant extracts

Vitek 2 characterisation

Euclea natalensis

Salvadora persica

Research Outputs

Emanating from this study, the following peer-reviewed conference proceedings have been published in accredited scientific journals:

1. Mustafa Esmaio, Pedro MDS Abrantes, Ahmed Hussein, Charlene WJ Africa. "Rare Fungal Species Isolated from the Oral Mucosa of Libyan Diabetic Patients" **Antimicrobial Resistance & Infection Control 6 (Suppl 1): 7-8 DOI10.1186/s13756-016-0153-0:** presented at 6th Infection Control Africa Network Congress 2016 Indaba Hotel & Conference Centre, Fourways, Johannesburg, South Africa 25 - 28 September 2016

   Background and objectives: The emerging resistance of *Candida* species to antifungals routinely used to treat candidiasis in HIV patients and in patients with diabetes mellitus (DM) has resulted in the frequent isolation of non-albicans *Candida* species. This study aimed to establish the prevalence and fluconazole resistance profiles of yeasts other than commonly identified Candida species which may be found colonizing the oral mucosa of Libyan patients with DM.

   Methods: Fungal species were isolated from the oral cavity of DM-positive patients attending a diabetes clinic in Misrata Diabetes Centre in Libya. This study included patients aged between 35 and 95 years and excluded subjects who had been on antifungal therapy within two weeks prior to sample collection. The identification of the isolated species was done by growing the isolates on selective and chromogenic media and by API ID 32C biochemical testing. Antimicrobial susceptibility testing of the isolates to the antifungal fluconazole was performed using disk diffusion. The study complied with the Declaration of Helsinki (2013).

Results: Forty-four rare fungal isolates representing ten fungal species were identified from the oral mucosa of 194 patients, with 28.6% of rare *Candida* species demonstrating resistance to fluconazole. *Saprochaete capitata* and *Cryptococcus humicola* isolates demonstrated high levels of resistance to fluconazole, with other yeast species showing lower resistance levels.

Conclusion: The methodologies used in this study allowed for the accurate identification of rare fungal species. The API 32 ID system was found to be a better identification method when compared to chromogenic media, as some species could not be identified with the latter. This study emphasizes the importance of accurate species identification and antifungal surveillance in patients with underlying chronic diseases such as DM who have higher morbidity and mortality rates due to less known and resistant fungal infections.

2. Mustafa HM Esmaioa, Charlene WJ Africaa, Ahmed A Husseinb. "The Antifungal Effect of 7-Methyljuglone against *Candida* Species Isolated from Oral Mucosa of Libyan Patients with Diabetes Mellitus". **South African Journal of Botany Volume 109, March 2017, Pages 332-333.** https://doi.org/10.1016/j.sajb.2017.01.049. Presented at 43rd Annual Conference of the South African Association of Botanists (SAAB), Lagoon Beach Hotel, Cape Town (8-11 January 2017).

Background: The emerging antifungal resistance of *Candida* species along with the toxicity contained within some of these drugs has necessitated the discovery and application of new treatment modalities and the re-evaluation of age-old traditional remedies. The objective of this study was to test the antifungal activity of 7-Methyljuglone against *Candida* species showing resistance to fluconazole.

Methods: *Candida* species were collected from patients attending a diabetes clinic in Misrata Diabetes Centre in Libya. *Candida* species were identified by culture on selective and chromogenic media and by API ID 32C biochemical testing. Antimicrobial susceptibility testing of the isolates to the antifungal fluconazole was performed using disk diffusion. The assay was performed using broth microdilution method with a 25µg/ml fluconazole as a positive control.

Results: Of the 194 *Candida* isolates, 41 (21.13%) isolates were fluconazole resistant, seventy species were identified as *C. albicans*, 5 of them are resistance to fluconazole (2.57%). All 5 isolates of *C. krusei* are resistance to fluconazole, and 19 of 21 *C. glabrata* are resistance (9.79%). In total, 41 species are resistance to fluconazole, and 32 species are intermediate-resistance (16.49%). The results

showed high activity of 7-methyljuglolne with MIC's ranging between 25 µg/ml – 0.390 µg/ml.

Conclusion: These findings emphasize the crucial need for accurate screening, identification and susceptibility testing to prevent further spread of nosocomial and community acquired resistance. The antifungal effect of 7-Methyljuglone from *Euclea natalensis* was evaluated as an alternative bioactive agent for azole-resistant oral *Candida* species in DM patients.

3. Mustafa H M H Esmaio, Pedro M D S Abrantes, Charlene WJ Africa. "Vitek characterisation of type 2 diabetes-associated *Candida* species. Presented at Conference: 11th International Symposium on Antimicrobial Agents and Resistance (ISAAR) / 3rd International Interscience Conference on Infection and Chemotherapy (ICIC), Combating and Emerging Infectious Diseases, September 14-16, 2017 At Busan, South Korea, Volume: **Int J Antimicrob Agents 2017 50 (Suppl 1)**, 118

Background: Type 2 diabetes mellitus (T2DM) predisposes patients to opportunistic infections, such as invasive candidiasis. Treatment of candidiasis is challenged by the emerging resistance of *Candida* species. In this study, the antifungal drug resistance patterns of *Candida* species present in the oral mucosa of T2DM Libyan patients was investigated.

Methods: Seventy-four (74) oral Candida isolates collected from T2DM patients in Misrata, Libya were characterised using the Vitek 2 Compact system.

Results: Prevalent species included *C. albicans, C. glabrata, C. dubliniensis, C. krusei, C. tropicalis, C. sake, C. kefyr, C. guilliermondii, C. parapsilopsis, C. membranifaciens and C. magnoliae*. Drug susceptibility showed an emerging resistance across representatives of all species for which breakpoints were available, with the exception of *C. parapsilopsis*. Although there are no established interpretative breakpoints for these species, three *C. sake* isolates and the *C. membranifaciens* isolate also had high MIC values for fluconazole. The tested isolates were found to be largely susceptible to caspofungin and micafungin. All C. albicans isolates were susceptible to the echinocandins, amphotericin B and 5-

flucytosine. Resistance to more than one drug class was seen in *C. dubliniensis, C. glabrata* and *C. krusei* isolates.

Conclusion: Although the susceptibility results for the echinocandins were encouraging, resistance against the azoles was apparent and should not be ignored. This was especially so in the case of fluconazole, which is often the only locally available antifungal drug for the treatment of disseminated candidiasis.

4. Mustafa H M H Esmaio, Pedro M D S Abrantes, Charlene WJ Africa. "*Candida* species carriage in diabetic patients in Misrata, Libya ***Southern African Journal of Infectious Diseases,* S93 2017 Volume 32 Supplement**: **93**". Presented at 7th FIDSSA Congress 2017, 09 – 11 November, Century City Conference Centre, Cape Town

Introduction: There is a paucity of studies describing the prevalence and antimicrobial profiles of *Candida* in Libya. Limited treatment choices in the antifungal armamentarium in public healthcare settings in the rest of Africa require a study of the prevalence and susceptibility of *Candida* species in Libya, where antifungals are not regularly distributed in public health care settings.

Methods: In this study, 170 diabetic mellitus type 2 (T2DM) patients were examined for *Candida* carriage in the oral mucosa, using differential Fluka and Oxoid chromogenic media and API 32 ID C biochemical testing. Fluconazole susceptibility was investigated by disc diffusion on YNBG agar. Isolates were graded as susceptible, intermediate or resistant according to their inhibition zone measurements and micro-colony scores.

Results: Thirteen species were identified from 182 isolates with a frequency of 69 *C. albicans,* 42 *C. dubliniensis,* 26 *C. humicola,* 20 *C. glabrata,* 5 isolates of each *C. krusei, C. tropicalis and C. kefyr,* 4 *C. sake,* 2 *C. parapsilopsis,* 2 *C. magnoliae* and 1 isolate each of *C. guilliermondii, C. globosa* and C. *membranifaciens*. Although largely susceptible to fluconazole, *C. albicans, C. dubliniensis, C. humicola* and *C. sake* demonstrated an emerging resistance with intermediate to total resistance observed in all the other species except for *C. magnolia* and *C. globosa* which were both susceptible to fluconazole.

Conclusion: Early recognition and treatment of rare or resistant species which may be contributing to patient morbidity and mortality in Libya is imperative

This study was funded by the Libyan government.

Manuscripts in preparation

1. Esmaio MHMH, Abrantes PMDS, Africa CWJ
   Application of Vitek 2 technology in epidemiological studies of *Candida* in type 2 dibetes mellitus patients in Libya
   To be submitted to:] Epidemiology and Infection

LIST OF ABBREVIATIONS

AST	Antifungal susceptibility testing
ATCC	American Type Culture Collection
AMB	Amphotericin B
BMI	Body Mass Index
BITC	benzyl isothiocyanate
BSA	Bovine serum albumin
CAS	Caspofungin
CLSI	Clinical laboratory standards institute
CSH	Cell surface hydrophobicity
CDR	*Candida* drug resistance
DCM	Dicholoromethane
DIW	Deionized water
DS	Denture Stomatitis
DMSO	Dimethylsulfoxide
EtOAc	Ethyl acetate
EtOH	Ethanol
E. natalensis	*Euclea natalensis*
FBS	Foetal Bovine Serum
FLU	Fluconazole
FCT	Flucytosine
HPLC	High Pressure Liquid Chromatography
HRMS	High Resolution Mass Spectroscopy
HDL	High Density Lipoprotein
IC_{50}	Half Maximal Inhibitory Concentration
INT	P-Iodonitrotetrazolium
IDDM	Insulin-dependent diabetes mellitus
MCF	Micafungin
MeOH	Methanol
MIC	Minimum Inhibitory Concentration

min	Minute(s)
MMC	Minimum Microbicidal Concentration
MS	Mass Spectroscopy
mL	Milliliter
mm	Millimeter
NIDDM	Non-insulin-dependent diabetes mellitus
NCPF	National collection of pathogenic fungi
NMR	Nuclear Magnetic Resonance
NPV	Negative predictive value
PPV	Positive predictive value
P. fruticans	*Psoralea fruticans*
PBS	Phosphate Buffered Saline
SDA	Sabouraud dextrose agar
S. Persica	*Salvadora percica*
SPSS	Statistical product and service solutions
Type 2 Diabetes Millitus	T2DM
TLC	Thin Layer Chromatography
v/v	Volume to volume
YNBG	Yeast nitrogen base agar
YO7	YeastOne 7
WHO	World Health Orgnisation
UWC	University of Western Cape
VRC	Voriconazole
µg	Microgram
µl	Microliter
°C	Degrees centigrade
7-MJ	*7-methyljuglone*

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CHAPTER 1

REVIEW OF THE LITERATURE

1. Introduction

The genus *Candida* is within the class Deuteromycetes and has been described as a "taxonomic pit" into which yeasts without a known sexual stage or other remarkable phenotypic character have been thrown (Parihar, 2011, Diekema *et al.*, 2012). There are currently over 150 species, and about 15 are known to cause infections in humans. These disease-causing *Candida* species include *Candida albicans, Candida glabrata, Candida dubliniensis, Candida tropicalis, Candida parapsilosis, Candida krusei, Candida guilliermondii, Candida lusitaniae, Candida pelliculosa, Candida kefyr, Candida lipolytica, Candida famata, Candida inconspicua, Candida rugosa,* and *Candida norvegensis*. However, the regularly isolated pathogens are *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis*, and *C. krusei* which were reported in 95% of patients with known *Candida* infections (Diekema *et al.*, 2012).

Candida species produce hydrolytic enzymes that harm the host tissues and play a role in disease causation. Cases of these enzymes comprise phospholipases, proteases, and haemolysin. The ability of *Candida* species to cause invasive infections is due to specific virulence factors which include evading the host immune system and ability to form biofilms on medical devices and on tissue surfaces (Calderone and Fonzi, 2001, Silva *et al.*, 2012).

The three most investigated factors are those which relate to the cell wall, adhesion,

and extracellular proteolytic enzyme production. The cell wall of the organism is essential for its success as a pathogen, since it is required for growth and protection against osmotic insult and is the site of contact between the organism and its environment. Cell-surface ligands and receptors promote colonization of host cells and tissues, whereas proteolytic enzymes are involved in tissue penetration (Naglik *et al.*, 2003, Naglik *et al.*, 2004, Karkowska-Kuleta *et al.*, 2009).

Colonization of the oral surface by *C. albicans* can cause damage to the oral tissues because of its ability to express several virulence factors including adherence to host cells or denture surfaces such as oral prosthesis, secretion of hydrolytic enzymes (Schaller *et al.*, 2005, Mishra *et al.*, 2007, Junqueira *et al.*, 2011), adaptation to different environmental conditions and biofilm formation (Silva *et al.*, 2010). Yeast adhesion to epithelial cell surfaces is deemed an initial and critical stage in the process of *Candida* colonization and further occurring infection (Wächtler *et al.*, 2012).

1.1. *Candida* Biofilm formation

Candida species occur and multiply either as planktonic (free-floating cells), or as structured, sessile communities which could be either mono or polymicrobial, enclosed in a self-created gel-like polysaccharide matrix known as a biofilm (Stickler, 2008). Fungal infections, particularly in immunocompromised persons and those on heavy courses of antibacterial compounds that are liable to super infection are a growing concern. This is because like bacteria, fungi such as *Candida albicans* have also been known to be capable of biofilm formation on medical implants (Busscher *et al.*, 1994, Ell, 1996, Busscher *et al.*, 1997, Silva *et al.*, 2012, Silva *et al.*, 2017). *Candida* species are often found in the normal microbiota of humans, which facilitates their encounter with most implanted biomaterials and host surfaces. Recent work has shed some

insight into the molecular mechanisms that control the development of fungal biofilms (Chandra *et al.*, 2001, Finkel and Mitchell, 2011, Mayer *et al.*, 2013).

Biofilms are defined as biological communities with a high-level configuration, in which micro-organisms form structured coordinated and functional communities (Donlan, 2001, Douglas, 2002). These biological communities are entrenched in a self-made extracellular matrix. The biofilm formation is also associated with a high level of antimicrobial resistance in the associated organisms (Sardi *et al.*, 2013). Recent evidence suggests that the majority of infections produced by this pathogen are associated with biofilm growth (Silva *et al.*, 2017).

Six different sequential stages of biofilm development have been described (Costerton *et al.*, 1999, Ramage *et al.*, 2001, Hall-Stoodley and Stoodley, 2005). These stages comprise of:

1. Initial adherence and attachment of yeast cells (0-2 h).
2. Germination and formation of micro colonies (2-4 h).
3. Filamentation (4-6 h).
4. Monolayer development (6-8 h).
5. Proliferation (8-24 h) and maturation (24-48 h).
6. Detachment of planktonic cells and formation of a new biofilm.

The mechanisms of *Candida* biofilm formation seem to be intricate, including a multiplicity of cell-to-protein and protein-to- protein interactions supported by components of biological fluids such as saliva or serum (Nikawa *et al.*, 1999). Accordingly, biofilms have a population of single or multiple species of microorganisms embedded in self-produced hydrated exopolysaccharides. These are

irreversibly adhered to a surface, and exhibit a phenotype considerably different from their planktonic counterparts (Donlan, 2002).

Cell surface hydrophobicity (CSH) and biofilm formation have been identified as vital virulence factors that have important roles in the pathogenesis of *Candida* spp. Previous studies (Samaranayake *et al.*, 1995, Panagoda *et al.*, 1998), also showed a positive correlation between CSH and adhesion of *Candida* to the buccal epithelial cells and inert polymeric surfaces such as denture prostheses. The adhesion of *Candida* spp., thus plays an initial and significant step in biofilm formation.

As mentioned above, the architecture of the biofilms formed by *C. albicans* is like that of bacterial biofilms. *Candida* species grown within a bacterial biofilm, have indicated an interaction between the prokaryotic and eukaryotic cells (Douglas, 2003) . Of greater interest, however, is the question of whether these fungi develop new physiological properties that are different from their planktonic counterparts. A study by Douglas have used classical assays to address this question (Douglas, 2003). Using their standard assay for biofilm formation in conjunction with electron microscopy, they showed that *C. albicans* biofilms became resistant to five antifungal compounds that are used routinely in clinical settings (Hawser and Douglas, 1995). This drug resistance phenotype is often associated with bacterial biofilms. Clearly, these workers have gone a long way towards establishing that a mode of life and developmental system long associated with bacteria may be a common strategy used by eukaryotic microorganisms as well.

The mechanisms of biofilm resistance to antimicrobial agents are not fully understood, although possible mechanisms have been proposed including : (1) restricted penetration of drugs through the biofilm matrix; (Arredondo-García *et al.*, 2009) phenotypic

changes resulting from a decreased growth rate or nutrient limitation; and (3) expression of resistance genes induced by contact with a surface (Mah and O'toole, 2001, Donlan and Costerton, 2002). Another recent suggestion is that a small number of 'persister'cells are responsible for resistance (Lewis, 2001). Multiple mechanisms appear to operate in bacteria, and these vary with the bacteria present in the biofilm and the nature of the antimicrobial agent being administered (Mah and O'toole, 2001).

1.2. Oral Candidiasis

Candidiasis is the most frequent oral fungal infection. Candidiasis may manifest with a combination of a pseudo membranous covering, erythema of the underlying tissues along with a burning sensation of the tongue or other intraoral soft tissues. *C. albicans* is a dimorphic fungus occurring in both yeast and hyphal forms; but, only the hyphal form is associated with oral candidiasis. It may be a component of the normal oral microflora in approximately 30% to 50% of the population and forms part of group of saprophytic fungi that causes infections of the oral cavity (Mohammad and Giannini, 2005). *Candida albicans* is the most common *Candida* species residing in the oral cavity of humans (Sardi *et al.*, 2013) and accounts for 70% to 80% of oral isolates (Vazquez and Sobel, 2002).

C. albicans is part of the human microbiota, but when the balance between the host and the microorganism is changed, *Candida* becomes an opportunistic pathogen (Tekeli *et al.*, 2004) and oral candidiasis is exhibited. Individuals at risk include those infected with the human immunodeficiency virus, those with nutritional deficiencies, malignancies, or with metabolic disorders like diabetes mellitus (DM) (Tekeli *et al.*, 2004). *Candida glabrata* and *Candida tropicalis* account for between 5 and 8% of oral isolates, respectively (Vazquez and Sobel, 2002).

1.2.1. Predisposing factors for oral candidiasis

Oral candidiasis has several predisposing factors, including systemic diseases that affect the immune status of the host, the local oral mucosal environment, and the specific strain of *C. albicans* implicated (Chi *et al.*, 2010).

The occurrence of these yeasts in the oral cavity does not ordinarily result in disease except if there are predisposing factors for the development of candidiasis, such as systemic diseases affecting host defences; physiologic conditions like old age, pregnancy, dietary factors, infancy, treatment with broad spectrum antibiotics, immunosuppressive drugs, and corticoids. Local factors such as wearing dentures are other examples of predisposing factors (Budtz-Jørgensen *et al.*, 2000, Maza *et al.*, 2002, Ruissen *et al.*, 2002, Torres *et al.*, 2002).

Persistent oral candidiasis is observed in patients with poorly controlled diabetes mellitus, human immunodeficiency virus (HIV) - positive patients, and patients with xerostomia.(Cassolato and Turnbull, 2003, Giannini and Shetty, 2011)

1.2.1.1. Xerostomia (Dry Mouth)

One of the most common causes of xerostomia, particularly among the elderly population, is the use of medications. Medications that are usually linked with predisposition to xerostomia include antidepressants, diuretics, and those that possess anticholinergic effects. Additional sources of xerostomia include radiation treatment to the head and neck region and Sjogren's syndrome. A reduction in the salivary flow leads to a decrease in the cleansing capability of the saliva, also to a decrease in secretory immunoglobulin A levels, establishing an environment that is more conducive to the growth of *C. albicans* (Almståhl *et al.*, 2003).

It has been demonstrated that salivary flow can be improved and boosted by exercising the masticatory function (Niedermeier *et al.*, 2000). Decreased secretion from the salivary glands cause increased incidence in oral conditions such as periodontal diseases and *Candida* infection. Partial relief may be gained by the frequent administration of artificial saliva (Chambers, 2007) .However, limited data is currently available on the effects of different treatment methods on complete denture wearers in xerostomia patients (Aframian *et al.*, 2001).

1.2.1.2. Administration of therapeutic agents

The use of broad-spectrum antibiotics that alter the normal microflora is another predisposing factor for oral candidiasis. Utilisation of topical corticosteroids (e.g., Clobetasol), steroid inhalers, and systemic steroids are regular iatrogenic causes of oral candidiasis (Battaglia *et al.*, 2014) . Physical disabilities that impair proper oral hygiene or nutrition are other predisposing factors for oral candidiasis (Bianchi *et al.*, 2016) .

1.2.1.3. Denture Wearers

Complete denture wearing patients with dry mouth syndrome who experience candidiasis may require lengthy antifungal therapy to eradicate the infection. Fungus-associated denture stomatitis is normally diagnosed by means of clinical findings, and often microscopy may be used to confirm the clinical diagnosis by supporting the presence of mycelia or pseudo hyphae in a direct smear (Ramage *et al.*, 2004, Zomorodian *et al.*, 2011, Iosif *et al.*, 2016, Dordevic *et al.*, 2017).

About 60% of *Candida*-associated denture stomatitis are carriers of a prosthesis and *C. albicans* is the major etiologic agent (Hoshing *et al.*, 2011, Salerno *et al.*, 2011), although other *Candida* species from patients with denture stomatitis have been reported. Giannini and Shetty (2011) have identified erythematous candidiasis as the

most common form of oral candidiasis and grouped it into several different forms depending on the cause and site of involvement, for example acute atrophic, chronic atrophic, angular cheilitis, median rhomboid glossitis, and chronic multifocal.

Surface *Candida* infections linked with implanted devices are much less serious but are encountered frequently and can be troublesome. The commonest is probably denture stomatitis, which is a *Candida* infection of the oral mucosa that is promoted by a close-fitting upper denture. A biofilm is formed on the surface of the acrylic denture and contains large numbers of bacteria, particularly streptococci, in addition to yeasts (Pawashe *et al.*, 2017). Silicone rubber voice prostheses which are fitted in laryngectomized patients can also become contaminated by polymicrobial biofilms containing *Candida* spp. These devices often fail within months because the biofilm causes a malfunction of the valve mechanism (Van Der Mei *et al.*, 2000). Biofilm formation is often linked to nosocomial infections, which complicates treatment and contributes to unacceptably high mortality rates (Pierce *et al.*, 2015).

1.2.1.4. Diabetes Mellitus type II

Diabetes mellitus (DM) is a chronic multi-systemic metabolic disorder characterized by hyperglycaemia ensuing from defects in insulin secretion and/or action. The incidence of diabetes mellitus is growing increasingly, mostly in people 60 years and older and is fast becoming one of the most significant chronic diseases globally with the number of people with diabetes estimated to rise from 171 million in 2000 to 366 million in 2030 (Wild *et al.*, 2004, Chen and Chen, 2012). This increase is due to population growth, ageing, urbanization, and the rising incidence of obesity and physical inactivity (Wild *et al.*, 2004).

Diabetic patients can be groped in two categories according to insulin need: type 1 or insulin-dependent diabetes mellitus (IDDM) represents 5% to 10% of the cases and mostly starts during childhood or adolescence. It is characterized by total deficiency on insulin production by the organism, caused by auto-immune attack to pancreatic beta cells. Type 2 diabetes (T2DM) or non-insulin-dependent diabetes mellitus (NIDDM) represents 80–90% of diagnosed cases and mostly affects middle-aged obese people (Bakoush and Elgzyri, 2006). This type of diabetes is nearly completely determined by genetic factors. Pancreatic beta cells are not destroyed and, hence, insulin is present at low levels (Adeghate *et al.*, 2006).

T2DM is a burden on both patients and society because of the high morbidity and mortality associated with infections and renal, retinal, and vascular complications (Casqueiro *et al.*, 2012, Elhwuegi *et al.*, 2012). Reports on the prevalence of DM in Libyan adult patients placed it at approximately 14.1% in Benghazi, Northeast Libya (Kadiki and Roaeid, 2001) and 7.9% in Tajura, Northwest Libya (National Board for Scientific Research, 2001) .

DM patients are more vulnerable to fungal infection, particularly *Candida* infections of the oral cavity (Belazi *et al.*, 2005, Khosravi *et al.*, 2008), due to increased salivary glucose in these subjects (Sashikumar and Kannan, 2010), and because of the heightened availability of *Candida* receptors (Brownlee *et al.*, 1988). As a result, buccal cells from DM patients have an increased adherence of *C. albicans* colonization being further enhanced by the hyposalivation associated with DM (Kadir *et al.*, 2002).

It has been established that most diabetic patients show at least one lesion or abnormality of the oral mucosa. Abnormalities include lingual varicosity, erythematous candidiasis, angular cheilitis, traumatic ulcer, fissured tongue, gingival hyperplasia,

mucocele, xerostomia, petechiae, hyperkeratosis and atrophy of lingual papillae, of which, the most frequent abnormalities were lingual varicosity and erythematous candidiasis (Vasconcelos *et al.*, 2008).

1.3. Treatment of *Candida* infections

Human infections caused by *Candida* range from relatively trivial conditions such as oral and genital thrush to fatal, systemic super-infections in patients who are already seriously sick with other illnesses (Mccullough and Savage, 2005).

In local systemic illnesses the antifungal itself is not effective, and the management should be directed to both antifungal use as well as the treatment of underlying diseases such as (1) diabetes mellitus, (Arredondo-García *et al.*) adequacy of dentures,(3) deficiency states (e.g., iron, folate or vitamin B12 deficiency) (4) drug history (e.g., antimicrobials, corticosteroids); and (5) medical history (e.g., immune deficiency) (Mccullough and Savage, 2005).

The antifungal drug, fluconazole, is broadly used in the treatment of *candidiasis, cryptococcosis*, and *coccidioidomycosis.* It is a second- line antifungal agent in the treatment of histoplasmosis, blastomycosis, and sporotrichosis (Sheehan *et al.*, 1999). Its availability in both oral and intravenous formulations; significantly reduced rates of hepatic, gastric, and endocrinological toxic reactions; and the ability to penetrate cerebrospinal fluid are among its advantages (Como and Dismukes, 1994).

Fluconazole is active against most *Candida* spp., including *C. neoformans*, with an intrinsic resistance reported for *C. krusei* (Dismukes, 2006). The fluconazole sensitivities of *Candida glabrata* strains, on the hand, vary widely. Even though some *C. glabrata* isolates may be dose-dependently susceptible to fluconazole, as many as 15% of the *C. glabrata* strains may show true resistance (Pfaller and Diekema, 2004).

Resistance to fluconazole has been reported in more than 10% of cases during the late stages of AIDS and other immunocompromised conditions including *C. albicans* (Lee *et al.*, 2012).

Fluconazole and itraconazole have been commonly used to treat superficial fungal infections caused by dermatophytes and, unlike the allylamines (such as terbinafine and naftifine), have been associated with resistance development (Ghannoum, 2015).

Side effects and toxic reactions due to fluconazole therapy, such as allergic reactions, angioedema (Karkowska-Kuleta *et al.*, 2009), thrombocytopenia (Villanueva *et al.*, 2002), and alopecia (Pappas *et al.*, 1995, Stevens *et al.*, 1997) have been observed. Reversible adrenal insufficiency has been reported in critically sick patients with multiple organ failures during high-dose fluconazole therapy (Albert *et al.*, 2001).

Fluconazole was shown to have a good safety profile and was accepted for the treatment of oropharyngeal, esophageal, vaginal, peritoneal, and genito-urinary *Candida infections*, disseminated candidiasis, and cryptococcal meningitis (Mendes *et al.*, 2000). Fluconazole's mode of action includes inhibition of the target enzyme sterol 14α demethylase, which is a cytochrome p450 enzyme undertaking 14α- demethylation (Lamb *et al.*, 2000) . Modification in the level of this enzyme is a consequence of enhanced transcription and amplification of the ERG11 gene which may result in reduced sensitivity of *C. albicans* to fluconazole (Franz *et al.*, 1998). Reductions in intracellular levels of ergosterol (main sterol in fungi) occur, which changes the sterol composition ultimately causing growth arrest (Kelly *et al.*, 1997). Growth arrest in the cell is associated with the accumulation of 14α-methyl-ergosta-8, 24(28)-dien-3β, 6α-diol in a yeast strain with sterol 14 demethylase disruptions (Kelly *et al.*, 1996, Parker *et al.*, 2008, Martel *et al.*, 2010, Hull *et al.*, 2012). It can be concluded that these

antifungals are selected as inhibitors of fungal enzymes (Podust *et al.*, 2001) . Fluconazole resistance also include the enhanced expression of proteins which cause multiple drug resistance. This also causes a significant increase of fluconazole efflux, out of the cell (Podust *et al.*, 2001, Basso *et al.*, 2010) .

Unlike ketoconazole, fluconazole is highly water soluble and can be administrated parenterally. In comparison, itraconazole has a broader spectrum that includes activity against *Aspergillus* species and some yeast strains that are intrinsically resistant to fluconazole, such as *C. krusei* and *C. glabrata* (Dismukes, 2006). Although the finding of fluconazole and itraconazole represented a major advancement in the management of systemic fungal infections, these triazole antifungal agents have some vital limitations including a narrow spectrum of activity, targeting mainly yeast (*Cryptococcus* neoformans, *C. albicans*) and dimorphic fungi, with no activity against molds (Dismukes, 2006).

1.4. The emergence of antifungal drug resistance

The use of broad-spectrum antimicrobial drugs has lead or attributed to the growing of fungal infections as well as the use of corticosteroids, anti-tumoural agents, and oral contraceptives. This is further exacerbated by the emerging resistance in opportunistic infection in the increasing number of immunocompromised patients (Bremenkamp *et al.*, 2011). For example, lack of regional surveillance of *Candida* prevalence and antifungal drug susceptibility, uncontrolled distribution and sale of medications, lack of patient access to medical treatment and limited resources in African laboratory and clinical settings have perhaps contributed to the emergence of *Candida* resistance to varies classes of antifungal drugs (Abrantes *et al.*, 2014). However, these factors need

to be addressed as part of an effort to reduce the high morbidity and mortality rates seen in these populations (Abrantes *et al.*, 2014).

Despite the introduction of new antifungal agents, antifungal resistance continues to grow and evolve and complicate patient management (Pfaller, 2012). The ability of *Candida* species to form drug resistant biofilms is an important factor in their contribution to human disease (Sardi *et al.*, 2013).

Many studies have incessantly reported the increased frequency of non-*albicans Candida* (Villanueva *et al.*, 2002) species (Pfaller *et al.*, 2001, Hajjeh *et al.*, 2004, Horn *et al.*, 2009), considered to be significant pathogens in the oral cavity, genitourinary system, and bloodstream. The growing number of NAC species might be related to previous exposure to azole and polyene drugs, malignancy disease and indwelling medical devices (Krcmery and Barnes, 2002, Pfaller and Diekema, 2010, Miceli *et al.*, 2011). However, differences in age groups and geographic areas might influence the distribution of NAC species (Falagas *et al.*, 2010). For instance, in North America, and Central Europe *C. glabrata* has a high incidence rate. *C. tropicalis* is commonly isolated in South America and Asia. Furthermore, *C. glabrata* predominates in the elderly (Blot *et al.*, 2001), while *C. dubliniensis* is associated with oral opportunistic infections in patients infected with human immunodeficiency virus (Chunchanur *et al.*, 2009).

1.5. Challenges of antifungal resistance

The emergence of antimicrobial resistance, which is one of the main reasons for antifungal treatment failure, has been observed as a serious clinical problem (Nucci and Perfect, 2008). An increase in the frequency of prophylactic treatment of high-risk individuals, coupled with extended exposure to the existing antifungals increases the

selection pressure and, as a result, drug resistance has become gradually common from initially sensitive species (Kontoyiannis and Lewis, 2002, Pfaller, 2012).

The increase in antimicrobial resistance and the restricted number of antifungal drugs, which retain several complications (Sardi *et al.*, 2013), creates a need for alternative methods of treating resistant oral infections.

Significant mortality is relevant with invasive candidiasis (Mcneil *et al.*, 2001, Gudlaugsson *et al.*, 2003), and is consequently, a serious danger to public health around the world (Eggimann *et al.*, 2003, Hobson, 2003, Pfaller and Diekema, 2007). The current shifting of the paradigm of *Candida* infections further complicates the situation and highlights the requirement for novel classes of antimicrobial agents, particularly those with new mechanisms of action (Pauw, 2000, Butts and Krysan, 2012).

1.6. Medicinal Plants and Oral Infections

Traditional medicinal treatment of various ailments has used medicinal plants as an important source of phytochemicals (Cruz *et al.*, 2007, Doss and Anand, 2012, Maobe *et al.*, 2013). For many years, different parts of medicinal herbs have been used to treat specific ailments in Kenya (Kiringe, 2006).

Medicinal plants comprise effective sources of antimicrobial and antioxidant natural products (Calixto, 2000), and might assist as a first treatment option (Scorzoni *et al.*, 2007). In addition, they characterize an economic alternative, are easily available and are applicable to several sicknesses (Rojas *et al.*, 2006), thus constituting an excellent alternative in the search for compounds or fractions that might be used to discover new antimicrobial drugs (Holetz *et al.*, 2002). It is necessary to find new antifungal agents, that lead to the disruption of resistant biofilms, by using several molecular mechanisms with reduced toxicity (Sardi *et al.*, 2011).

Herbal medication is the study of medicines derived from botanical sources materials that have least or no manufacturing processing (Tilburt and Kaptchuk, 2008). A dissimilarity of antimicrobial activity is due to active compounds of plants (Shai *et al.*, 2008). Flavonoids alkaloids, tannin, and phenolic compounds, amongst others, are supposed to be vital active substances of various plants (Thirumurugan, 2010). Medicinal plant extracts have several traditional uses which contain lotion for wounds, curing of venereal and skin diseases as well as for relief of inflammation of the eyes (Van Wyk *et al.*, 2008). The Khoi-san also chews on this plant to relieve toothache (Van Wyk *et al.*, 2008), while the antifungal potential of these flavonoids against human pathogens should be considered (Vries *et al.*, 2005).

1.6.1. *Euclea natalensis*

1.6.1.1. Taxonomy, Morphology, and Distribution of Euclea natalensis.

The angiosperm family, Ebenaceae or ebony family, to which *Euclea natalensis* belongs are woody plants (trees, shrubs, or subshrubs) and mainly found in the tropical and subtropical regions of the world where they grow in a variety of vegetation types. Even though Asia and the Indo-Pacific regions have the highest concentration of species, the African and Madagascan regions display the greatest morphological diversity (Wallnöfer, 2004).

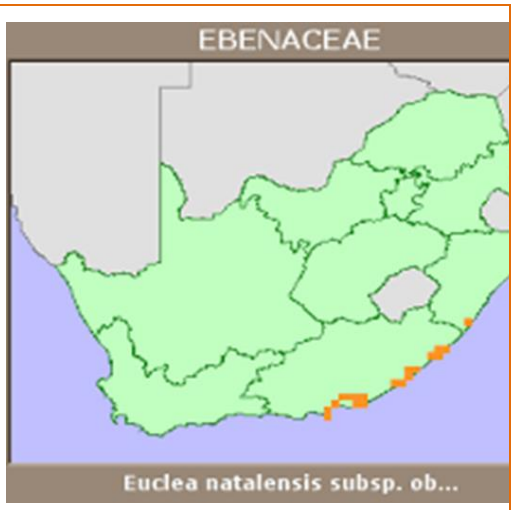
The *Ebenaceae* is divided into two subfamilies, the Lissocarpoideae and the Ebenoideae based on differences in floral morphology, pollen morphology and wood anatomy (Duangjai *et al.*, 2006). *Euclea* with approximately 20 species (of which 16 occur in southern Africa) is the second largest genus in the family (Table 1.1).

Euclea natalensis is a bushy tree found in many parts of South Africa, such as Clanwilliam in the Western Cape, along the coast between the Eastern Cape and

KwaZulu-Natal and in Mpumalanga, Limpopo, Gauteng, and neighbouring countries such as Swaziland, Mozambique, Zambia, Zimbabwe and further North, Ethiopia. Used for many years by the locals, it goes by many names such as guarri (Khoisan) swartbasboom (Afrikaans) in Kunzane (Zulu).

Table 1.1: Taxonomy of *Euclea natalensis*

Kingdom	Plantae
Class	Magnoliopside
Order	Ericales
Family	Ebenaceae or ebony family
Genus	*Euclea*
Synonym(s)	*Euclea* multiflora Hiern, Royena macrophylla
Derivation of specific name:	*natalensis: of Natal, South Africa; acutifolia: with acute (pointed) leaves.*
Binomial name	*Euclea natalensis*
Description	Shrub or small tree, 2-7 m, or sometimes a suffrutex, less than 1m tall. Bark greyish, rough, and fissured, flaking in thick pieces. Leaves elliptic up to 12 × 4 cm, densely hairy below. Flowers in dense, branched axillary heads, greenish-white or cream, most parts covered in dense rusty woolly hairs. Fruit in clusters, round c. 7 mm in diameter, sometimes rusty hairy, red to black.



Distribution map of *Euclea natalensis* along South African costal area.

Figure 1.1: Collection of Euclea natalensis plant material by researcher and Botanist (Prof. Christopher Cupido) along South African costal area approximately 25 km east of Clanwilliam area.

Euclea natalensis has many uses including the use of its twigs as toothbrushes, the roots and bark are used as remedies for worms, stomach disorders, toothache, headache, chest infections, urinary tract infections, venereal diseases, schistosomiasis, dysmenorrhoea, and skin infections. It is also used for protective sprinkling to ward off evil. Its application to improve oral hygiene has shown that it is able to suppress the growth of both aerobic and anaerobic oral bacteria (Stander and Van Wyk, 1991, Van Wyk and Van Wyk, 1997) as well as *Candida* (More *et al.*, 2008).

In South Africa, ethnobotanical use of *Euclea* is extensively popular such as hardening the chickens' eggshells by feeding the chickens *Euclea pseudebenus* fruit (Van Wyk and Van Wyk, 1997). *E. natalensis* roots are used for dyes in basket weaving because of the dark brown or black stains produced when pounded and boiled. The source of the pigmentation can be linked to the presence of compounds such as *7-methyljuglone* and diospyrin as well as other quinones (Figure 1.2) (Van Wyk and Gericke, 2000).

Traditionally, the root is chewed for cleaning teeth and the gums with a chew, in the belief that it benefits the health of the mouth and teeth (Stander and Van Wyk, 1991). The root of *E. natalensis* contains naphthoquinones which are bactericidal (Stander and Van Wyk, 1991). Regular use of *E. natalensis* might control the formation and activity of dental plaque and therefore reduce the incidence of gingivitis and possibly of dental caries due to the inhibitory effect of the ethanol extract of *E. natalensis* against *Actiomyces naeslundii, Actimyces israelii, Porphyromonas gingivalis, Prevotella intermedia* and *Streptococcus mutans* (More *et al.*, 2008). Its use as an alternative to the use of conventional antifungals has not been adequately investigated.

Among the traditional other uses of *Euclea* species are as a treatment for chest illness, bronchitis, chronic asthma, pleurisy , venereal diseases, and urinary tract infections (Pujol, 1990). Furthermore, Zulu people practice it as a remedy for scrofula a form of tuberculosis characterised by glandular swellings. Additionally, the roots are burned and the smoke inhaled as a hypnotic (Van Wyk and Van Wyk, 1997, Van Wyk and Gericke, 2000). Moreover, extraction of the roots of *E. crispa* are swallowed orally for epilepsy (Van Wyk and Gericke, 2000).

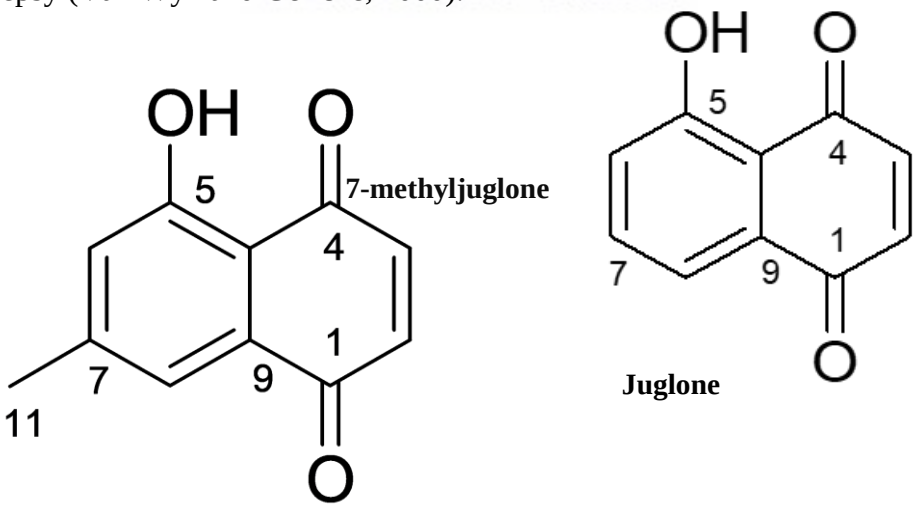


Figure 1.2: Chemical structure of the isolates 7-methyljuglone (7-MJ) and Juglone (5-Hydroxy-1, 4 naphthoquinone), using (NMR)

1.6.2. *Salvadora persica* (Miswak)

1.6.2.1. Taxonomy, Morphology, and Distribution of *Salvadora Persica*

Salvadora persica (Table 2) is a plant growing in the dry areas from the region of Western India to Africa (Ezoddini-Ardakani, 2010). Also called the Arak tree, *Salvadora persica* is an evergreen shrub or small tree that can reach a height of 6-7 m. It has an erect trunk with slightly rough bark and a wide crown of profuse, crooked, and dropping branches. Saltbush leaves are opposite, oblong-elliptic to almost circular, 3 x 7 cm, light to dark green, rather fleshy, borne on a 1 cm long petiole. The inflorescence is a 10-cm long panicle that bears very small, greenish to yellowish flowers. The fruit is pink to scarlet, spherical, fleshy, 5-10 mm in diameter. It contains one seed that turns from pink to purple to semi-transparent at maturity (Orwa *et al.*, 2009) chewing stick, natural toothbrush and Miswak or Siwaak, *S. persica* has been reported to avoid dental caries if it is used for a long periods (Ezoddini-Ardakani, 2010, Sofrata *et al.*, 2011).

Additionally, a number of antimicrobial substances have been produced using roots of *S. persica*, with the benzyl isothiocyanate (BITC) being the main antimicrobial ingredient with high killing activity against Gram-negative periodontal pathogens (Sofrata *et al.*, 2011). Antibacterial effects against *S. mutans* and *S. faecalis* have also been reported (Almas, 1998).

Salvadora persica is found in several geographical areas and was used by Arabs thousands of years ago to whiten and polish the teeth (Halawany, 2012). Although used in pre-Islamic times, as the Islamic culture expanded to different geographical regions, so also did the use of Miswak spread (Almas, 2001). Despite the fact that there are more than 182 appropriate plants species for use as tooth brushing sticks, its twigs, stems,

and roots are still the most widely used for achieving dental health and oral hygiene (Elvin-Lewis, 1982, Ezoddini-Ardakani, 2010).

Al-Bayati and Sulaiman (2008) have revealed in their research that Miswak consists of natural chemical compounds or fractions which play a vital part in promoting good oral hygiene and more studies should be investigated to evaluate its efficacy in treating oral pathogens (Who, 2000, Al-Bayati and Sulaiman, 2008). The following compounds are alkaloids (salvadorine), benzyl isothiocyanate (BIT), calcium, chloride, essential oils, fluoride, resin, silica, sulphated compounds, tannins, salicylic acid, sterol, trimethylamine, saponins and flavonoids (Al-Bayati and Sulaiman, 2008, Poureslami *et al.*, 2008). It is cost effective and is easily obtainable in various regions of the world (Poureslami *et al.*, 2008).

The sour taste and chewing effects of Miswak may stimulate saliva secretion in the oral cavity, thereby raising its buffering capacity (Hattab, 1997). *In vitro* trials, (Sofrata *et al.*, 2007) have demonstrated that using Miswak extracts as a mouth wash increased parotid gland secretion, thereby raising the plaque pH; this effect can possibly protect from dental caries by reversing the acid challenge of cariogenic bacteria. In rural areas of Zanzibar, the caries prevalence rate was found to be lower where Miswak was traditionally and regularly used than in the city areas (Petersen and Mzee, 1998).

Darmani *et al.* (2006) have reported in a comparative study of *S. persica* extracts and derum (different type of chewing stick obtained from walnut tree Juglans regia) that both were capable of inhibiting the growth of cariogenic bacteria (Darmani *et al.*, 2006), probably, due to the fluoride contained in *S. persica* extracts (Ezoddini-Ardakani, 2010). However, because of the negligible total soluble content of fluoride in Miswak

soaked in water, (0.07 l g / ml) the possible influence of fluoride was unclear (Hattab, 1997).

One of the other beneficial components of *S. persica* that might have positive effects on oral health is the availability of sulfur in the *S. persica* roots, measured to be as high as 4.73% (Galletti *et al.*, 1993, Halawany, 2012). Sulfur has a bactericidal activity (Alsamh, 1996), while calcium in saliva as a result of the practicing of chewing sticks promotes enamel remineralization (Gazi *et al.*, 1992). Resins may form a coating on enamel that defends against dental carries (Ababneh, 1995). Salvadorine, an alkaloid present in *S. persica*, may have a bactericidal effect and improve the gingival condition (Almas, 1993), while silica acts as an abrasion and was found to remove the pigmentations from teeth surfaces (Ababneh, 1995). The astringent effect of tannins may help to reduce clinically noted gingivitis by inhibiting the action of glucosyl transferase (Gazi *et al.*, 1992).

Numerous toothpastes containing *S. persica* extracts are in the market (Guile *et al.*, 1996, Almas and Al-Bagieh, 1999), and when compared with Oral-B toothpaste, was found to be more potent in cleaning dental plaque (Hattab, 1997). Moreover, the mixed impact of mechanical cleaning and enhanced saliva excretion with the regular use of Miswak, was found to be more functional than other toothbrushes in removing dental plaque (Wu *et al.*, 2001).

Table 1.2: Taxonomy of *Salvadora persica*

Kingdom	Plantae Trees, shrubs or subshrubs
Division	Magnoliphyta
Class	Magnoliopsida
Order	Brassicales
Family	Salvadoraceae
Genus	Salvadora Otholobium
Synonym(s)	Toothbrush tree, mustard tree, arak, Miswak, Swak, Saltbush,
Species	*persica oleoides*
Binomial name	*Salvadora persica (Khari Jaal) Salvadora oleoides (Meethi Jaal)(Khatak et al., 2010)*

1.7. Summary and Objectives

With *Candida* species showing on increasing prevalence of antifungal resistance, and diabetes being a predisposing factor for *Candid* carriage, primary prevention of T2DM and treatment of diabetic complications such as *Candida* species, are of great practical importance.

A literature search showed that there is an absence of published data regarding the prevalence of *Candida* infection in T2DM in the elderly Libyan population. The Libyan population is approximately 5.5 million, with one in five Libyans having DM II. A high percentage of risk factors including obesity, family history of diabetes, hypertension and microvascular complications necessitates a Libyan national policy for the surveillance, prevention and control of diabetes and its complications (Elhwuegi *et al.*,

2012). Misrata is a fast-growing city in the north of Libya with a population of just over 550,000 in 2006 and the third largest city in Libya after Tripoli and Benghazi.

Since natural compounds such as those contained in *S. persica* and *E. natalensis* have demonstrated good antibacterial activity, particularly in the oral cavity, the objectives of this study were to:

i. Investigate *Candida* species prevalence in Libyan T2DM patients.
ii. Examine *Candida* isolates for antifungal susceptibility profiles.
iii. Investigate the antifungal activity of *S. persica* and *E. natalensis* for use against fluconazole resistant *Candida* species.

CHAPTER 2

ISOLATION AND CHARACTERISATION OF *CANDIDA* SPECIES

2.1. Introduction

Accurate identification of *Candida* species frequently associated with infections is of paramount significance for increasing the understanding of *Candida* pathogenicity Manfredi *et al.* (2006). Rapid identification of *Candida* species can assist suitable treatment, to reduce fungal infections often related with patient mortality and morbidity (Pincus *et al.*, 2007). Several factors such as immunosuppression, behavioural patterns, diet and socio-economic status and distribution of *Candida* species varies according to different geographical regions which could have an effect on their prevalence (Dos Santos Abrantes *et al.*, 2014).

Recently, *Candida* species are progressively becoming the predominant commensal in the oral cavity (Sharma *et al.*, 2017). It could be a constituent of the normal oral microflora in approximately 30% to 50% of the population (Giannini and Shetty, 2011). *Candida albicans* is the most frequently isolated of *Candida* species from the oral cavity, although a range of non- *albicans Candida* species are being increasingly observed. The basic management of candidiasis is to classify and eliminate any underlying host predisposing factor (Lewis and Williams, 2017).

With the increasing incidence of *Candida* infections in African countries, the understanding of their pathogenicity as well as the association predisposing factors to enhance *Candida* growth is needed. This chapter describes the prevalence of *Candida*

in T2DM patients in Libya and the importance of species differentiation in treatment modalities.

Material and Methods

2.1.1. Sample collection

Three hundred and thirty (330) samples were collected between March 2015 and November 2015 from Type 2 Diabetes Mellitus (T2DM) patients at Misrata Diabetes Centre which is in the northern central part of Libya. Samples were collected by swabbing the patient's oral mucosa and tongue with a sterile cotton swab.

This study included:

i. T2DM positive patients presenting with and without clinical oral thrush (white patches in the mouth) were included in this study, as these patients had a higher chance of harbouring oral *Candida* species.

ii. 35-95 years old

Excluded from the study were any patients who had undergone antifungal therapy two weeks prior to sample collection.

Prior to sample collection, the reasons for, and nature of the study were explained to the patients who willingly consented to participate by signing appropriate consent forms (Appendix 1). Data from the patients' hospital folders were used to establish their diagnosis and they were asked to submit some personal information by completing a questionnaire which provided data such as gender, age, race, whether or not they wore dentures, duration of wearing dentures, smoking, date diagnosed with T2DM, antifungal treatment and duration of treatment prior to taking the samples (Appendix 2). Ethical clearance for this project was granted by the Ethics Committee at University

of Western Cape. Authorisation from the Ministry of Health in Libya was obtained for sample collection in Misrata city.

2.1.2. Isolation of *Candida* species

Samples were cultured in the Medical Microbiology laboratories at MCH (Misrata Central Hospital) onto Sabouraud dextrose agar (Cat. no. BO0408T, Oxoid, UK) and incubated at 37˚C for 24 hours. Plates showing no growth were re-incubated for an additional 24 hours before being discarded as negative. All isolated *Candida* strains were stored at -80 ˚C in Pro-Lab Microbank microbial preservation vials (Cat. no. PL.170/M, Pro-Lab, Canada), and slant agar in 2.5 mL Eppendorf tubes, allowing them to be resuscitated as and when needed. Samples were transported from Libya in these frozen preservation vials to UWC for characterisation.

2.1.3. Characterisation of isolates

Presumptive identification of species was achieved by inoculation onto Fluka and Oxoid chromogenic agars (Cat. no. 94382, Sigma-Aldrich, USA and Cat. no. CM1002A, Oxoid, UK), Gram staining, and germ tube testing. The identification of the isolated species was done via biochemical testing, using API ID 32 C (bioMérieux, Marcy l'Etoile, France). Further confirmation of species resistant to fluconazole were done by using VITEK 2 compact system YST 07 (bioMérieux, Marcy l'Etoile, France).

2.1.3.1. *Candida* species identification using chromogenic media

Presumptive identification of *Candida* was done on chromogenic agar. Selective agar and chromogenic media included Sabouraud's agar, modified Fluka chromogenic *Candida* identification agar, (Cat. no. 94382, Sigma-Aldrich, USA) with respective

selective supplement (Cat. no. 68067, Sigma-Aldrich, USA), Oxoid chromogenic *Candida* agar (Cat. no. CM1002A, Oxoid, UK), with respective selective supplement (Cat. no. SR0231E, Oxoid, UK). Type strains of *C. albicans* (ATCC 90028 and NCPF 3281), *C. tropicalis* (ATCC 950), *C. dubliniensis* (NCPF 3949a), *C. glabrata* (ATCC 26512), *C. krusei* (ATCC 2159), *C. parapsilosis* (ATCC 22019), *C. kefyr* (ATCC 4135) and *C. lusitanae* (ATCC 3449) were used as positive controls for the chromogenic species differentiation.

2.1.3.2. Microscopy

Purity of cultures was confirmed by microscopy of colonies picked from agar plates. The Gram stain was used for the staining of *Candida* isolates prior to light microscopy observation. A single colony of each isolate was smeared on a glass slide, heat fixed and covered with crystal violet (primary stain, iodine (which acts as a mordant), alcohol (used for decolourization) and counterstained with dilute carbol fuchsin.

2.1.3.3. Germ Tube Test

Presumptive *C. albicans* and *C. dubliniensis* cultures were incubated at 37˚C for 2-3 hours in fetal bovine serum (Cat. no. A15-101, PAA Laboratories, Austria), for the stimulation of germ tube production. Germ tube formation was observed microscopically in a wet mount preparation. Type strains of *C. albicans* (ATCC 90028) and *C. dubliniensis* (NCPF 3949a) were used as positive controls, whereas *C. tropicalis* (ATCC 950) and *C. glabrata* (ATCC 26512) served as negative controls.

2.1.3.1. *Candida* Species Identification using Chromogenic Media

Presumptive identification of *Candida* was done on chromogenic agar. Selective agar and chromogenic media included Sabouraud's agar, modified Fluka chromogenic

Candida identification agar, (Cat. no. 94382, Sigma-Aldrich, USA) with respective selective supplement (Cat. no. 68067, Sigma-Aldrich, USA), Oxoid chromogenic *Candida* agar (Cat. no. CM1002A, Oxoid, UK), with respective selective supplement (Cat. no. SR0231E, Oxoid, UK). Type strains of *C. albicans* (ATCC 90028 and NCPF 3281), *C. tropicalis* (ATCC 950), *C. dubliniensis* (NCPF 3949a), *C. glabrata* (ATCC 26512), *C. krusei* (ATCC 2159), *C. parapsilosis* (ATCC 22019), *C. kefyr* (ATCC 4135) and *C. lusitanae* (ATCC 3449) were used as positive controls for the chromogenic species differentiation.

2.1.3.2. API ID 32 C Identification System

This technique consists of a disposable plastic strip with 32 wells containing substrates for 29 assimilation tests (carbohydrates, organic acids, and amino acids), one sensitivity test (cycloheximide), one colorimetric test (esculin), and negative control. The yeast identification procedures were conducted according to the manufacturer's instructions. A single colony of mature growth (24-48h) sub cultured species was transferred to sterile distilled water 10 mL in test tubes to prepare a suspension with a final turbidity comparable to McFarland standard 3. A 250 µl of this suspension was then dispensed to an ampule of C medium provided by the manufacturer and homogenized to prepare an even dispersion of inoculum. After homogenizing, the inoculum suspension was used to inoculate the wells in the strip (135 µl x 32 wells), the lid of the strip was replaced, and the system was incubated in a shaking incubator (LBOTEC Orbi Shake, South Africa) in speed 75 at 29 ^{0}C ±2 ^{0}C for 24 h for the first reading, followed by a second reading at 48 h for confirmation. Turbidity indicated positive growth and the clear wells indicated negative growth (Figure 2.1). The results were transformed (+, -) into the bioMérieux websites.

(https://apiweb.biomerieux.com/servlet/Authenticate?action=preparelogin).

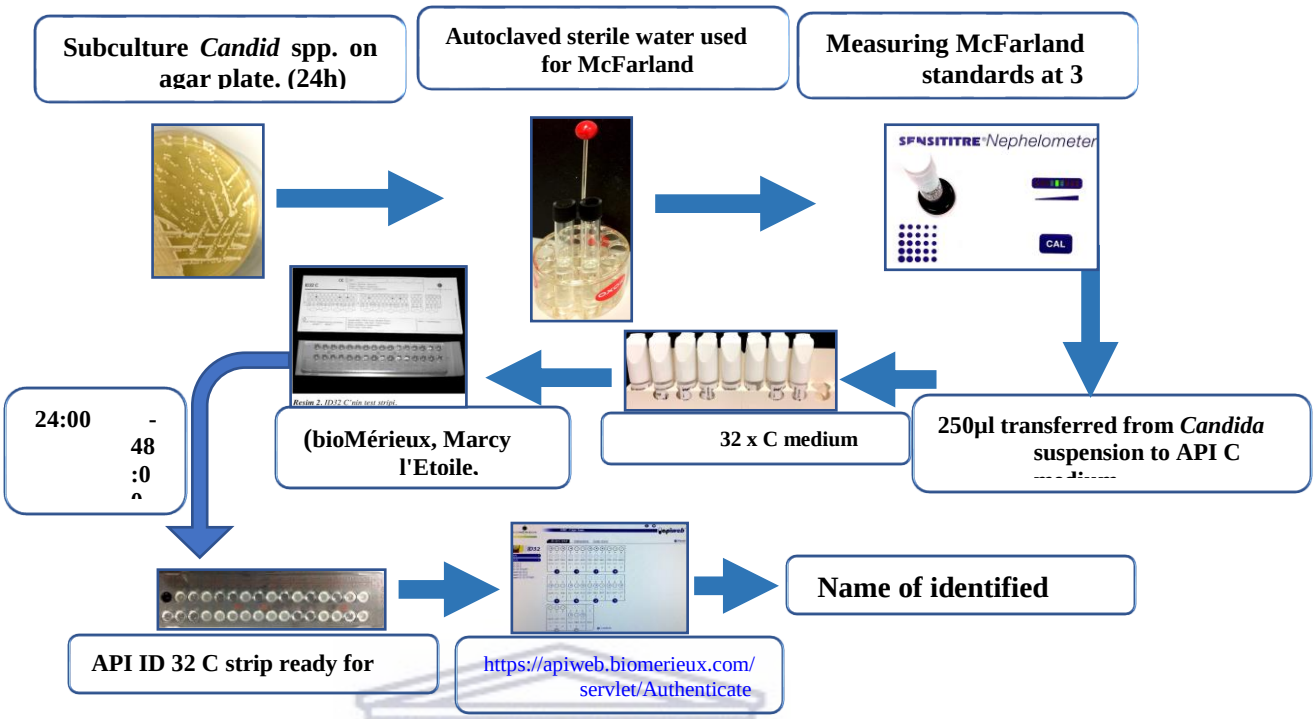


Figure 2.1. Flow diagram demonstrating API ID 32 C processing to identify *Candida* spp.

2.1.3.4. Vitek 2 Compact Identification System

The Vitek 2 Yeast identification card (Vitek 2® Compact, Marcy l'Etoile, bioMérieux, Franc) is projected for use with Vitek 2 compact Systems for the automated identification of most clinically significant yeasts and yeast-like organisms. There are 14 biochemical tests measuring carbon source utilization, nitrogen source utilization, and enzymatic activities. Final results are available in roughly 18 hours with YST - yeasts and yeast-like organisms. The Vitek 2 YST identification card (bioMerieux, Inc. Hazelwood, MO) is a single-use disposable card.

Nine known *Candida* type strains *C. albicans* (ATCC 90028 and NCPF 3281), *C. tropicalis* (ATCC 950), *C. dubliniensis* (NCPF 3949a), *C. glabrata* (ATCC 26512), *C. krusei* (ATCC 2159), *C. parapsilosis* (ATCC 22019), *C. kefyr* (ATCC 4135) and *C. lusitanae* (ATCC 3449) served as controls for the Vitek 2 compact system.

2.1.3.5. Phenotype Microarrays

Biolog, Inc. has developed a new proprietary technology called Phenotype Microarrays (PMs). PMs allow biologists, for the first time, to globally analyse the phenotypes of cells. The recent PM technology is manufactured for use with fungi cells. PMs are sets of 96-well microtiter plates with each well comprising a different cell culture medium that is designed to test a unique phenotype or cell function.

Phenotype Microarrays (Bochner, 1989, Funchain *et al.*, 2000, Oresnik *et al.*, 2000, Bochner *et al.*, 2001) measure the carbon (PM1-2), nitrogen (PM3), and phosphorus/sulfur (PM4) metabolism of a strain. As the PMs are incubated under appropriate conditions, color forms in the wells to reflect the Phenotypes of the strain being tested. PM1-2 measures carbon utilization in a fully supplemented medium and should not require any supplements to the inoculating fluid other than menadione,

thioglycolate or salicylate. PM3-4 measure sulfur, phosphorus, and nitrogen utilization in a minimal defined medium. If the cells display an expected phenotype and can grow in a well, they respire normally to give a dark purple color. If the phenotype and growth are weak, they respire more slowly and give a light purple color. If the phenotype and growth are negative, the well will remain colorless.

The constituents of the culture media were dried onto the bottom of each well. After inoculation, the cells were incubated, usually for 24 to 48 hours. Suspensions were prepared to determine the target cell density using turbidity standards available from Biolog (e.g. 85%T or 40%T). A 100-mL volume was pipetted per well, and the PM (1, 2, 3, 4) incubated for 24 hours at the recommended temperature (35-37 ^{0}C) in a humidified incubator to prevent drying of the outer wells, PM3 and PM4 were incubated for 36-48 hr. Reading and scoring the results were according to the formation of purple color which indicates utilization of the C, N, P, or S basis in a particular well. Any well with more color than the reference well is measured “positive”. Usual reactions in PM1-4 for the species listed in are posted on Biolog's website (www.biolog.com). Different strains will vary, but the result with non-mutated strains should be similar.

RESULTS

2.1. Descrptive analysis of patients sampled.

The study sample included 330 patients of whom 17 (5.2%) showed clinical signs of candidiasis on examination. Statistics for Age, weight, height, BMI and denture wearers are discribed in Table 2.1.

2.1.1. Age.

The ages of the 330 patients studied, ranged between 18-94 years with a mean (SD) of 60.6 (±9.7) (Table 2.1).

Table 2.1: Descriptive statistics of patients

		Age in years	Weight in Kg	Height in cm	Calculated BMI	Duration of wearing dentures in years	Date diagnosed in years
N	Valid	330	330	330	330	99	329
	Missing	0	0	0	0	231	1
Mean		60.65	83.85	161.42	32.32	7.53	13.11
Std. Error of Mean		.537	.867	.576	.371	.702	.484
Median		60.00	80.00	160.00	31.25	5.00	12.00
Std. Deviation		9.754	15.758	10.461	6.747	6.980	8.777
Range		76	120	76	68	37	49
Minimum		35	50	110	19	1	1
Maximum		94	170	186	87	38	50
Sum		20014	27672	53269	10667	745	4314

2.1.2. Weight and height

The mean weight and height were 83.8 kg (±15.7) and 161.4 cm (±10.5) respectively (Table 2.1). These were used to calculate the Body Mass Index (BMI) of the participants.

2.1.3. Body Mass Index (BMI)

According to WHO (International classification) (Pasco *et al.*, 2012) participants were classified into five groups using BMI: normal BMI range (18.50-24.99) was the lowest (7.9%) of which 5 and 9 male patients were positive for *Candida*. The mean BMI was 32.32 (±6.74).

2.1.4. Denture wearers

Denture wearers constituted 99 (30%) of participants (Table 2.1). The duration for wearing dentures ranged between 1-38 years with a mean (SD) of 7.5 (± 6.9) years.

2.2. Isolation and identification of *Candida* from clinical samples

Samples were initially plated onto Sabouraud dextrose agar for the isolation and of the 330 patients, one hundred and seventy (51.5%) samples resulted in positive *Candida* growth on Sabouraud dextrose agar (Figure 2.2), with 12 patients carrying >1 *Candida* species. *Candida* and species differentiation achieved using chromogenic agar, API biochemical testing and Vitek 2 compact system.

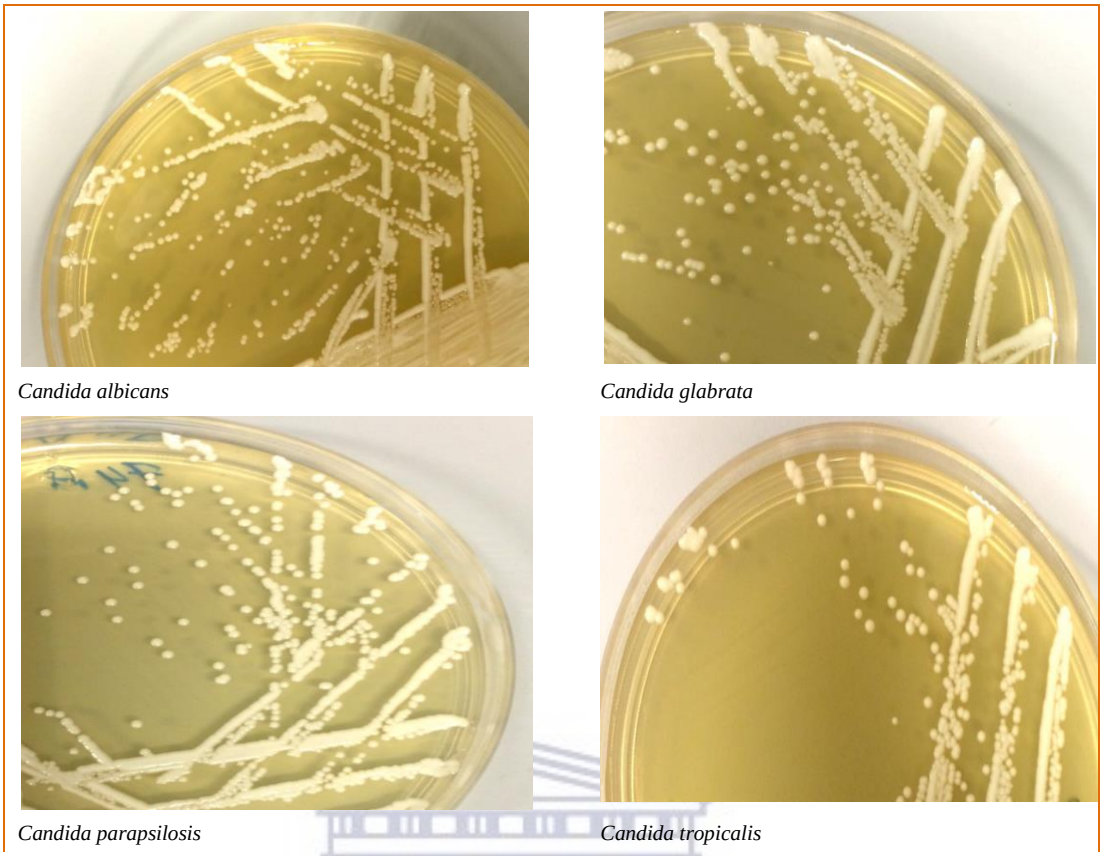


Figure 2.2: Growth of *Candida* species on Sabouraud dextrose agar

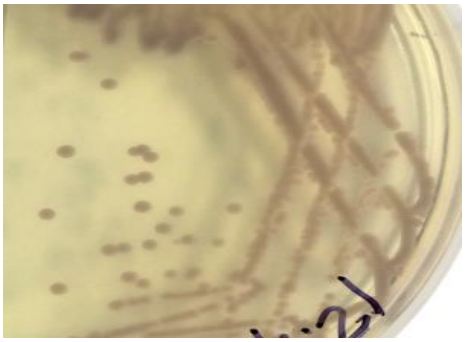
2.2.1. Frequency of *Candida* Carriage

Of the 330 patients, 170 (51.5%) samples resulted in positive *Candida* growth on Sabouraud dextrose agar with 12 patients carrying > 1 *Candida* species. *Candida* species differentiation was achieved using chromogenic agar, API ID 32C and Vitek 2 compact system.

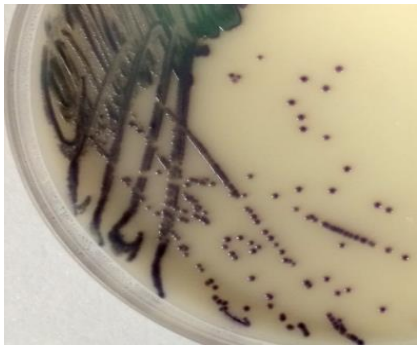
2.2.2. Species differentiation using chromogenic media

Isolates were inoculated onto chromogenic agar and incubated at 37˚C for 24 hours. The different colours and textures that distinguish the various species are clearly demonstrated for selected species in Figure 2.3, using Fluka and Oxoid chromogenic media. The *Candida* species differentiation results are shown in accordance with the colours/textures expressed by the *Candida* type strains on both Oxoid and Fluka media.

(A) **(B)**

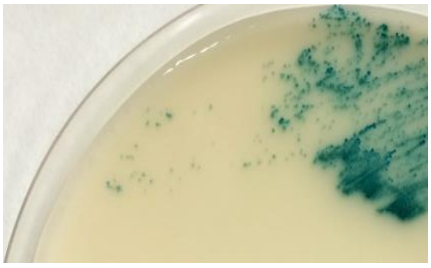


C. multisgemmis

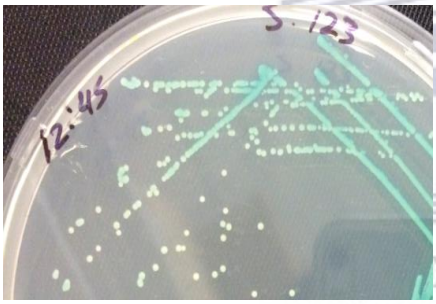


C. multisgemmis

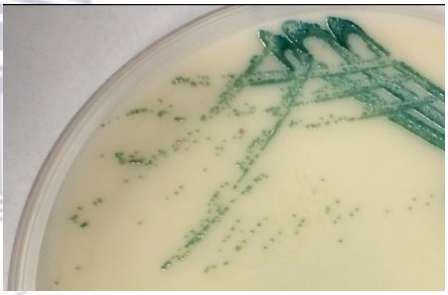
C. albicans



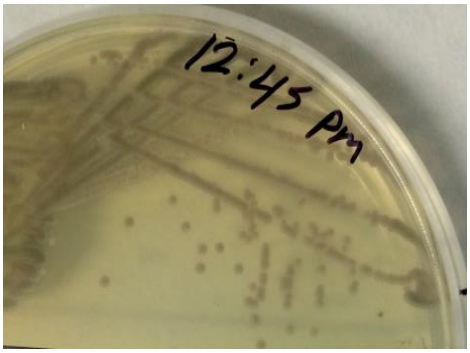
C. albicans



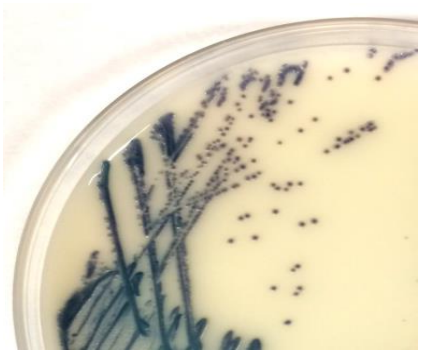
C. dubliniesis



C. dubliniesis



C. maritima

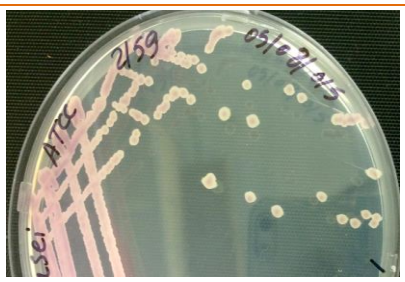


C. maritima

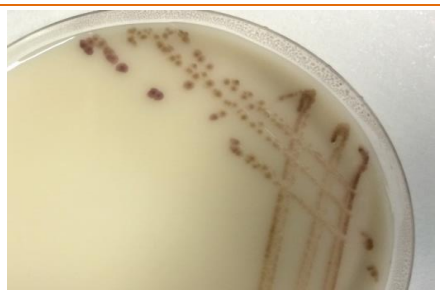
Figure 2.3: Growth of clinical *Candida* species on Fluka **(column A)** and Oxoid **(column B)** chromogenic media

With Fluka chromogenic agar, *C. albicans* (ATCC, NCPF) grew as opaque light green colonies, *C. dubliniensis* distinctly dark green shiny colonies, and *C. tropicalis* as metallic blue colonies. *C. glabrata* grew as beige-cream to very light brown colonies and *C. krusei* grew as pink with beige to brown, fuzzy colonies. The identification of *C. kefyr/krusie/parapsilopsis/ lusitaneae* is not described in the Fluka catalogue (Figure 2.4).

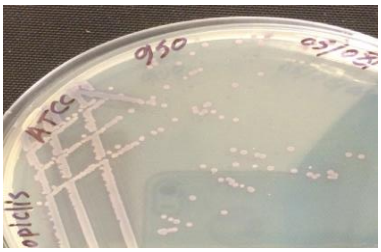
(A) **(B)**



C. krusei (ATCC 2159)



C. krusei (ATCC 2159)

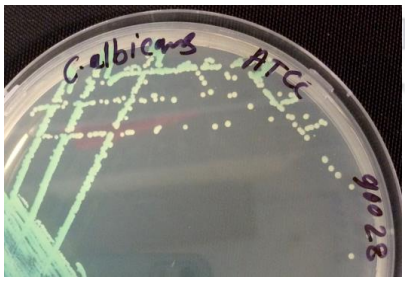


C. tropicalis (ATCC 950)

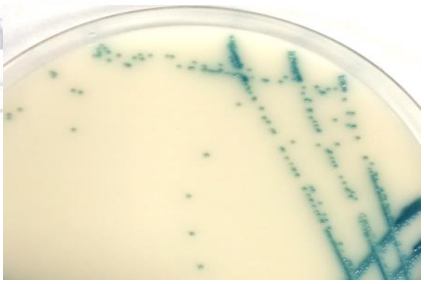
C. tropicalis (ATCC 950)

C. kefyr (ATCC4135)

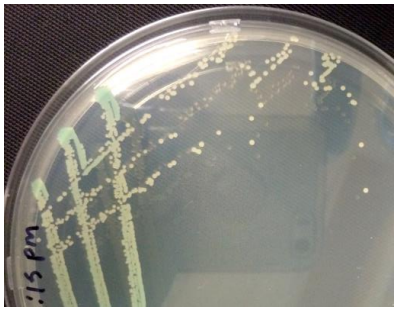
C. kefyr (ATCC4135)



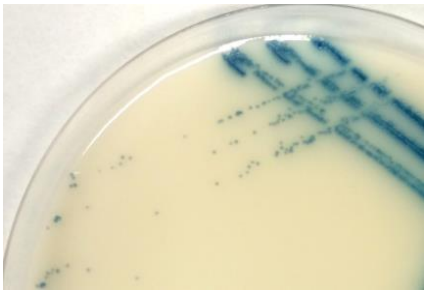
C. albicans (ATCC90028)



C. albicans (ATCC90028)



C. dubliniesis (NCPF3949)



C. dubliniesis (NCPF3949))

Figure.2.4.: Growth of *Candida* type strains on Fluka **(column A)** and Oxoid **(column B)** chromogenic media.

Fluka chromogenic media identified 113 (61.1%) isolates as *C. albicans*, 20 (11.0%) as *C. dubliniesis*, 4 (2.2%) as *C. glabrata*, 1 (0.5%) as *C. kefyr*, 2 (1.1%) as *C. krusei*, 2 (1.1%) as *C. lusitaneae*, 1 (0.5%) as *C. parapsilosis*, 2 (1.1%) as *C. tropicalis* and 37(20.3%) as *C.* unidentified (Figure 2.5).

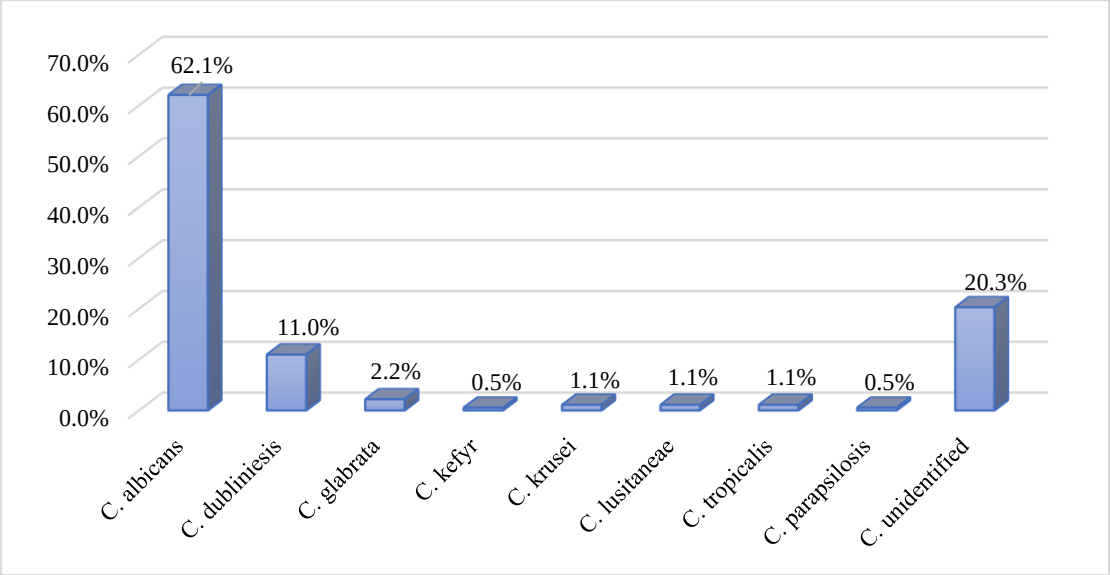


Figure 2.5: Distribution of *Candida* species using Fluka chromogenic agar

On Oxoid chromogenic media, *C. albicans* in both ATCC and NCPF grew as light opaque green colonies, *C. dubliniensis* as dark shiny green colonies, *C. glabrata* as smooth beige or creamy/yellow/brown colonies, *C. kefyr/parapsilopsis/lusitaneae* as variable, natural pigment colonies and *C. tropicalis* as metallic blue colonies, while C. *krusei* grew as pink/brown, fuzzy colonies.

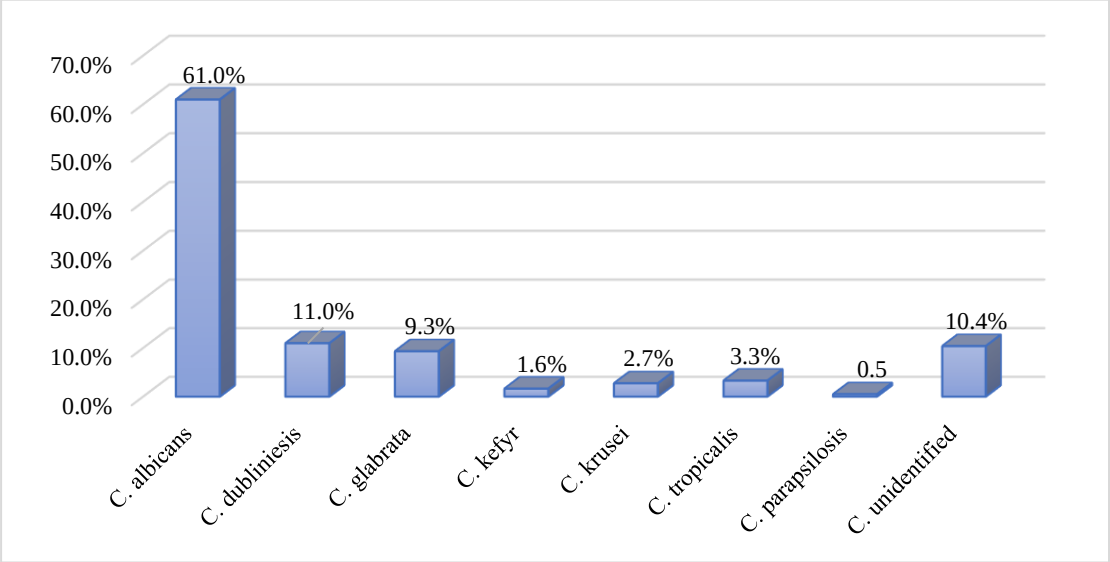


Figure 2.6 Distribution of *Candida* species using Oxoid chromogenic agar

Oxoid chromogenic agar identified 111 (61%) *C. albicans*, 20 (11.0%) *C. dubliniensis*, 17 (9.3%) as *C. glabrata*, 3 (1.6%) as *C. kefyr*, 5(2.7%) as *C. krusei*, *19 (*10.4%) as *C.* others, 1 (0.5%) as *C. parapsilosis*, and 6 (3.3%) as C. *tropicalis* (Figure 2.6)

In the case of *C. albicans* and *C. dubliniensis*, although a slight colour difference could be seen in both chromogenic agars, neither medium was able to give clear results. In order to ensure accurate identification, other techniques have been used for confirmation such as API ID 32 C, Vitek 2.

2.2.3. Biochemical *Candida* species identification by API ID 32 C

The presumptive species identification on chromogenic media was confirmed by biochemical testing using API ID 32 C (Figure 2.8). API ID 32 C results were recorded as positive when the wells in the API strip showed turbidity, and negative if they appeared clear.

Nine reference *Candida* type-strains served as controls namely, (*C. albicans* (ATCC 90028 and NCPF 3281), *C. tropicalis* (ATCC 950), *C. dubliniensis* (NCPF 3949a), *C. glabrata* (ATCC 26512), *C. krusei* (ATCC 2159), *C. parapsilosis* (ATCC 22019), *C. kefyr* (ATCC 4135) and *C. lusitanae* (ATCC 3449)) using API ID 32C.

The data were processed by the associated software and the clinical samples were compared to the reference type strains (Figure 2.7). Each sample was tested in duplicate to ensure specific identification of the isolates. Where there were discrepancies, the samples were repeated.

Figure 2.7: API 32 ID C and YST cards using Vitek 2 compact system aided in the accurate differentiation of *C. albicans* and *C. dubliniesis* as well as others such as *C. kefyr/krusie/parapsilopsis/glabrata*.

Thirty seven point three percent (37.3%) of the patient's isolates were identified as *C. albicans* (68 isolates), 11.1% as *C. glabrata* (20 isolates), 23% as *C. dubliniensis* (42 isolates), 2.70% as *C. krusei* (5 isolates), 2.74% as *C. tropicalis* (5 isolates), 2.74% as *C. kefyr* (5 isolates), 1.10% as *C. parapsilopsis* (2 isolates), 2.2% a *C. sake* (4 isolates),

14.3% as *C. humicola* (26 isolates), 1.1% as *C. magnoliae* (2 isolates), 0.54% as *C. guilliermondii* (1 isolate), 0.54% as *C. globosa* (1 isolate), and 0.54% as *C. membranifaciens* (1 isolate) (Figure 2.8).

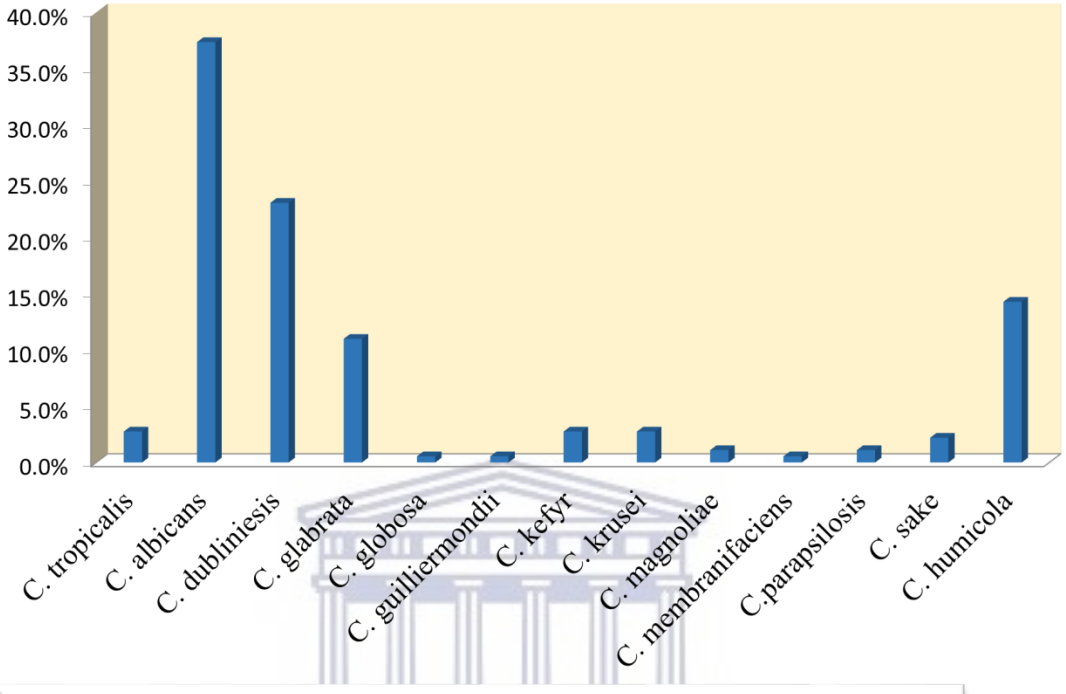


Figure 2.8: Distribution of *Candida* species as identified by API ID 32 C

Candida humicola was the most frequently isolated species that coexisted with other *Candida* species in the oral cavity (6 out of 26), followed by five of 42 *C. dubliniensis* isolates being found in the presence of a second species. *Candida albicans* and *C. kefyr* (3 of each) were also found to be growing with other *Candida* species, with *C. glabrata*, *C. parapsilosis*, *C. membranifaciens*, *C. krusei*, *C. globosa*, *C. tropicalis*, and *C. magnoliae* only having one representative colonizing the patient's mouth with another *Candida* species.

2.2.4. Comparison of chromogenic media with API ID 32 C.

Fluka and Oxoid chromogenic media were compared with API ID 32C which was chosen as the gold standard.

Using ID API 32 C as the gold standard, better sensitivity was shown with Oxoid than Fluka for all species with the exception of *C. lusitaneae* which was not detected by Oxoid but showed 62.5% sensitivity with Fluka (Table 2.2)

Fluka on the hand, demonstrated better specificity and PPV were higher for *C. glabrata. C. kefyr*, *C. krusei* and *C. tropicalis* than Oxoid (Table 2.2).

Table 2.2: Sensitivity, specificity, PPV, and NPV for Oxoid and Fluka chromogenic media using ID API 32 C as a gold standard

Candida spp. Name	Sensitivity Oxoid / Fluka		Specificity Oxoid / Fluka		Positive Likelihood Ratio Oxoid / Fluka		Negative Likelihood Ratio Oxoid / Fluka		PPV Oxoid / Fluka		NPV Oxoid / Fluka	
C. albicans	61.26%	60.18%	50.67%	61.03%	1.24	1.54	0.76	0.65	37.99%	37.57%	72.61%	79.73%
C. dubliniesis	65.62%	60.18%	87.50%	89.01%	5.25	5.97	0.39	0.39	67.74%	67.74%	86.42%	88.04%
C. glabrata	86.96%	65.62%	90.50%	97.59%	9.16	23.06	0.14	0.46	54.05%	83.33%	98.18%	91.01%
C. kefyr	71.43%	55.56%	98.33%	99.44%	42.86	98.89	0.29	0.45	62.50%	83.33%	98.88%	97.79%
C. krusei	100.00%	55.56%	97.25%	98.88%	36.4	55.94	0	0.38	50.00%	71.43%	100.00%	98.33%
C. parapsilosis	66.67%	0.00%	99.45%	98.91%	120.67	92	0.34	0	66.67%	50.00%	99.45%	100.00%
C. tropicalis	100.00%	100.00%	96.72%	98.88%	30.5	55.94	0	0.38	45.45%	71.43%	100.00%	98.33%
C. lusitaneae	0.00%	62.50%	0.00%	98.90%	0.00%	0	0.00%	1.01	0.00%	0	0.00%	98.90%

"PPV = Positive Predictive Value, NPV = Negative Predictive Value"

2.2.5. Application of Vitek compact system for species confirmation

Isolates were cultured on blood agar as part of the protocol for application of the Vitek 2 compact system characterisation. Differences in colonial morphology were observed for different speciecs.

For instance, *C. krusei* grew as smutched opaque colonies, while *C. sake* grew as shiny well-formed colonies, and *C. himucola* appeared as opaque well-formed colonies (Figure 2.9).

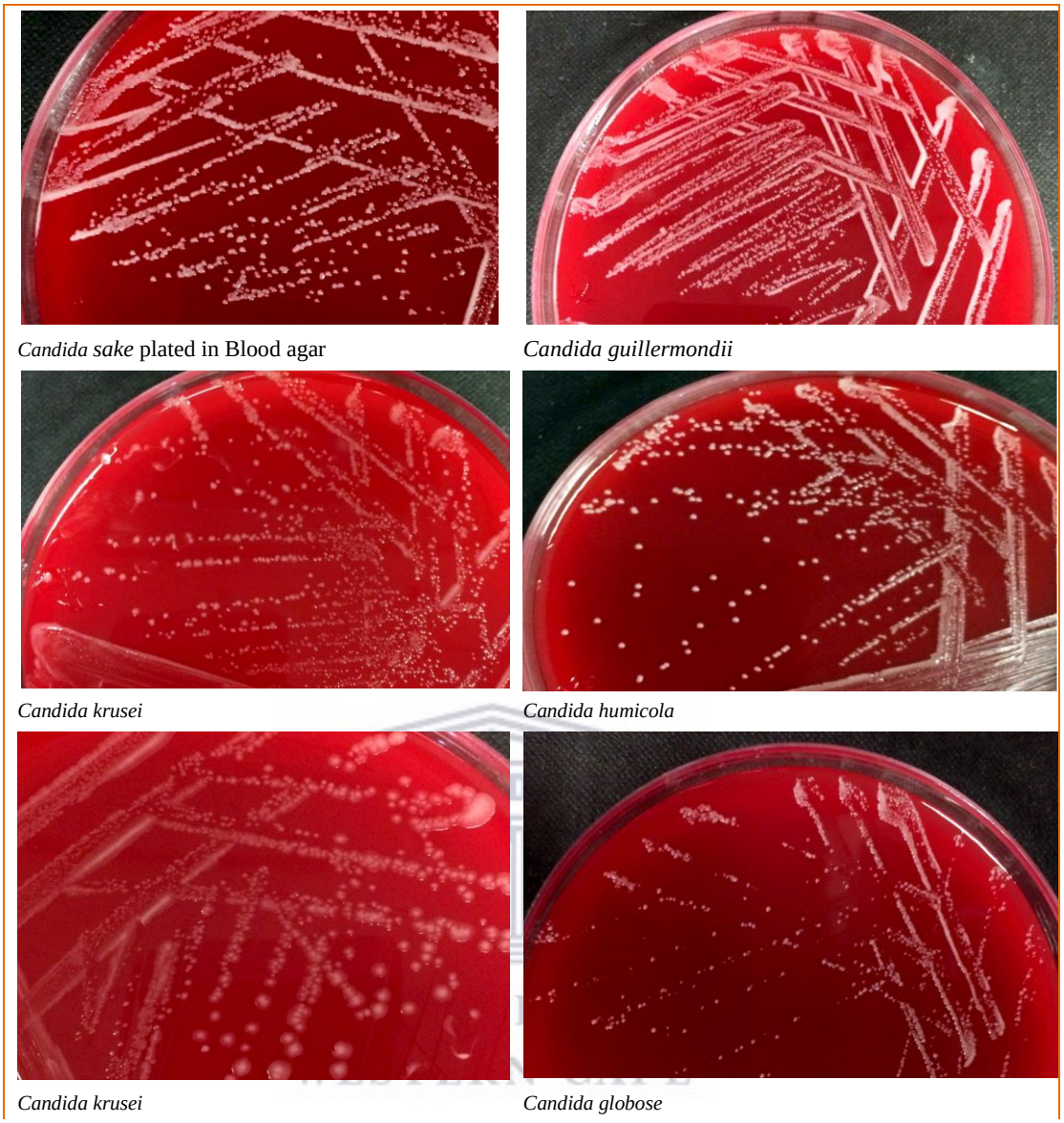
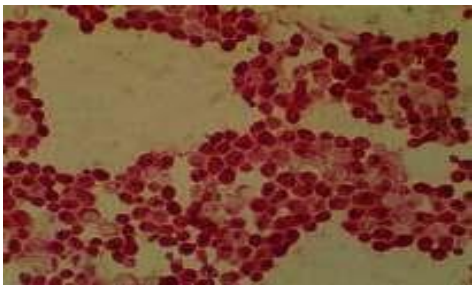


Figure 2.9: Colonial morphology on blood agar also differed for species.

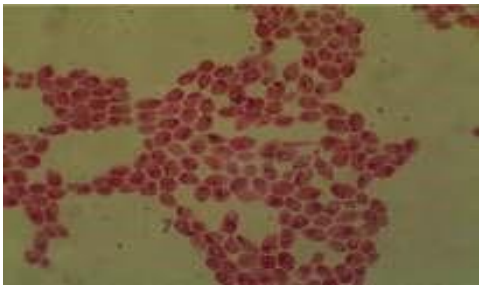
2.2.6. Microscopic cell Morphology

Candida krusei was the only *Candida* species that could be presumptively identified by microscopy, due to its noticeably larger cells.

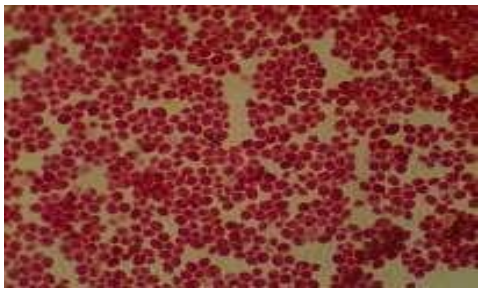
C. albicans and *C. dubliniensis* appeared as spherical to sub-spherical budding blastoconidia. Figure 2.10 shows the different *Candida* type strains stained with carbol fuschin and examined by oil immersion microscopy. With bacteria- like cells, Gram stain was preformed to confirm either bacteria or fungi. Figure 2.11 shows some of the different *Candida* cell morphologies isolated from clinical samples in this study.



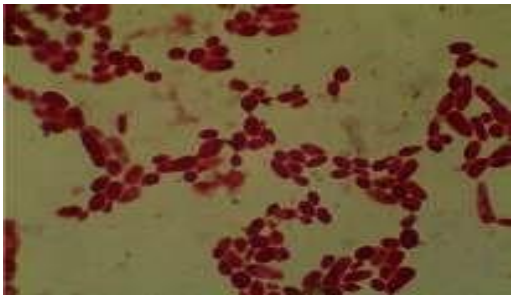
C. albicans ATCC (90028)



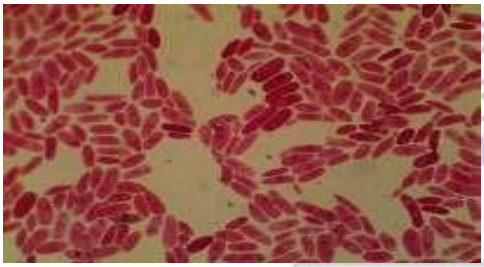
C. dubliniensis (NCPF 3949)



C. glabrata ATCC 26512

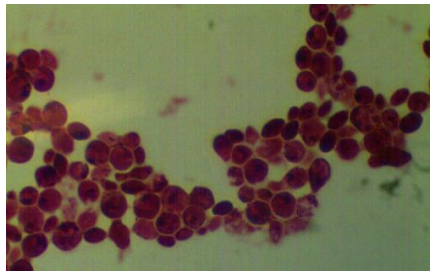


C. tropicalis (ATCC 950)

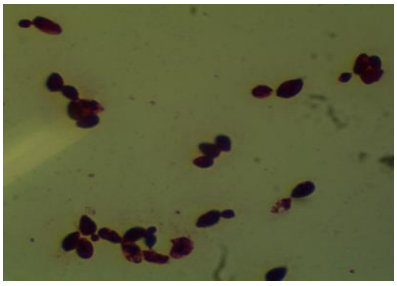


C. krusei ATCC (2159)

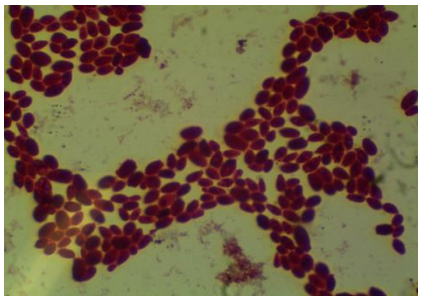
Figure 2.10: *Candida* type strain cell morphologies (1000X) using an Optikam B3 camera attached to an optical microscope.



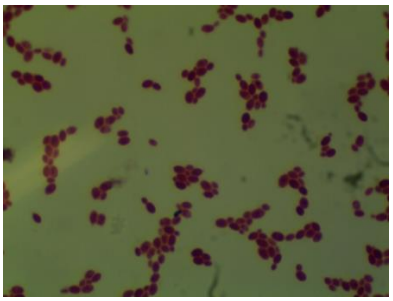
C. sake



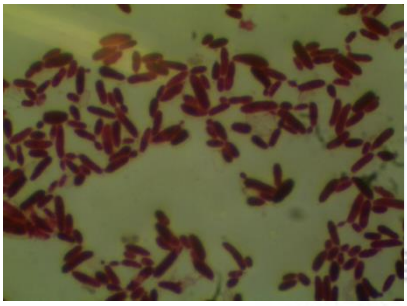
C. globose



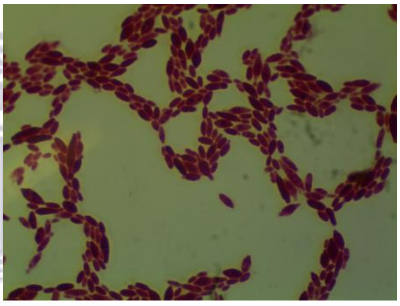
C. kefyr



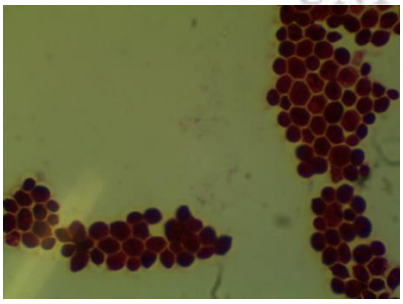
C. glabrata



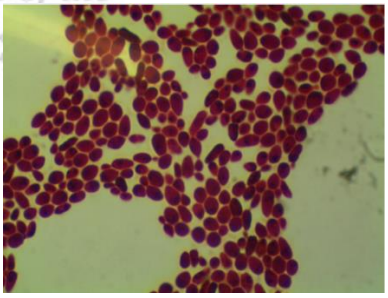
C. krusei



C. humicola



C. albicans

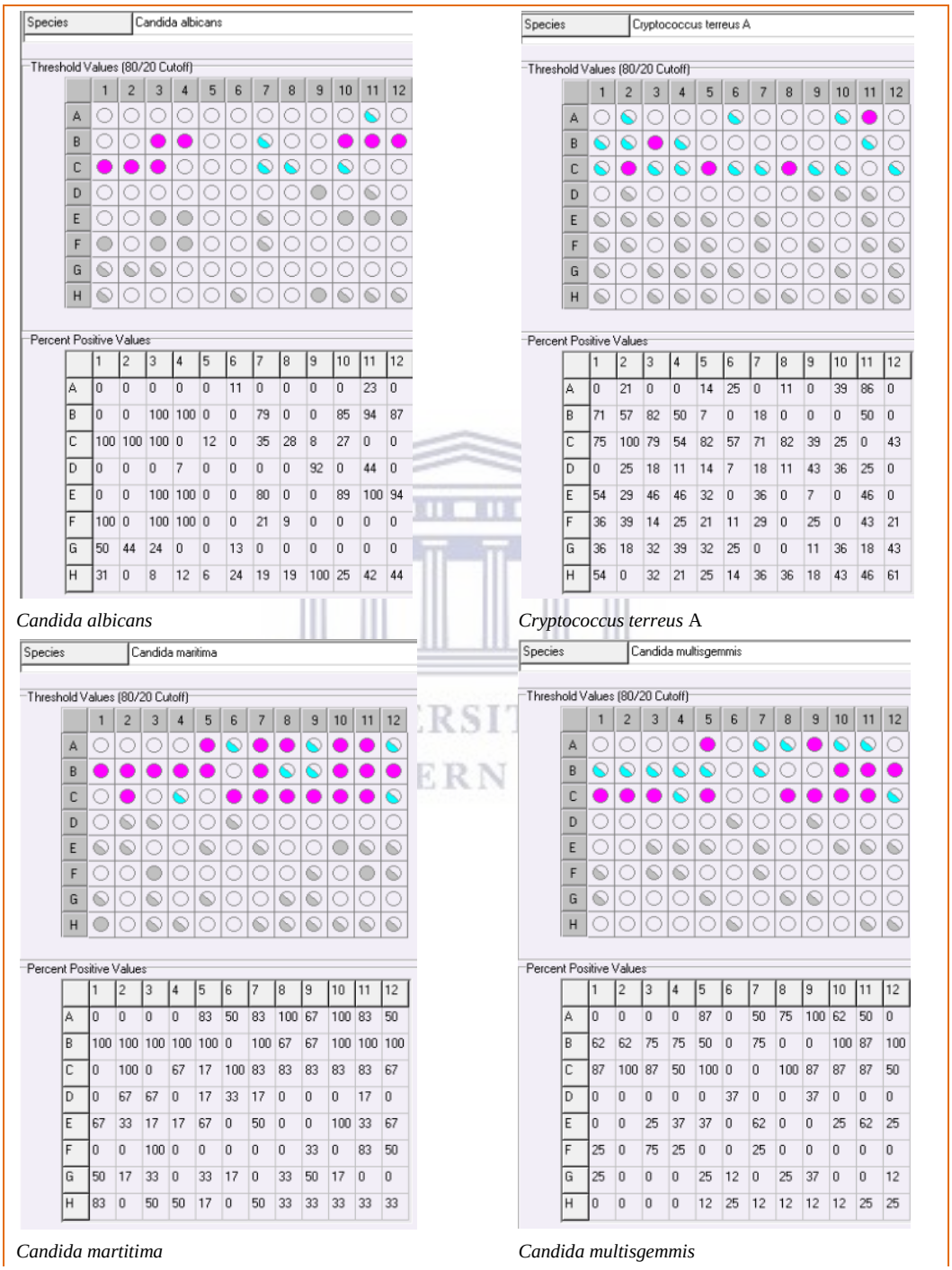


C. dubliniensis

Figure 2.11: *Candida* clinical strain cell morphologies (1000X).

2.2.7. Species identification using Phenotype Microarrays

Inconclusive results obtained with eight isolates tested with ID API 32 C and Vitek 2 compact system were confirmed with Phenotype Microarray (Figure 2.12)



Candida albicans *Cryptococcus terreus* A

Candida martitima *Candida multisgemmis*

Figure 2.12: Growth of *Candida* species on 96 wells plate of Phenotype Microarray, the purple colour shows positive results and gray show negative results

2.3. *Candida* carriage compared with patient demography

2.3.1. Gender

Females were represented by 205 (62.1%) of the sampled group and 125 (37.9%) were males (Table 2.3). *Candida* was detected more frequently in female patients (57.1%) than in the male patients (42.4%) in all sites sampled p = 0.012 using Chi squared test and constituting 68.8% and 31.2% of the total isolates respectively.

Table 2.3: Patients Demography

Table 2: Patients Demography		N & Percentage	*Candida* positive	% of total isolates	% within category	significant
Gender	Female	205 (62.1%)	117	68.8%	57.1%	P= 0.012
	Male	125 (37.9%)	53	31.2%	42.4%	
Denture wearing	Yes	99 (30.0%)	60	35.3%	60.6%	P = 0.020
	No	231(70.0%)	110	64.7%	47.6%	
Smoking	Yes	44 (13.3%)	17	10.0%	38.6%	P= 0.047
	No	286 (86.7%)	153	90.0%	53.49%	
Age group	Youth (15-34)	4 (1.2%)	3	1.8%	75%	P = 0.289
	Adults (35-64)	204 (61.8%)	99	58.2%	48.5%	
	Elderly (≥65)	122 (37.0%)	68	40.0%	55.7%	
BMI	Normal-range (18.50-24.99)	26 (7.9%)	14	8.2%	53.8%	P = 0.296
	Overweight (25.00-29.99)	104 (31.5%)	47	27.6%	45.2%	
	Obese (≥30)	200 (60.6%)	102	64.1%	54.5%	
Obesity as determined by WHO criteria	Normal range (18.50-24.99)	26 (7.9%)	14	8.2%	53.8%	P = 0.506
	Pre-obese (25.00-29.99)	104 (31.5%)	47	27.6%	45.2%	
	Obese class I (30.00-34.99)	103 (31.2%)	54	31.3%	45.6%	
	Obese class II (35.00-39.99)	65 (19.7%)	37	21.8%	56.9%	
	Obese class III (≥40.00)	32 (9.7%)	18	10.6%	56.2%	
Total		**330 (100.0%)**				

2.3.2. Age

Four (1.2%) fell within the category described as youth, 204 (61.8%) as adult and 122 (37%) as elderly (Table 2.3). Of these, 75% of the youth, 48.5% of the adults and 55.7% of the elderly tested positive for *Candida* (Table 2.3). This constituted 1.8%, 58.2% and 40% respectively of total *Candida* isolates. The species differences between the three groups were not significantly different (Table 2.3), although, *C. albicans* was most prevalent in the 35 to 64-year age group (Appendix 4), as well as *C. dubliniensis* and *C. glabrata*

23.3. Denture Wearers

Candida was detected in 60/90 denture wearers (60.6%) and in 110/231 non-denture wearers (47.61%) constituting 35% and 64.7% of total isolates respectively. This difference was found to be significant ($p = 0.020$) using Chi-Squared Tests (Table 2.3). The reported duration for wearing dentures ranged between 1-38 years with a mean of 7.53 ($\pm$69). No association was found between the duration of wearing dentures and *Candida* carriage ($p = 0.103$) using Fisher's Exact Test. The prevalence of *C. albicans* in denture wearers was (25/68; 36.76%). The predominant non-*albicans* species found, was *C. glabrata* (10/20; 50%), followed by *C. dubliensis* (9/42; 21.42%), *C. humicola* (6/26; 23%), *C. tropicalis* (3/5; 60%), *C. kefyr* (1/5; 20%), *C. parapsilosis* (1/2; 50%), *C. magnoliae* (1/2; 50%), *C. membranifaciens* (1/1; 100%), and other genus of yeast which were identified, and they were associated with denture wearers *Saprochaete capitata* (4/4; 100%), *Kloeckera japonica* (1/1; 100%), *Kloeckera apis/apiculata* / (1/1; 100%).

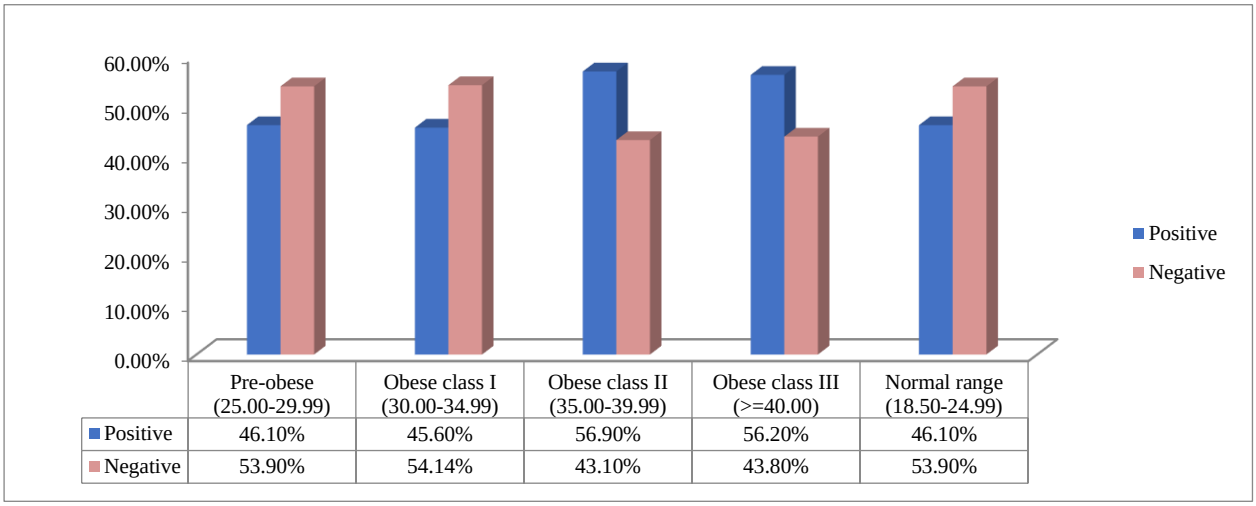
2.3.4. Smoking

Only 44 of 125 (35.2%) male patients were positive smokers and 38.6% of smokers were positive for *Candida* carriage. None of the female subjects were smokers (Table1). The frequency of *Candida* isolation was significantly higher in smokers than in the non-smokers P=0.047 using Fisher Exact Tests (Table 2.3).

2.3.5. Body Mass Index (BMI)

Classification of BMI into normal (18.50-24.99), overweight (25.00-29.99) and obese (≥ 30.00) showed that of the 330 patients in this study 26 (7.9%) fell within the normal range, 104 (31.5%) were overweight (pre-obese) and 200 (60.6%) were obese. This constituted 8.2%, 27.6% and 64.1% of the total isolates respectively. Further breakdown of obesity 103 (31.2%) could be classified as class I (30.00-34.99), 65 (19.7%) as class II (35.00-39.99) and 32 (9.7%) as class III (≥ 40.00).

Although *Candida* isolation increased with increased BMI from normal to obese, this increase was not statistically significant (p = 0.296), nor were the comparisons of the different classes of obesity found to differ significantly regarding *Candida* carriage (Table 2.3).



	Pre-obese (25.00-29.99)	Obese class I (30.00-34.99)	Obese class II (35.00-39.99)	Obese class III (>=40.00)	Normal range (18.50-24.99)
Positive	46.10%	45.60%	56.90%	56.20%	46.10%
Negative	53.90%	54.14%	43.10%	43.80%	53.90%

Figure 2.13: Distribution of Candida carriage according BMI, classified according to WHO classification.

DICUSSION

This was a cross-sectional study carried out from March 2015 to November 2015 among 330 diabetic mellitus type 2 patients (T2DM) at Misrata Diabetes Centre. The aim of this study was to isolate and characterize *Candida* species from Libyan subjects diagnosed with T2DM. This is very important, as no similar study of the prevalence of Candida in T2DM has previously been done in Libya.

The characterization of *Candida* species in this study employed the use of chromogenic/selective media for presumptive species identification and differentiation. Other factors, such as patient gender, and age were also considered.

Nine type strains served as controls namely, (*C. albicans* (ATCC 90028 and NCPF 3281), *C. tropicalis* (ATCC 950), *C. dubliniensis* (NCPF 3949a), *C. glabrata* (ATCC 26512), *C. krusei* (ATCC 2159), *C. parapsilosis* (ATCC 22019), *C. kefyr* (ATCC 4135) and *C. lusitanae* (ATCC 3449), for all identification tests. In our finding, *C. albicans* was the predominant species, followed by *C. dubliniensis* and *C. glabrata*.

Chromogenic media were compared to API 32 C and Vitek identification of the individual species, as in some cases two or three different species presented with the same colour on certain media. For instance, Oxoid agar identification of *C. krusei* matched exact number of isolates identified with API 32C, while only one of five isolates identified as *C. krusei* on Fluka agar was positive with API. In addition, Oxoid agar identified 17 of 20 isolates as *C. glabrata*, whereas Fluka agar only identified four isolates. Also, 3 of 5 isolates were identified by Oxoid agar as *C. kefyr* while API 32 C identified all five isolates as *C. kefyr*. In contrast, only one of the five was identified as *C. kefyr* on Fluka agar compared to API 32 C. Moreover, isolates initially categorised

as *C. unidentified* were less frequently found on Oxoid agar than on Fluka agar. When compared with API ID 32 C, Oxoid chromogenic agar more accurately identified *Candida albicans* compared with Fluka chromogenic agar, but both Oxoid and Fluka chromogenic agar equally identified *C. dubliniesis.* Overall, Oxoid chromogenic agar better identified species than Fluka agar when using API ID 32 C as a gold standard. Similar results were obtained with YST identification cards of the Vitek 2 compact system. Additionally, in the case of *C. albicans* and *C. dubliniensis* differentiation, although a slight colour difference could be seen in both chromogenic agars, neither medium was always able to give clear precise results. Thus, inconclusive results on chromogenic agar (which demonstrated a darker tonality) were confirmed using API 32 C and YST identification cards of Vitek 2 compact system. The use of these techniques aided in the precise differentiation of these *Candida* isolates.

One hundred and seventy samples were *Candida* positive out of the 330 swabs collected from Libyan patients. Because 12 of the 170 patients harboured more one species, the total number of *Candida* isolates was 182. With diabetes rapidly becoming a major public health problem worldwide (Kim, 2011), with the prevalence of oral *Candida* infections among T2DM patients in this study is consistent with previous studies, which have shown that diabetes mellitus is a major predisposing factor for oral candidiasis (De Leon *et al.*, 2002, Soysa *et al.*, 2006, Lamster *et al.*, 2008, Al-Maskari *et al.*, 2011), and suggest that diabetes mellitus enhances *Candida* colonization and propagation (Manfredi *et al.*, 2002, Manfredi *et al.*, 2004, Soysa *et al.*, 2006, Dorocka-Bobkowska *et al.*, 2010, Sardi *et al.*, 2010). Yar Ahmadi *et al.* (2002) have proposed that 40.2% of diabetics carry *C. albicans* in the oral cavity , but along with , Gonçalves *et al.* (2006) (Willis *et al.*, 1999, Manfredi *et al.*, 2002, Yar Ahmadi *et al.*, 2002, Gonçalves *et al.*,

2006), showed no statistical difference between the prevalence of different *Candida* species and DM.

Our findings show that the prevalence of *Candida* carriage was 51.5%, which is similar to that found in other studies (Guggenheimer *et al.*, 2000, Ship, 2003, Belazi *et al.*, 2005). In this study, *Candida albicans* was more frequently isolated than any other *Candida* species (68 isolates of 182 *Candida* spp. 37.4%) followed by *C. dubliniensis* (42 isolates 23%) and *C. glabrata* (20 isolates, 10.9%).

Other species isolated from the Libyan samples were *C. himucola* (26 isolates), twenty of *C. glabrata* (20 isolates), five each of species categorized as *C. kefyr*, *C. krusei*, *C. tropicalis*, *Candida sake* (4 isolates), two *C. parapsilopsis* (2 isolates), and one each of *C. guilliermondii*, *C. magnoliae*.

Studies from other middle-eastern countries reported similar findings. With a similar sample size to ours, a study from Kuwait, reported *Candida* distribution in the order of *C. albicans* (63.7%), *C. dubliniensis* 14.3%, *C. krusei* (8.1%), *C. tropicalis* (7.5%) and *C. glabrata* (6.2%) from T2DM patients (Ellepola *et al.*, 2011). *C. albicans*, followed by *C. dubliniensis* , *C. tropicalis* and *C. glabrata* have also been reported in a study from Saudi Arabia with *C. albicans* reported as a highest frequently with an absence of *C. krusei* (Al Mubarak *et al.*, 2013). A similar recent study conducted in hospitals in Tunisia, a neighbouring country to Libya, where the majority of Libyan patients seek medical treatment, revealed that *C. albicans* was the dominant species (43.37%), followed by *C. glabrata* (16.55%), with fewer *C. dubliniensis* (4.96%) isolates (Eddouzi *et al.*, 2013), than reported in this study. A study conducted in Iran reported *C. albicans* as the predominant species, followed by *C. dubliniensis*, *C. krusei*, *C. kefyr*, *C. parapsilopsis*, *C. guilliermondii*, and *C. glabrata* (Zomorodian *et al.*, 2016).

A Jordanian study confirmed that various yeast species are more prevalent in the oral cavity of diabetics (58.3%) than non-diabetics (30%) and identified *Candida* species, such as *C. albicans, C. tropicalis, C. parapsilosis,C. glabrata, C. kefyr and C. krusei* (Abu-Elteen *et al.*, 2006).

The distribution of *Candida* species appears to differ according to geographic region and sometimes within the same region (Mareş and Rusu, 2008, Khalaf *et al.*, 2009). Studies from Brazil reported different species prevalence e.g. a study was done by Gonçalves et al. (2006), isolated a total of 93 yeast strains from diabetic patients including 89 *Candida* species of which *C. albicans* represented 56%, and non-*albicans Candida* and other genus of yeast were 39.8% and 4.3%, respectively. *C. albicans* was the predominant *Candida* species, followed by *C. parapsilosis, C. tropicalis, C. glabrata, C. krusei, C. rugosa* and *C. guilliermondii* (Gonçalves *et al.*, 2006).

Another study from Brazil reported a higher prevalence of *C. dubliniensis* followed by *C. albicans*, *C. glabrata* and *C. tropicalis* (Sardi *et al.*, 2011), while yet another from the same region found that *C. albicans* was the most frequently isolated, followed by *C. stellatoidea, C. parapsilosis, C. tropicalis, C. lipolytica, C. glabrata,* and *C. krusei* , with an absence of *C. dubliniensis* (Bremenkamp *et al.*, 2011). An absence of *C. dubliniensis* was also reported by Sanitá *et al.* (2011) with *C. albicans, C. tropicalis and C. glabrata* being the predominant species isolated.

A more recent study in India identified *C. albicans*, *C. glabrata*, *C. dubliniensis, C. krusei, C. parapsilosis* except for *C. tropicalis* which presented a significantly higher ($p<0.001$) incidence in the type 2 diabetic group compared to the healthy group (Sharma *et al.*, 2017). Several studies, report an increase in *Candida* carriage and colonization in the mouth in diabetic patients compared with non-diabetic subjects (Willis *et al.*,

2000). However anti-diabetic therapy and glycemic control in diabetes were found to bear no relation with *Candida* carriage in the oral cavity (Kumar *et al.*, 2005).

In a study done in 2010 United State of America, with poorly controlled T2DM, 53% were positive for C. *albicans*, 20% for *C. glabrata*, 6% for *C. tropicalis*, and 6% for *C. parapsilosis* (Melton *et al.*, 2010). While a study was done from Poland on 77 diabetic patients reported the most frequently identified yeasts as *C. albicans* (55.2%), followed by *C. glabrata* (12.4%), *C. parapsilosis* (10.5%) and *C. tropicalis* (9.5%) respectively (Maciejewska *et al.*, 2004).

Currently, the non-*albicans Candida* species have become documented as a significant cause of infection and oral colonization with association of varies species in a number of immunosuppressed patients (Rossoni *et al.*, 2017). Species such as *C. krusei, C. parapsilopsis* and *C. tropicalis*, have been described in invasive *Candida* infection (Arredondo-García *et al.*, 2009, Chen *et al.*, 2011) and were observed also in the Libyan subjects.

New technologies continue to identify *Candida* albicans, as the predominant species in most research subjects, although in lower numbers than before with an increase in non-*albicans Candida* reported as a result of these developed technologies (Rossoni *et al.*, 2017).

C. dubliniensis (with a prevalence of 18%) was first isolated from the oral mucosa of patients with DM as the second most prevalent species after *C. albicans* (Willis *et al.*, 1999). Using either phenotypic or molecular methods, other reports of *C. dubliniensis* as the second most prevalent species after *C. albicans* in patients with DM have emerged (Kumar *et al.*, 2005, Gonçalves *et al.*, 2006) as in our study. However, *C. dubliniensis* is often hard to differentiate from *C. albicans* by conventional methods, and many earlier studies might have identified this isolate as *C. albicans* (Martinez *et*

al., 2013), thus it is speculated that the prevalence of *C. dubliniensis* has been underestimated due to misidentification Although, most frequently reported from patients suffering from AIDS (Topley and Carlton, 2005), there is a paucity of information concerning the prevalence of *C. dubliniensis* in healthy subjects or persons with other sicknesses (Manfredi *et al.*, 2002, Manfredi *et al.*, 2006, Al Mubarak *et al.*, 2013).

In the current study, the mean age of subjects with T2DM was 60.65 years, which is comparable with other T2DM studies (Gonçalves *et al.*, 2006, Neppelenbroek *et al.*, 2008, Dorocka-Bobkowska *et al.*, 2010) but differed with the opinion that only elderly patients had an increased risk of fungal infection, since a higher percentage of *Candida* species was isolated from the 35-64 year age group with 58.2% *Candida* carriage among this group. Similar results within the same age group were reported in the recent studies of candidiasis in T2DM patients in Saudi Arabia (Al Mubarak *et al.*, 2013) and in Iran (Ayatollahi Mousavi *et al.*, 2013).

Our data analysis showed no significant association between *Candida* carriage and Body Mass Index (BMI), as reported in other studies (Vitkov *et al.*, 2003, Maciejewska *et al.*, 2004). In addition, it has been suggested that Diabetes mellitus with lower BMI are risk factors for *Candida* colonization in critically ill patients (Mojazi Amiri *et al.*, 2012), with a positive relationship reported between glycemic control and *Candida* colonization (Lydia Rajakumari and Saravana Kumari, 2016).

Candida is able to secrete its products and reach the pancreas via the bloodstream, thus indirectly affecting insulin secretion (Berman and Sudbery, 2002). It is important to maintain a balance between bacteria and *Candida* since bacterial infections can reduce insulin secretion, leading to hyperglycemia (Nikolić, 2011), while *C. albicans* infections may increase insulin secretion, causing hypoglycemia and insulin resistance.

This may lead to glucose intolerance and insulin resistance, sometimes resulting in the development of type 2 diabetes (Nikolic, 2014).

The association between denture wearers and *Candida* colonization is consistent. Dentures are inert and non-shading surfaces and are therefore easily colonized by *Candida* species, especially in denture wearers with a dry mouth (Murakami *et al.*, 2015).

Colonization of the oral surface by *C. albicans* can result in damage to the oral tissues because of its ability to express virulence factors such as an ability to adhere to host cells or surfaces such as oral prosthesis, secretion of hydrolytic enzymes (Schaller *et al.*, 2005, Junqueira *et al.*, 2011, Goswami *et al.*, 2017), adaptation to changes in environmental conditions (e.g. smoking) biofilm formation (Silva, 2010), and an imbalance of normal oral bacterial micro-flora (Mojazi Amiri *et al.*, 2012), results in more complications in relation to oral health conditions therapy affecting. quality of life (Molania *et al.*, 2017). Subsequent *Candida* biofilm formation may lead to denture stomatitis (DS) and candidiasis (Prakash *et al.*, 2015), thereby affecting the quality of life of the patient (Molania *et al.*, 2017)

Denture stomatitis is typically asymptomatic, with a proposed prevalence of 75% in denture wearers and, although the etiology may be multifactorial, *C. albicans* has been strongly associated, along with other risk factors such as denture irritation and inadequate denture hygiene (Puryer, 2016).

As in the present study, *Candida albicans* was the most prevalent species isolated among diabetics and non-diabetics with Denture Stomatitis DS (Javed *et al.*, 2017), while a study comparing dentate diabetic patients with denture wearing diabetic patients (Manfredi *et al.*, 2002) revealed that denture wearing diabetic patients were colonized by more non-*albicans Candida* species than dentate diabetic patients. Manfredi *et al.*

(2002) reported a prevalence of 15.5% for *C. dubliniensis* and found a close association between its prevalence and denture users. Our findings reported a prevalence of 23% for *C. dubliniensis* with 21.4 % of patients wearing dentures and carrying *C. dubliniensis.* A recent study showed no significant association between DS and gender, age, nor the type of dentures (Čanković *et al.*, 2017).

In a study by Lydia Rajakumari and Saravana Kumari (2016) conducted in India, on 95 patients, 57 (60.0%) were found to wear complete or partial dentures and 38 (40.0%) had their own dentition. Their results showed that *Candida albicans* was more frequently found in patients with dentures than in those without dentures ($p = 0.0003$), as was *C. glabrata*, with *C. tropicalis* reported as the most predominant of the non-*albicans* species.

Candida species prevalence was significantly higher in denture wearers with *C. albicans, C. tropicalis, C. dubliniensis,* and *C. glabrata* most frequently found in a study by Prakash *et al* (2015). Increasing age among denture wearers was associated with an increase in *Candida* prevalence, with multispecies of *Candida* colonizing the dentures thus presenting a higher risk of candidiasis, especially with increasing age (Prakash *et al.*, 2015).

Our results showed that *Candida* carriage rate among denture wearers was significantly higher than *Candida* carriage rate among non-denture wearers with 35 isolates from 60 denture wearers found to be non-*albicans Candida* species, and 39 denture wearers showing negative *Candida* carriage (Table 2.2).

Mycological findings from the present study indicate that dentures have a significant effect on oral colonization by *Candida albicans* or other species of *Candida*, which agrees with a study done in 2006 by (Daniluk *et al.*, 2006), reporting a statistically

significantly association between *C. albicans* and denture wearers with diabetes mellitus (p = 0.0207).

Smoking was reported to be a predisposing factor for *Candida* carriage particularly in the oral cavity (Kadir *et al.*, 2002, Soysa *et al.*, 2006). This present study reported that 38.6% of smokers were positive for *Candida* indicating that smoking may be a risk factor for the presence of *Candida* in the oral cavity. There was a significant association between smoking and *Candida* carriage as revealed by Chi squared test (Table 2). A study done in Turkey concluded that smoking in DM patients was found to be one of the significant factors for yeast colonization (Sahin *et al.*, 2005), as was also indicated in studies done in Iran (Khosravi *et al.*, 2008), Spain (Sheth *et al.*, 2016), and in Australia (Mun *et al.*, 2016). Secretion of *Candida* histolytic enzymes and adherence to denture surfaces are reported to be significantly heightened by cigarette smoke condensate (Baboni *et al.*, 2009). In contrast, a study in Saudi Arabia showed no significant association between *Candida* carriage and smokers among T2DM individuals (Al-Attas and Amro, 2010).

In summary, no specific differential chromogenic culture media can always give always a precise *Candida* species identification even though it is a very helpful tool for differentiating species in mixed samples. Species confirmation using other methods of identification such as Vitek 2 and API ID 32C are recommended.

This study did not compare T2DM patients with non-T2DM patients. T2DM patients were selected on the basis of previous association studies of T2DM and *Candida* carriage. Since there is no data currently available on the prevalence of *Candida* in T2DM Libyan patients, we elected to investigate this group.

The higher colonization of no-*albicans Candida* species in the present study supported other reports of studies in the Middle East and confirmed that diabetics have a higher susceptibility to colonization with these species. Unfortunately, non-*albicans* species are less susceptible to common antifungal drugs than *C. albicans* (Al-Karaawi *et al.*, 2002). This highlights the importance of screening for oral colonization in diabetic patients in countries such as Libya where treatment for candidiasis not routinely administrated.

Candida colonization was not significantly associated with BMI, nor age and the significance obtained with gender was largely due to the increased number of females compared with males. *Candida* carriage was significantly associated with the wearing of dentures which provides for easy colonization of *Candida* and with smoking, which enhances *Candida* colonization.

Having established what the predominant species were, we proceeded to investigate their antimicrobial profiles as described next chapter.

CHAPTER 3

ANTIFUNGAL SUSCEPTIBILITY TESTING

3.1 Introduction

Several superficial and systemic infections are caused by *Candida* species in individuals with superficial infections being of clinical significance in both immunocompetent and immunocompromised patients. In addition to the common clinically relevant *Candida* species, a relatively diverse spectrum of fungi is now isolated in clinical mycology laboratories.

In addition, recent studies have demonstrated the increasing resistance of *Candida* infections to antifungals routinely used to treat oral candidiasis in HIV-positive patients (Dos Santos Abrantes *et al.*, 2014) and in patients with DM (Goswami *et al.*, 2006), in particular, fluconazole. This has been attributed to the emergence of resistant *C. albicans* strains as well as the more frequent isolation and detection of non-*albicans Candida* species with innate resistance.

Drug classes routinely used in the fight against *Candida* infections include the routinely used triazoles such as fluconazole, that affect ergosterol production in the fungal cell membrane; the echinocandins (e.g. micafungin and caspofungin), that inhibit β1-3 glucan synthesis in the fungal cell wall; 5-flucytosine, a fluorinated analogue pyrimidine that inhibits DNA and protein synthesis and amphotericin B, a polyene antifungal used for systemic infections that binds to ergosterol in the fungal cell membrane.

There is a great demand for novel agents with reduced toxicity and widened spectra of activity, and the development of novel antifungal therapy is now enjoying a period of rapid growth.

In this chapter, antifungal susceptibility of *Candida* species will be examined using the disc diffusion method and the Vitek 2 compact system.

Material and Methods

3.2.1 Media preparation

3.2.1.1 Yeast Nitrogen Base agar with Glucose

A 5X solution of Yeast Nitrogen Base in powder (REF NO. 239210, Difco, USA) form (3.35 g/500 mL) and dextrose (2.5 g / 500 mL) was filtered using a 0.45 µm disposable filter (Ref. no. 25NS, MSI filters, USA) attached to a 50mL sterile plastic syringe into a sterile bottle containing Difco granulated bacteriological agar (3.25 g / 500 mL) (Cat. no. 214530, Difco, USA), according to the manufacturer's instructions.

3.2.1.2. Preparation of Nutrient Blood Medium agar

Nutrient agar (Lot no. 382448, L20 1EA, MAST groub Ltd, UK) was added to distilled/ deionized water and volume brought to 1.0 L. thoroughly, mixed, and gently heated until boiling for proper dissolving. After autoclaving for 15 min at 15 psi pressure 121^0C, the media was cooled to 45-50 ^{0}C before adding, 50 ml of sterile horse blood was added to 950.0 ml of cooled, sterilised nutrient medium. (5% of blood agar). Prepared blood medium plates were used for AST Vitek antifungal tests.

3.3. Antifungal Susceptibility Testing

3.3.1. Disk Diffusion Susceptibility Testing

Antifungal drug susceptibility was determined by placing 25 µg fluconazole discs (Cat. no. X7148, Oxoid, UK) on yeast nitrogen base agar with glucose (YNBG) plates (Cat. no. 239210, Beckton, Dickinson and Company, UK) previously spread with a 0.5 McFarland standard suspension of the organisms using a sterile cotton swab and incubated at 37°C for 24 hours. Isolates were only tested against fluconazole because of the emerging increased resistance to fluconazole.

After inoculation, the inhibition areas around the fluconazole disks were measured from the edge of the disk to the edge of the susceptibility area and the presence of microcolonies within the susceptibility zone noted and scored as follows; 0 = a clear zone with no microcolonies, 1 = a few microcolonies present, 2 = moderate growth of microcolonies and 3 = many microcolonies in the susceptibility area. Tests were done in triplicate.

When differences in susceptibility / microcolony growth were seen, the susceptibility test was repeated. In random samples, microcolony and outer growth areas were stained and observed microscopically for *Candida* species confirmation. These were subsequently grown in chromogenic media and were shown to be the same species. Samples with susceptibility areas less than 7 mm in YNBG and with the presence of microcolonies were regarded as resistant, including samples with more than a microcolony score of 2.

Samples with susceptibility areas higher than 12 mm in YNBG and a microcolony score of 2 or less were regarded as susceptible to fluconazole. Strains with a susceptibility area ranging from 7 to 12 mm in YNBG were regarded as intermediate (susceptible dose dependent) strains. All susceptible dose dependent and resistant species to 25 µg / ml fluconazole disk were screened with the Vitek 2 antifungal testing (AST-YS07). The reason for only intermediate and resistant strains being tested with Vitek was that we wanted to confirm their antimicrobial profiles accurately, while keeping costs low. The sample numbers were too high to include all susceptible strains were as well, however, 15 susceptible strains were included as controls.

Figure 3.1 shows the sequence of tests used for the characterization of isolates

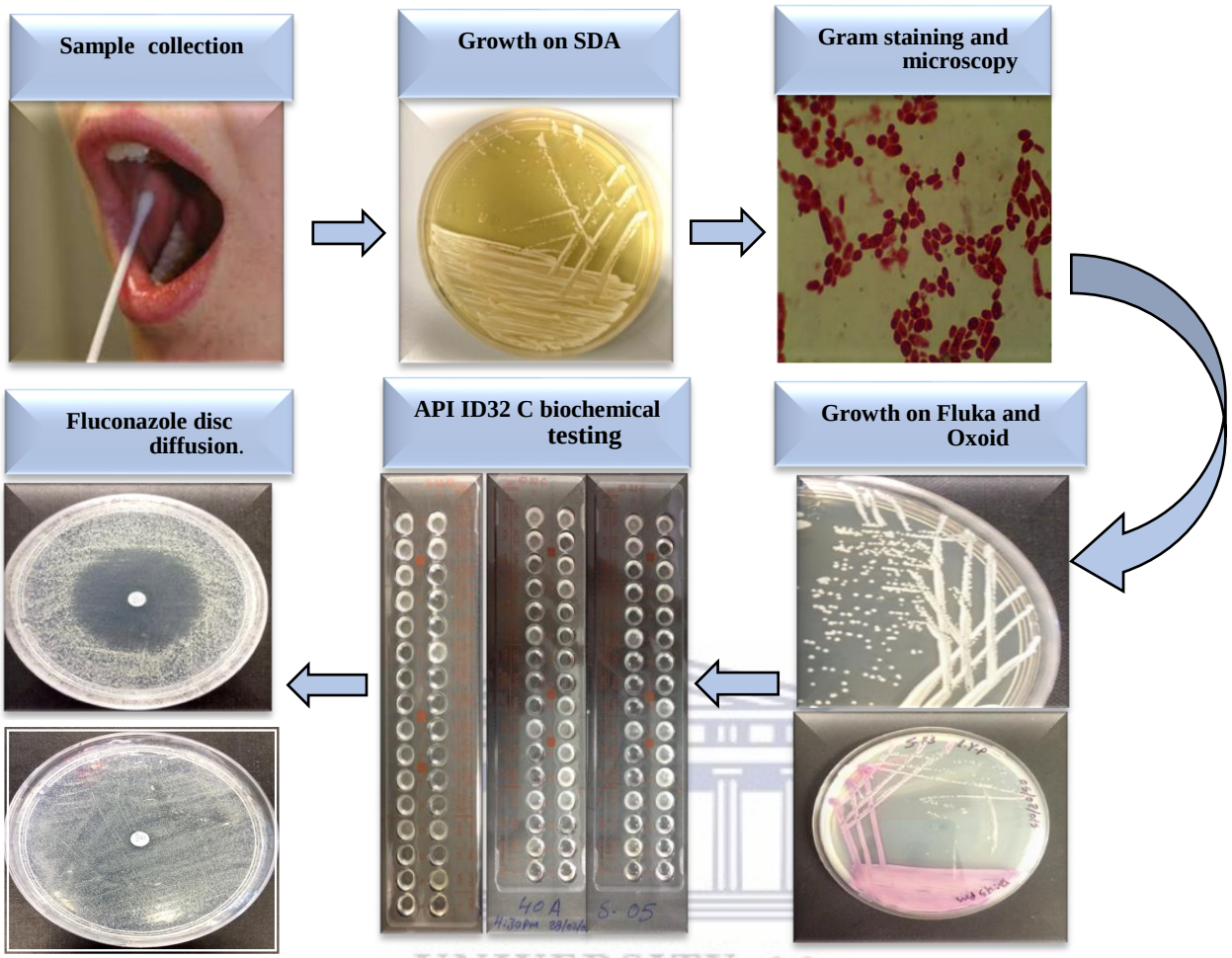


Figure 3.1: Sequence of sample isolation, identification and drug susceptibility techniques used in this study.

3.3.2. The Vitek 2 Antimicrobial Susceptibility Tests

The antimicrobial susceptibility tests (AST) card is a complete system for routine sensitivity testing. The Vitek compact (Cat. No. 69280 Marcy-l'Etoile - France) AST-Y07 system, the AST card is essentially a miniaturised and abbreviated version of the doubling dilution technique for the minimum inhibitory concentration (MICs) determined by the microdilution method and consists of a card set in with six different drugs [amphotericin B (AB), caspofungin (CAS), fluconazole (FLU), flucytosine (FCT), micafungin (MFC) and, voriconazole (VRC)] in ascending concentrations. Each AST card contains selected antifungals in changing concentrations, dried with a microbiological selected culture media. The drug concentration ranges on the AST-YS07

wells are 1, 4, 16, 32 µg/ml for AB; 1, 4, 8 µg/ml (CAS); 1, 4, 8, 16 µg/ml (FLU); 4, 8, 16, 64 µg/ml (FCT); 0.06, 0.25, 1, 4 µg/ml; and 0.5, 1, 4, 8 µg/ml for (VRC).

Each AST card contained a control well with only microbiological culture medium. The remaining micro-wells contained premeasured amounts of specific antifungal drugs combined with dried culture medium.

The reagent cards have 64 wells, each containing an individual test substrate. Substrates measure various metabolic activities such as acidification, alkalinisation, enzyme hydrolysis, and growth in the presence of inhibitory substances. An optically clear film present on both sides of the card allows for the appropriate level of oxygen transmission while maintaining a sealed vessel that prevents contact with the organism-substrate admixtures. Each card has a pre-inserted transfer tube used for inoculation (described below). Cards have bar codes that contain information on product type, lot number, expiration date, and a unique identifier that can be linked to the sample either before or after loading the card onto the system.

Nine *Candida* type strains *C. albicans* (ATCC 90028 and NCPF 3281), *C. tropicalis* (ATCC 950), *C. dubliniensis* (NCPF 3949a), *C. glabrata* (ATCC 26512), *C. krusei* (ATCC 2159), *C. parapsilosis* (ATCC 22019), *C. kefyr* (ATCC 4135) and *C. lusitanae* (ATCC 3449) were applied in the Vitek 2 compact system MIC as controls.

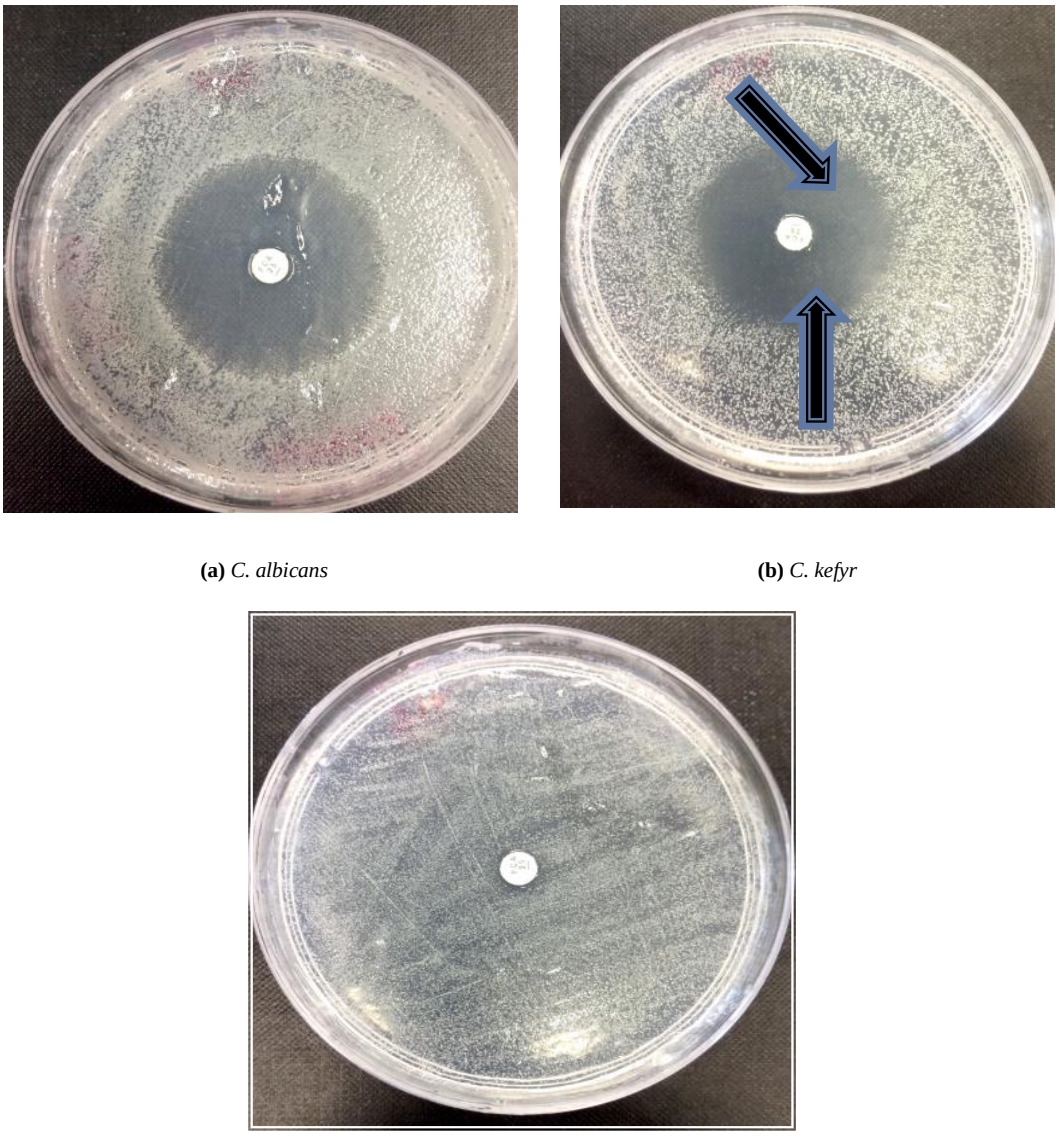
Running of the samples on the Vitek 2 compact system were done by inoculation of a fresh 24 h *Candida* subculture into sterile saline tubes using a sterile cotton swab to transfer morphologically similar colonies to the saline tube with a density equivalent to McFarland standard ranging in 1.80 – 2.20, using the supplied Vitek nephelometer. The species suspension to be tested must be diluted to a standardised concentration in 0.45% saline before being used to rehydrate the antifungal

medium within the card. The card is then filled, sealed, and placed into the machine incubator/reader automatically. The instrument monitors the growth of each well in the card over a defined period of time up to 36 hours for the yeast. At the completion of the incubation cycle, MIC values or test results were determined for each antifungal contained on the card. MICs were defined as the lowest concentrations that inhibited growth at 100%. 15 random susceptible isolates to fluconazole were tested as a negative control to confirm their susceptibility.

RESULTS

3.1. Fluconazole Disc Diffusion Susceptibility Testing

Figure 3.2, Demonstrates susceptibility or intermediate resistance or resistance to fluconazole. Susceptibility is demonstrated by a zone of inhibition around the fluconazole-impregnated disc (Fig. 3.2 a), intermediate resistance is indicated by the growth of fungal micro-colonies in the susceptibility area (b) and resistance is indicated where the fungal growth grew over the impregnated disc, (c) when cultures were incubated in YNBG at 37˚C for 24 hours Appendix 5.



(a) *C. albicans*

(b) *C. kefyr*

(c) *C. glabrata*

Figure.3.2: Inhibition of *Candida* growth in YNBG media in the presence of a fluconazole disc. Intermediate resistance indicated by the arrow showing presence of microcolonies within the zone of inhibition (b)

Figure 3.3 shows the fluconazole susceptibility of *Candida* isolates using disc diffusion on YNBG agar. Sixty-two-point six percent (62.6%) of the isolates were susceptible to fluconazole, while 20.3% of the isolates showed resistance and 17 % (31 isolates) showed intermediate resistance (Figure 3.3).

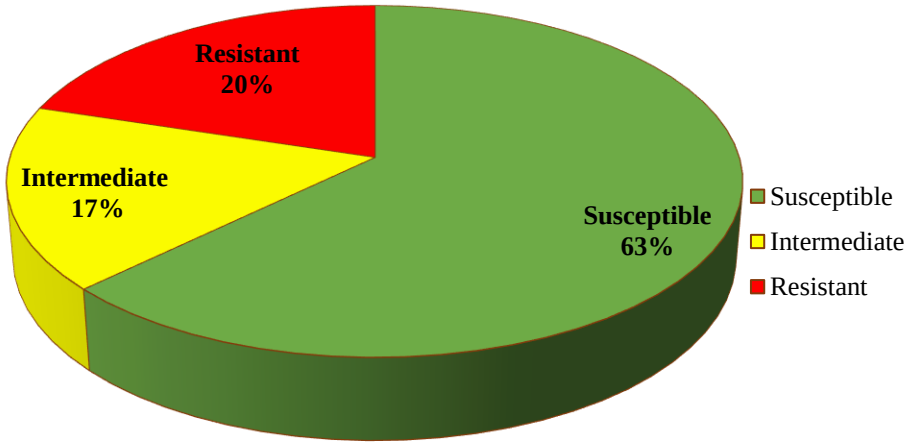


Figure 3.3: Fluconazole susceptibility of clinical isolates using disc diffusion.

Table 3.1. represents susceptibility results of *Candida* species grown on YNBG media: Chi squared analysis revealed a significant difference in susceptibility to fluconazole ($p < 0.001$, $X^2 = 106.305705$, $df = 6$).

Table 3.1: Susceptibility results of *Candida* spp. grown on YNBG agar.

***Candida* isolates**	**Susceptible**	**Intermediate**	**Resistant**	**Total**
C. albicans	52	12	4	68
C. dubliniesis	33	8	1	42
C. glabrata	0	0	20	20
C. humicola	20	3	3	26
Total	105	23	28	156

C. albicans, C. *dubliniesis*, *C. humicola*, and *C. magnoliae* were mostly susceptible to fluconazole, while *C. glabrata*, *C. guilliermondii*, *C. membranifaciens* and *C. krusei* were resistant or mostly resistant to fluconazole. Others were split in their susceptibility profiles (Table 3.2).

Table 3.2: Summary of species susceptibility of Fluconazole

	Susceptible	Intermediate	Resistant	Total
C. albicans	52	12	4	68
	76.8%	17.4%	5.8%	100.0%
C. dubliniensis	33	8	1	42
	78.6%	19.0%	2.4%	100.0%
C. glabrata	0	0	20	20
	0.0%	0.0%	100.0%	100.0%
C. globose	1	0	0	1
	100.0%	0.0%	0.0%	100.0%
C. guilliermondii	0	0	1	1
	0.0%	0.0%	100.0%	100.0%
C. humicola	20	3	3	26
	76.9%	11.5%	11.5%	100.0%
C. kefyr	2	3	0	5
	40.0%	60.0%	0.0%	100.0%
C. krusei	0_a	$0_{a, b}$	5_b	5
	0.0%	0.0%	100.0%	100.0%
C. magnoliae	2	0	0	2
	100.0%	0.0%	0.0%	100.0%
C. membranifaciens	0	1	0	1
	0.0%	100.0%	0.0%	100.0%
C. parapsilosis	0	1	1	2
	0.0%	50.0%	50.0%	100.0%
C. sake	2	0	2	4
	50.0%	0.0%	50.0%	100.0%
C. tropicalis	2	3	0	5
	40.0%	60.0%	0.0%	100.0%
TOTAL	114	31	37	182
	62.8%	16.9%	20.2%	100.0%

More than 1 *Candida* species were simultaneously detected in 12 patients (Table 3.3). *Candida humicola* was found to be the most frequently isolated species that coexisted with other *Candida* species in the oral cavity (6 out of 26), followed by five of 42 *C. dubliniensis* isolates being found in the presence of a second species. *Candida albicans* and *C. kefyr* (3 of each) were also found to be growing with other *Candida* species, with *C. glabrata, C. parapsilosis, C. membranifaciens, C. krusei, C. globosa, C. tropicalis,* and *C. magnoliae* only having one representative colonizing the patient's mouth with another *Candida* species.

Table 3.3: Simultaneous species combinations observed in 12 Libyan patients

Species name	Fluconazole susceptibility	Species name	Fluconazole susceptibility	Patient No.
C. humicola	S	*C. kefyr*	I	17
C. humicola	S	*C. krusei*	R	20
C. humicola	I	*C. dubliniensis*	I	29
C. humicola	R	*C. tropicalis*	I	40
C. humicola	S	*C. kefyr*	I	109
C. humicola	S	*C. albicans*	S	146
C. dubliniensis	I	*C. membranifaciens*	I	24
C. dubliniensis	S	*C. globosa*	S	59
C. dubliniensis	S	*C. parapsilosis*	I	74
C. dubliniensis	S	*C. albicans*	S	142
C. albicans	S	*C. glabrata*	R	52
C. magnoliae	S	*C. kefyr*	I	151

R = Resistant, I = Intermediate, S = susceptible

Each one of the two *C. dubliniensis* species which coexisted with *C. membranifaciens* and *C. humicola* (both reported as rare *Candida* species), showed intermediate resistance to fluconazole

on YNBG agar. Three of 5 *C. kefyr* isolated, presented as intermediate resistance in this study and co-colonized the patient's mouth with other susceptible *Candida* species (Table 3.3). One of the 3 *C. himucola* was resistant to fluconazole and coexisted with *C. tropicalis* which showed intermediate resistance to fluconazole.

3.2. Susceptibility Testing using Vitek 2 Compact System

Identification of *Candida* species by API 32 C were confirmed and tested for their resistance to antifungals using Vitek 2 compact system (Table 3). These included *C. albicans* (20 isolates), *C. glabrata* (20 isolates), *C. dubliniensis* (13 isolates), *C. krusei* (5 isolates), *C. tropicalis* (4 isolates), *C. sake* (4 isolates), *C. kefyr* (2 isolates), *C. guilliermondii* (1 isolate), *C. parapsilopsis* (1 isolate), *C. magnoliae* (1 isolate), and *C. membranifaciens* (1 isolate).

The AST cards of Vitek 2 compact system drug susceptibilities of *Candida* species are summarised in (Table 3.4). These results demonstrate the differences in susceptibility patterns of different *Candida* species when exposed to the different antifungal drugs using the Vitek system.

Some discrepancies were seen when comparing the Vitek 2 results to those obtained with fluconazole disk diffusion in YNBG agar: Twenty-seven isolates were classified as intermediate resistant on the Vitek, while only nine isolates were resistant to fluconazole. Twenty eight out of the thirty-seven YNBG resistant isolates in Vitek were intermediate, with nine isolates being classified as resistant to fluconazole.

Two of 68 *Candida albicans* isolates were resistant to fluconazole with one of these two resistant to voriconazole in Vitek and another showed intermediate fluconazole resistance. Three of 42 *C. dubliniesis* were resistant to fluconazole with one of them also resistant to 5-flucytosine and

voriconazole. All identified *C. glabrata* demonstrated intermediate resistant to fluconazole and micafungin with only two of 20 isolates were resistant to amphotericin B.

All the 182 *Candida* isolates were tested for fluconazole susceptibility with 68 *Candida* species (total *Candida* species showing resistance and intermediate resistance were tested to six antifungal agents. In the table 3.4, 63 *candida* species tested with their interpreted results with 5 more rare species (four *C. sake* and only one *C. membranifasciens*).

Thirty-two (32) of the 68 isolates were categorized as susceptible by the Vitek 2 compact system, with the remaining 36 being confirmed as either intermediate or resistant. Most of the tested *Candida* isolates demonstrated low resistance levels to the different antifungal drugs (table 1), including some of the less common species.

Four rarer species, for which no interpretative breakpoints have been established, showed high fluconazole MIC values, three *C. sake* isolates (one with MICs: 4 µg/ml and two: 8 µg/ml), the other two of the four had a lower MIC ≤ 1 µg/ml and the *C. membranifasciens* isolate had a MIC 4 µg/ml. Only one *C. guilliermondii* isolate had MIC: 2µg/ml.

Table 3.4: Vitek compact 2 system susceptibility results of *Candida* species obtained from the Libyan T2DM patients.

Antifungal Drugs	Interpretation	*C. albicans* n=68	*C. glabrata* n=20	*C. dubliniensis* n=13	*C. krusei* n=5	*C. tropicalis* n=4	*C. kefyr* n=2	*C. guilliermondii* n=1	*C. parapsilosis* n=1
Amphotericin B	Susceptible	68	17	13	4	4	2	1	1
	Intermediate	0	0	0	0	0	0	0	0
	Resistant	0	3	0	1	0	0	0	0
5-Flucytosine	Susceptible	68	20	12	0	4	2	1	1
	Intermediate	0	0	0	0	0	0	0	0
	Resistant	0	0	1	5	0	0	0	0
Caspofungin	Susceptible	68	0	13	5	4	1	1	1
	Intermediate	0	20	0	0	0	1	0	0
	Resistant	0	0	0	0	0	0	0	0
Micafungin	Susceptible	68	20	12	5	4	2	1	1
	Intermediate	0	0	1	0	0	0	0	0
	Resistant	0	0	0	0	0	0	0	0
Fluconazole	Susceptible	64	0	10	0	2	1	0	1
	Intermediate	2	20	1	0	2	1	1	0
	Resistant	2	0	2	5	0	0	0	0
Voriconazole	Susceptible	67	20	11	5	4	2	1	1
	Intermediate	0	0	0	0	0	0	0	0
	Resistant	1	0	2	1	0	0	0	0

DISCUSSION

3.1. Antifungal susceptibility testing

The treatment of fungal infections can in certain cases be more adequately performed using alternative classes of antifungals, as opposed to the empirical usage of fluconazole. Thus, the identification of fungal species and susceptibility testing are increasingly important, especially in the face of emerging resistance to commonly dispensed drugs.

In triplicate, all 182 isolates were screened by the YNBG agar disc diffusion test. The *in vitro* antifungal activities of fluconazole were determined by the agar disk diffusion test, while the susceptibility to additional antifungal agents was determined with the Vitek 2 compact system. Our results showed low incidence of azole resistance in *C. albicans* (2.7%), *C. dubliniensis* (2.7%) *C. tropicalis* (1.1%) and *C. glabrata* (11.1%), with all *C. glabrata* susceptible to voriconazole. In addition, compounding was active against most isolates of the collection except 19 of 20 isolates of *C. glabrata* which showed intermediate resistance. Micafungin was active against most isolates except one of *C. dubliniensis* which showed intermediate resistance. Amphotericin B was active against most isolates with the exception of three *C. glabrata* isolates and one each of *C. krusei* and *C. kefyr*. Furthermore, 5-flucytosine was active against most isolates with an exception of all of *C. krusei* 5 isolates and one *C. dubliniensis* which showed resistance. Finally, voriconazole was also active against of most *Candida* species except one of each of *C. albicans* and *C. dubliniensis* which showed resistance. This is the first report to describe these antifungal resistant *Candida* isolates in Libyan patients.

Antifungal sensitivity testing of isolates from Libya results in YNBG demonstrated very high resistance to fluconazole levels (either resistant or dose dependent) in 100.0% of the *C. glabrata*

(20 of 20) isolates, followed by 100% *C. krusei* (5 of 5 isolates), 11.5 % *C. humicola* (3 of 26 isolates) as resistance and (3 of 26) 11.5 % as intermediate resistance, 100% *C. guilliermondii* (1 of 1 isolate) , 75% *C. sake* (3 of 4 isolates), 50% *C. parapsilosis* (1 of 2, the other one showed intermediate resistance). With *C. albicans* being the most frequent species in this study, 5.9% showed resistance and 17.6% intermediate resistance. *C. dubliniensis* demonstrated 2.4% resistance and 19.1 % intermediate resistance. The intermediate (dose-dependent) result for *C. kefyr* and *C. tropicalis* were each 60% (3 of 2 isolates each).

When using the disk diffusion method, thirty-seven (37) isolates were found to be resistant to fluconazole, while 31 isolates were categorized as intermediate. These 68 isolates were then tested on the Vitek 2 system in an effort to confirm the fluconazole MICs and determine the susceptibility of these resistant isolates to other antifungal drugs.

Some discrepancies were seen when comparing the Vitek 2 results to those obtained with fluconazole disk diffusion in YNBG agar. It showed with the Vitek 2 compact system –AST different results than the previous results with fluconazole disc diffusion in YNBG agar. Twenty-seven isolates were intermediate resistance on Vitek, while only nine isolates were resistant to fluconazole drug. Twenty- eight (28) of 37 (YNBG DD resistant isolates) isolates in Vitek were intermediate, and 9 isolates were resistant to fluconazole.

All positive *Candida* isolates were tested in duplicate for their susceptibility to fluconazole, the most widely prescribed antifungal drug for localized and disseminated candidiasis, using the disc diffusion method.

In the disc diffusion method, fifteen isolates of *Candida humicola* showed susceptibility to fluconazole, with the remaining one having different degrees of resistance (6 resistant and 5

intermediate resistant). The two isolates of *C. magnoliae* and only one *C. globosa* isolate were susceptible to fluconazole, while one isolate of *C. membranifasciens* showed intermediate resistance to fluconazole. None of these species currently have established susceptibility breakpoints on the Vitek system, so no finite conclusion can be drawn.

The Vitek 2 Compact system (bioMérieux, Marcy l'Etoile, France), an automated miniaturized microdilution system for routine clinical laboratory identification and sensitivity testing, was used with the Vitek AST-Y07 card format for the testing of the isolates that expressed resistance patterns for fluconazole. The susceptibility to 6 antifungal drugs was tested, namely amphotericin B, caspofungin, micafungin, fluconazole, voriconazole and flucytosine.

The high resistance levels of all *C. krusei* isolates to fluconazole are to be expected, since this species is intrinsically resistant to this drug. However, all *C. krusei* isolates were also found to be resistant to 5-flucytosine and one showed resistance to amphotericin B. Consequently, all tested *C. glabrata* isolates showed intermediate resistance to both fluconazole and caspofungin, with three of these isolates showing resistance to amphotericin B.

On the other hand, a slight difference in resistance levels was agreed in this study. Cross-resistance to a range of clinically used antifungals may also be attributed to the antifungal agents used (Sojakova *et al.*, 2004). *C. dubliniensis* was detected as the second most frequent species in this study 23% (42 isolates). *C. dubliniensis* is normally recovered from the oral cavity of HIV-infected and AIDS patients (Sullivan and Coleman, 1998), but in recent times this *Candida* species has been isolated from the oral cavity of other immunocompromised patients such as DM (Willis *et al.*, 2000).

The results from the Vitek 2 compact system showed a 2.19% overall fluconazole resistance (including intermediate drug resistance isolates) and with voriconazole (0.54%) of *Candida* species in T2DM candidates in Misrata city of Libya. Although *C. albicans* was the most prevalent species in the present study, drug resistance in Libyan subjects of T2DM patients was much more prominent in non-*albicans* species.

The emerging fluconazole resistance levels seen in the present study, can be partly blamed on the prolonged prescription of this drug in clinical candidiasis cases. In Libya, where the sale of medicines is not controlled, the distribution of antifungal drugs by untrained persons and/or self-medication by the patients is common.

In vitro studies have been proposed for *Candida* species (Magaldi *et al.*, 2001), and this cross-resistance may occur with fluconazole and other azole compounds (Pfaller *et al.*, 2002, Pfaller *et al.*, 2004). In several studies, resistance mechanisms involve the upregulation of genes encoding the ATP-binding cassette efflux transporters or CDR pumps (Sanglard *et al.*, 1997, Sanguinetti *et al.*, 2005).

In our samples, for each one of the thirty-six isolates that were fluconazole resistant, two were voriconazole resistant, probably due to secondary resistance through such a mechanism. However, primary resistance cannot be ruled out unless specific antifungal hospital treatment for each patient is analyzed and correlated with resistance. Cross resistance to a range of clinically used antifungals may also be attributed to the antifungal agents used (Sojakova *et al.*, 2004). Interestingly, in *C. krusei*, whenever resistance to fluconazole was detected, resistance to 5-flucytosine was also seen.

Non-*albicans Candida* species such as *C. glabrata* have been implicated as the causative agents of 46% of systemic *Candida* infections (Wingard, 1995) . Since very high levels of *C. glabrata* isolates were found to be either resistant or intermediate to fluconazole in immunocompromised

The occurrence of resistant *C. glabrata* and *C. krusei* in immunocompromised individuals is dramatically increasing. (Bodey *et al.*, 2002, Alborzi and Davarpanah, 2010)

Recently developed echinocandins and third generation azole compounds have shown a better efficacy in combating certain *Candida* infections (Pemán and Almirante, 2008) and could be considered as second-line drugs to deal with fluconazole-resistant fungal infections.

With intrinsic resistance in *Candida* infection included in most species, proper treatment choice can mainly be made on the foundation of identification (Arendrup *et al.*, 2013), for example *C. glabrata* and *C. krusei* which are resistant to fluconazole. Resistance in *Candida* that is acquired during antifungal therapy is reported less commonly. *C. glabrata* regularly displays echinocandin resistance, as has also been frequently found in other *Candida* species that are usually completely susceptible to this kind of drug's class, for instance, *C. krusei*, *C. albicans* and *C. tropicalis* (Pfaller *et al.*, 2010, Dannaoui *et al.*, 2012, Pfaller, 2012, Arendrup *et al.*, 2013).

The Vitek 2 system has the advantage of allowing both fungal identification and MIC determination rapidly and simultaneously. It is also a faster and easier alternative to the antimicrobial susceptibility testing procedures developed by either the CLSI or the EUCAST, while still giving very similar results (Cuenca-Estrella *et al.*, 2010).

One of the Vitek system's limitations is that the Vitek 2 Yeast identification card cannot be used with direct clinical samples or other sources containing mixed flora. Pure colonies must be sub cultured and any change or modification in the procedure may affect the results. In addition, newly described or rarer species may not be included in the Vitex's YST database. Although selected species are periodically added over time, testing of uncategorized species may result in an unidentified result or a misidentification.

The accuracy of susceptibility of clinical isolates using Vitek 2 compact system are very similar compared to CLSI and EUCAST methods. However, both CLSI and EUCAST concluded that Vitek 2 system is an easy technique to determine antifungal sensitivity testing of those *Candida* isolates, and that it can be clinically useful for determining the susceptibility of both *Candida* spp. and other yeast species, such as *C. neoformans* and emerging species. It is also a reliable technique for identification of azole and amphotericin B resistance *in vitro*.(Cuenca-Estrella *et al.*, 2010). In this study of Libyan subjects, both *C. albicans* and *C. dubliniesis* presented susceptibility levels to echinocandin drugs. Very high azole resistance levels were noted in *C. glabrata* and high azole resistance in the case of *C. krusei* isolates. *C. albicans* isolates responded well to azole drugs, with the exception of four of 68 isolates.

A hundred percent (100%) of *C. krusei* and 2.3% of *C. dubliniesis* species were found to be resistant to 5-flucytosine. All *C. albicans, C. glabrata, C. kefyr, C. tropicalis, C. guilliermondii,* and *C. parapsilosis* isolates were susceptible to this drug.

When using the Vitek 2 compact system, a high level of drug resistant *Candida* isolates was found. Isolate LY 16B, a *C. dubliniensis* isolate, was found to be resistant to three drugs on the Vitek. The only exception was amphotericin B and caspofungin, which inhibited growth of this isolate in concentrations ≤ 0.25 µg/ml, also susceptible with micafungin at concentration ≤ 0.06 µg/ml. This is as the first report of resistance of this isolate among T2DM patients.

Resistance to amphotericin B was observed with respect to non-*albicans Candida* species, demonstrating especially resistance levels, namely *C. glabrata* and one each of *C. krusei* and *C. kefyr* of Libyan population.

It is possible that these species formed part of the oral microbial biofilm in these patients, which might thus have resulted in an increased pathogenicity. The coexistence of twelve *Candida* species demonstrating the same resistance or intermediate resistance to fluconazole seen in three patients could signal the exchange of genetic information related to drug resistance and predispose the host to a higher degree of fungal colonization and infection.

The laboratory identification and susceptibility testing of fungal infections is not commonly performed in Libya, with patients being treated empirically according to their clinical symptoms. Clinicians usually request laboratory tests such as microscopic examination using potassium hydroxide for the identification of suspected *Microsporum* or *Trichophyton* infections, with no susceptibility testing being performed.

Consultation with dentists in Misrata, Libya, confirmed that diagnosis of oral Candidiasis infections in their clinic is accompanied by prescribing either Flagyl (for bacterial and fungal infection), or Nystatin as an antifungal agent with no laboratory susceptibility testing. In some cases, no antifungals are administrated. Differences in the treatment guidelines between various geographical areas can result in the emergence of strains resistant to different classes of antifungal agents. Furthermore, the treatment of seemingly similar fungal infections can vary according to antifungals for each isolate.

CHAPTER 4

THE ANTIFUNGAL ACTIVITY OF INDIGENOUS PLANTS *EUCLEA NATALENSIS* AND *SALVADORA PERSICA*

4.1. Introduction

Complementary alternative medicine is becoming increasingly popular in several developed countries (Who, 1998) . The secondary metabolites of the medicinal plants have an important role in conventional western medicine. They are obtained after the processing of fresh or dried plant material. Natural medicinal plants are the source of several biologically active compounds, many of which have been used for the development of new chemicals for pharmaceuticals (Palombo, 2011).

For thousands of years, medicinal plants have been used as traditional therapy for numerous human diseases around the world. In developing countries' rural areas, people continue to use medicinal plants as the primary source of medicine (Chitme *et al.*, 2004). Approximately 80% of the people in these developing countries used traditional medicines for their health care (Kim, 2005). Medicinal plants compose the main component of traditional medicine. In other words, about 3300 million people regularly use medicinal plants (Farnsworth, 1994).

In Latin America, the World Health Organization Regional Office for the Americas reported that 71% of Chileans and 40% of Colombians used traditional medicine for treatment. In the US,, patients prefer to use complementary alternative medicine than western medicine while in Japan, traditional medicines are prescribed for patients in clinics by up to 70% of allopathic doctors (Bussmann *et al.*, 2010).

Euclea natalensis was recorded as one of the most important plants used in South African folk medicine for the treatment of oral diseases (More *et al.*, 2008, Babula *et al.*, 2009). Chemical and pharmacological studies indicated that one of the chemical constituents *viz* 7-methyljuglone is the most active compound (Mahapatra *et al.*, 2007), showing a wide spectrum of antibacterial activities (Sinha *et al.*, 2009).

Salvadora persica, on the other hand is well documented for the treatment of oral diseases in oriental counties and North Africa (Almas, 1999, Abdelrahman and Skaug, 2002). The biological activity of the plant being attributed to the glucosinolate fraction (Sofrata *et al.*, 2011).

In this chapter we will focus on the preparation of both plants total extracts as well as purification of 7-methyljuglone (from *E. natalensis*) and total enriched glucosinolate enrich fraction from *S. persica* as well as their biological activities against *Candida* species resistant to fluconazole antifungal agent.

With the increased morbidity and mortality of invasive fungal infections and the emergence of severe antifungal drug resistance, there is a great demand for novel new antifungal agents with reduced toxicities and widened spectra of activity.

4.2. Methodology for the use of plant materials for antimicrobial assays

4.2.1. Collection of plant materials

Euclea natalensis

Euclea natalensis root material was collected in 2014 from two localities on the West Coast, Clan William 25 km east of town, Western Cape, next to the R364 road. GPS coordinates: 32°8'58.47"S, 19°1'44.64"E. Voucher specimens were identified by and deposited in the Compton Herbarium Collector and number: Cupido s.n (Figure 4.1).

Plant sample collection and Identification

Euclea natalensis was collected from known two sites, approximately 20 km east of Clanwilliam (the pigmented hand was a primary identification of the plant)

Roots were sampled, and voucher specimens were collected for deposit in the Compton Herbarium

Figure 4.1: plant collection and identification

Salvadora persica (Miswak)

Approximately 2 Kgs of sticks of miswak trees (*S. persica*) were obtained from a market in Libya.

4.2.2. Extraction of plant material and identification of active compounds

E. natalensis

The root bark was separated by mechanical means (1.5 kg) and blended with 3.5 L of dichloromethane (DCM) and left overnight for extraction. After filtration, the plant residue was extracted overnight with fresh DCM. The combined filtrate was evaporated under reduced pressure at 40 ℃ to give 27.43g of total extract.

Salvadora persica

The plant materials were powdered mechanically (1.9 kg) and extracted with 80% aqueous ethanol for two days at room temperature. After filtration, the total extract was evaporated under reduced pressure at 50 ^{o}C to give 431.2 g.

4.2.3. Purification and identification of compounds.

4.2.3.1. 7-methyljuglone from E. natalensis

There are different techniques which are available for the purification and identification of natural products. Through this study, the isolated compounds were purified by means of column chromatography and identified using different spectroscopic means.

A 30 X 7 cm dry column technique was employed to fractionate the crude extract. The dry extract (281.59 g) was re-dissolved in a minimum amount of chloroform and added to dry silica gel powder in a suitable glass container. This mixture was left at room temperature until all the chloroform evaporated, after which it was added on top of the silica gel inside the glass column. The column was eluted in order of increasing polarity (Table 4.1). Thirty-two fractions (500 ml each) were collected and pooled together to 8 main fractions (I-IIIV) according to their Thin-layer chromatography (TLC) profile (Figure 4.2).

Table 4.1: Solvent system of chromatography column for purifying the 7-methyjuglone

NO.	HEXANE	DCM.	ACETONE	VOLUME
1.	**100%**	0%		1L
2.	**95%**	5%		1L
3.	**90%**	10%		1L
4.	**85%**	15%		1L
5.	**80%**	20%		1L
6.	**70%**	30%		1L
7.	**50%**	50%		1L
8.	**40%**	60%		1L
9.	**25%**	75%		1L
10.	**10%**	90%		1L
11.	**0%**	100%		1L
12.		**90%**	10%	1L
13.		**70%**	30%	1L
14.		**50%**	50%	1L
15.		**30%**	70%	1L
16.		**80%**	20%	1L
17.		**70%**	30%	1L
18.		**0%**	100%	1L

4.2.3.1.1. High performance liquid chromatography (HPLC) of sub-fraction II-3

Final purification of subfraction-II-3 (1.59g mg) was carried out using semi-prep HPLC Agilent Technologies 1200 series, equipped with UV detector, manual injector, quaternary pump (G1311A), vacuum degasser (G1322A), and column compartment (G1316A) and reversed phase C18 column SUPELCO (25 X 2.1 cm). The flow rate was set at 1.5 ml/min and detection wavelength at λ_{254} nm. Solvent system of methanol: water (80% to 100 MeOH in 30 minutes) was used. A Prominent peak of 7-methyljglone was collected (Figure 4.3), purity and structure were proved using ^{1}H- and ^{13}C NMR

4.2.3.1.2.1. Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectra of the isolated compound from the previous fraction were recorded at 25 °C in $CDCl_3$, using Bruker Avance 400 NMR spectrometer (^{1}H at 400 MHz, ^{13}C at 100 MHz). Chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in ppm were determined relative to solvent signal (Figures 4.5, 4.6, 4.7).

4.2.3.2. Preparation of Total Glucosinolate Fraction from *S. persica*

The total glucosinolate fraction was prepared according to (Ezmirly and El-Nasr, 1981). A 3.0 g of total extracts of *S. persica* were dissolved in 30 mL of methanol, 170 ml of methanol were added again and total *glucosinolate* was precipitated using 170 ml dichloromethane, (DCM). The precipitate was collected and re-dissolved in methanol and again precipitated using DCM, this process was repeated three times to obtain partially purified total glucosinolate enriched fraction.

4.2.4. Screening of Plant Extracts for Antifungal Activity

Candida glabrata and *Candida albicans* type strains were sub-cultured over night at 37 ^{0}C, the density was adjusted at 0.5 OD at 450 nm (McFarland standard) using a nephelometer reader.

4.2.4.1. Kirby- Bauer Disk Diffusion method for screening the antifungal activities of *Euclea natalensis* extracts

The Kirby-Bauer disc diffusion method using type strain *C. albicans* (ATCC 90028) at concentrations of 2 and 5 mg/ml were used. Acetone disks as (50%) and 25 µg fluconazole antimicrobial susceptibility test disks were used as negative and positive controls respectively. The 50% acetone was also used as a diluent to prepare the total extractions of *Euclea natalensis* (bark and inner parts) and the Juglone (5-Hydroxy-1, 4-naphthoquinone) (Commercial compound) at various concentrations by carrying out a 2-fold serial dilution.

4.2.4.2. Kirby- Bauer Disk diffusion for screening the antifungal activity of *Salvadora persica* extracts

S. persica was prepared and dissolved in sterile autoclaved water. The extract solution was applied onto previously autoclaved 9 mm filter disks in serially diluted concentrations ranging from 500 µg/ml – 3.9 µg/ml. Extract solutions of 40 µl were loaded onto the filter disks and dried overnight at 37°C. A sterile swab was then dipped into the cell suspension, previously standardized, and used to inoculate YNBG Agar plates. Appropriate premade disks were then impregnated on the inoculated plates using a sterile needle and the plates were inverted and incubated for 24 hours at 37°C. Zones of inhibition were measured form the edge of the disk to the edge of the zone

4.2.5. Antifungal susceptibility using broth microdilution Tetrazoluim Microplate Assays.

4.2.5.1. Roswell Park Memorial Institute media (RPMI- 1640) Medium preparation

RPMI 1640 medium was prepared by adding (10.43g with L-glutamine) (Lot SLBP7558V, Sigma Aldrich, UK) to 18g glucose (2%) diluted into 900 mL of distilled water. The medium was buffered to a pH of 7.0 at 25^0C with MOPS (morpholine propane sulfonic; acid final molarity at pH 7.0, 0.165) and dissolved using a sterile magnetic pellet on a Crison GLP21 magnetic stirrer (Barcelona, Spain). Once dissolved completely, the pH was adjusted to pH 7.2 and sterilised using a 0.22 µm disposable filter (Ref. no. 25NS, MSI filters, USA) attached to a 50 mL sterile plastic syringe into an autoclaved sterile bottle.

4.2.5.2. Broth Microdilution Assay using Total Extracts of *Euclea natalensis*, compounds 7-methyljuglone, and Juglone (5-Hydroxy-1, 4-naphthoquinone)

Microplate assays were performed in a 96 well plate. (INT) p-iodonitrotetrazolium chloride was used as a colour indicator to measure the growth of the test microorganism treated with various extracts to determine the MIC (Perumal *et al.*, 2012). The colourless tetrazolium salts (yellowish, clear not turbid in colour) act as electron acceptors and is reduced by biologically active organisms resulting in the colour change observed (Eloff, 1998). One mg of each extract was dissolved in one mL of dried Dimethyl sulfoxide (DMSO) using a water bath at 45°C and vortexed (Vortex Mixer, Labnet, USA), then 200 µl of stock solution were added to 800 µl of RPMI broth. A volume of 100 µl of the RPMI broth was dispensed into microtiter plate wells.

An inoculum of *Candida glabrata.* was prepared in RPMI -1640 medium with L-glutamine and a pH indicator and without sodium bicarbonate plus 2 % glucose (Himedia, India), and the turbidity was adjusted to 0.5 McFarland. A volume of 100 µl of the RPMI broth media was dispensed into

microtiter plate wells with different concentrations of each compound, the plate sealed with sealing film and incubated for 24 hours at 37°C. Post incubation, some of the plates were read using Anthos 2010 plate reader at the wave length 450 OD (Biochrom Ltd, Cambridge CB4 OFJ, England)

A hundred µl of solution of plant compound and 100 µ L of *Candida glabrata* inoculum were placed into the wells of the microtiter plate and the plate sealed with sealing film and incubated in Labotech shaking incubator 90 rpm (Orbi Shake, Johannesburg, South Africa) for 24 hours at 37°C h. After 24 hours' incubation, the MIC was determined by adding 40 µl of 0.2 mg/ml INT to microplate wells and incubated in a shaking incubator at 75 rpm at 37°C for 1.5-2 hours and the plate sealed with sealing film. Post incubation, plates were read using a plate reader for checking the absorption of the compounds. The MIC was calculated as the lowest concentration of compound that inhibited 100% growth of the *Candida* spp.

4.2.5.3. Determination of MIC using total extraction of *Salvadora persica* and total extraction of *Glucosinolates*

The microdilution technique using 96-well plates as described by Elof (1998) was used to gain the minimum inhibitory concentration (MIC) values of the crude extract of total extraction of *Salvadora persica* as well as total extraction of Glucosinolates against *Candida spp*. Both total extractions were prepared to dissolve in 1 mL each of RPMI medium (Balouiri *et al.*, 2016). The active extracts were serially diluted in 96-well plates. An inoculum of *Candida spp.* was prepared in 10 mL of RPMI 1640 medium in an autoclaved test tube, and the turbidity was adjusted to 0.5 McFarland.

The positive control was 25 µg /ml fluconazole. Twenty-four-hour cultures of *Candida* spp. were

added to the 96-well plate and incubated for 24 hours at 37°C. *Candida* growth inhibition was initiated by adding 40 µl (using multi-channel pipette) of 0.2 mg/ml INT to microplate wells and incubated at 37°C for 1.5-2 hours.

4.3. Statistical analysis

Statistical analysis was done using the SPSS 24 statistical software and confirmed with Stata 15 software. Some of the statistical results were from Excel, tests coming from NCSS. Descriptive statistics, chi-squared tests and T test were used for the comparison of different patient data. Analysis of fluconazole and other antifungal drug susceptibility results was also done by means of chi-squared tests ($p < 0.05$)

RESULTS

4.4. Biological Antimicrobial Activity of *Euclea natalensis*

4.4.1. Purification and identification of 7-methyljuglone

The DCM extract of the *Euclea natalensis* root bark was chromatographed on silica gel and 7-methyljuglone enrich fraction was selected (according to preliminary NMR data) for further purification using silica gel (Figure 4.2) and a gradient of hexane: ethyl acetate mixture (1-5%) of increasing polarity (Figure 4.3). The sub fraction 3 was further purified on the semi-prep HPLC using methanol / water (7:3) to (9:1) in 30 minutes and detected at λ_{254} nm (Figure 4.4). The purification process yielded pure 7-methyljuglone as indicated by the NMR spectra (Figures 4.4 – 4.6).

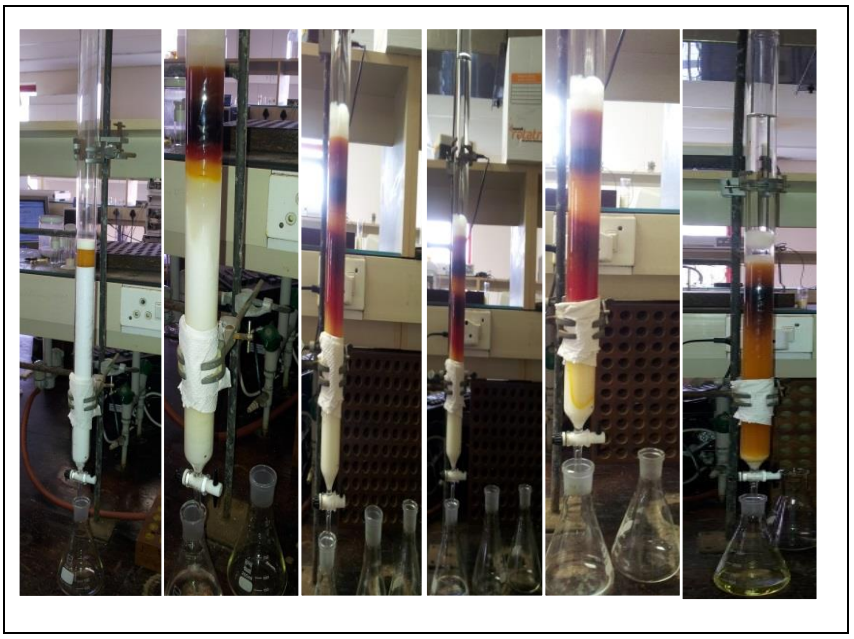


Figure 4.2: Column chromatography setup (7 X 30 cm, 500g silica gel) for collecting fractions which were screened using HPLC. Each colour in the column represented different fraction.

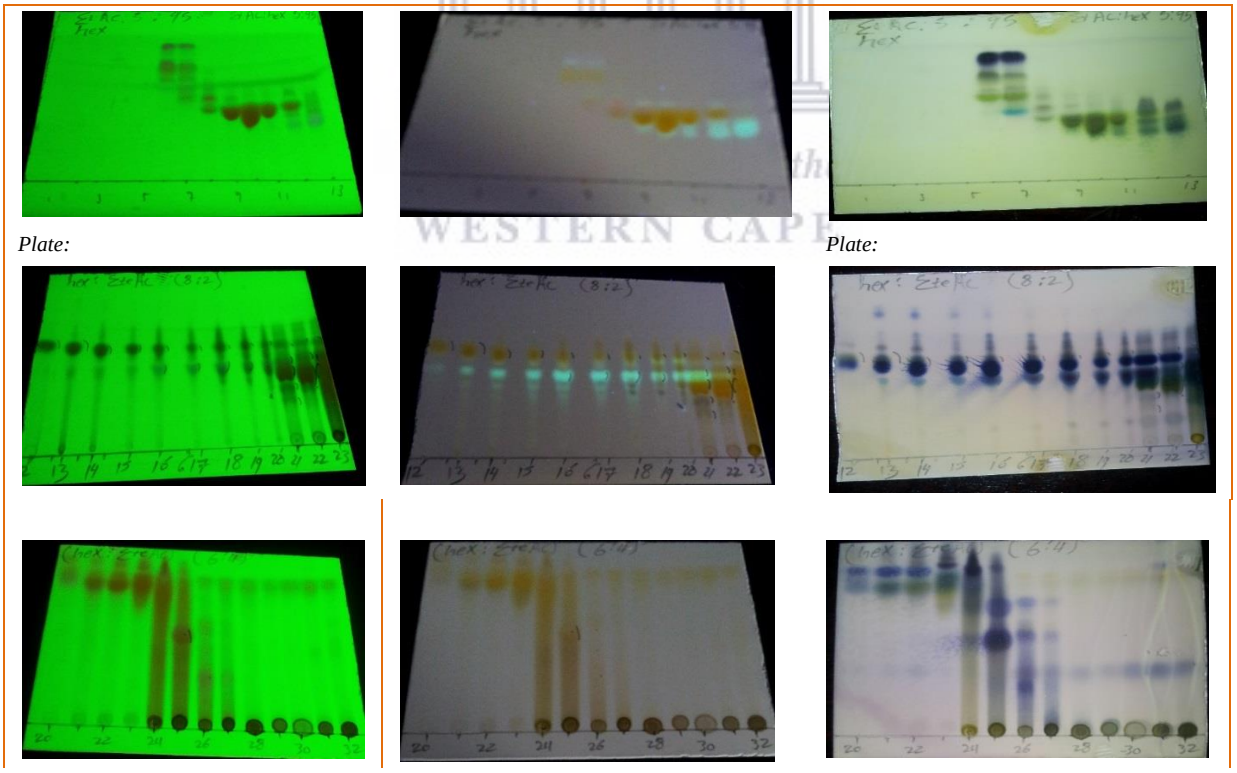


Plate:

Plate:

Figure 4.3: TLC chromatogram of the first column for the total extract showing different chemical constituents as detected by $UV_{254/360}$ and vanillin / H_2SO_4 reagent. Fractions 9-11 (1.59 g) were selected for further chromatography.

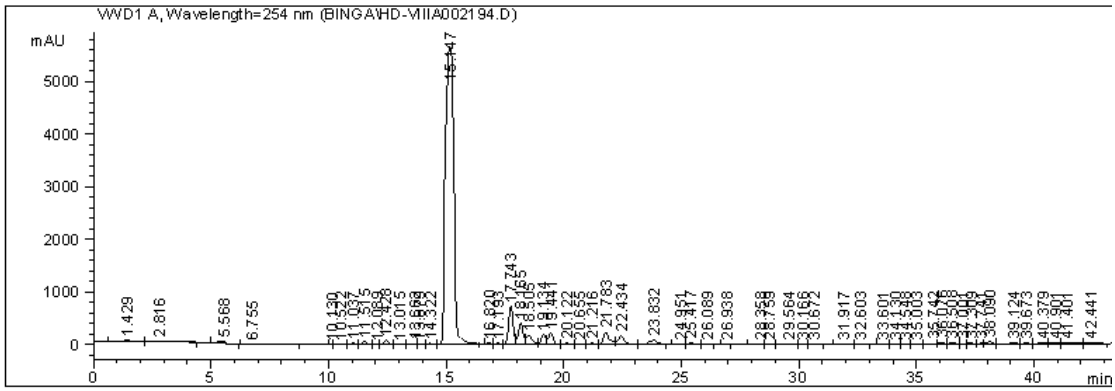


Figure 4.4: HPLC chromatogram of sub-fraction II-3, it shows a major peak at 15.15 minutes.

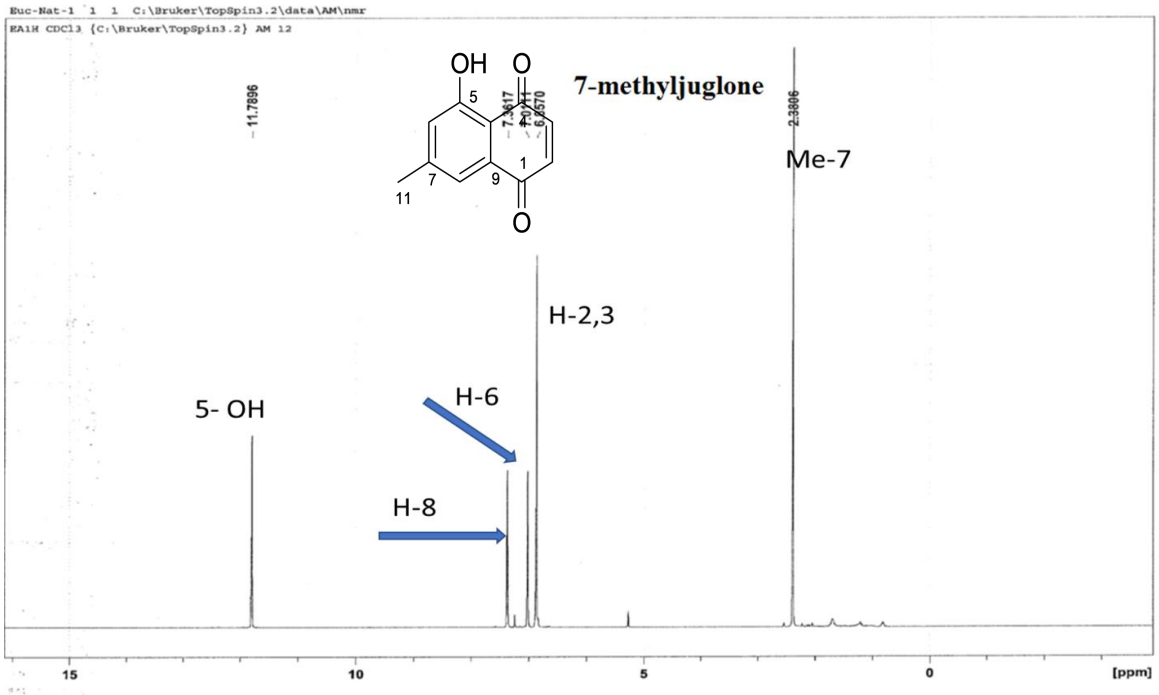


Figure 4.5: ^1H NMR of 7-methyljuglone in $CDCl_3$

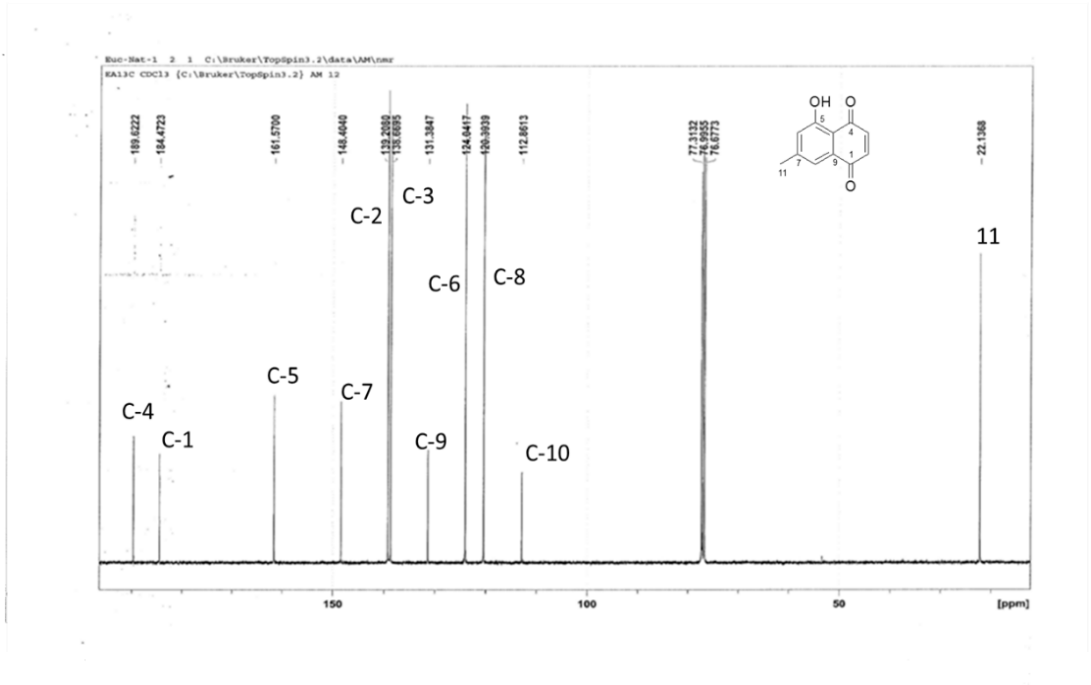


Figure 4.6: 13C NMR of 7-methyljuglone in CDCl3

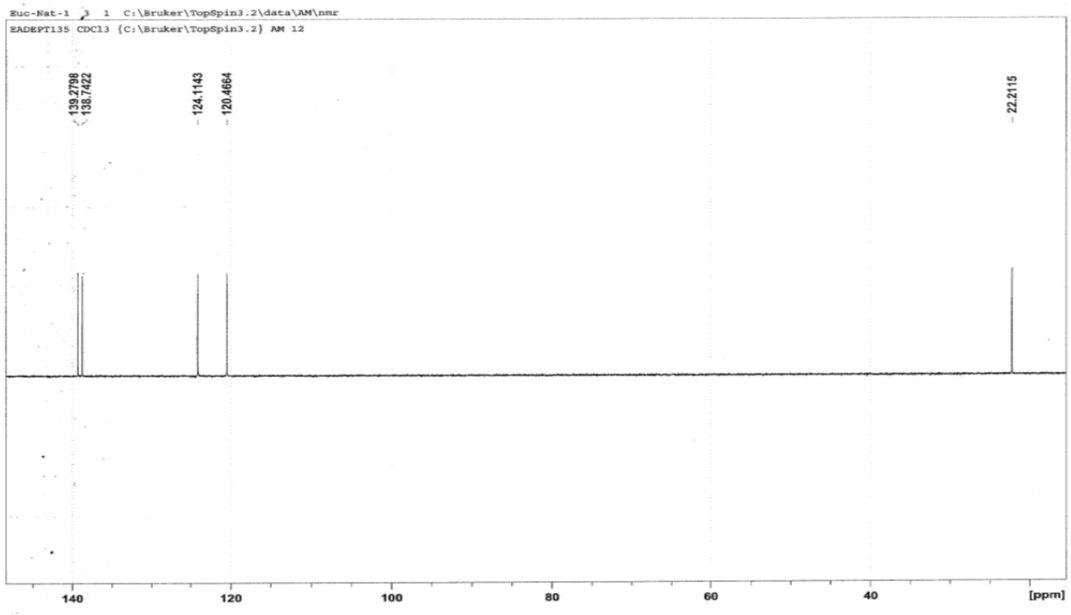


Figure 4.7: DEPT-135 spectra of 7-methyljuglone in CDCl3

The peaks (^{1}H NMR) showed a methyl group at 2.38 and four unsaturated protons two of them are aromatics at 7.01 (H-6) and 7.36 (H-8); a singlet at 6.85 for two olefinic proton (H-2, -3) in addition to OH signal at 11.78. The carbon-13 NMR and DEPT-135 (figures (4.6 and 4.7) showed 11 carbon

signals, assigned as one methyl at 22.14, two conjugated carbonyl groups at 184.47 (C-1) and 189.62 (C-4) in addition 8 unsaturated carbons of the aromatic ring and the double bond at 161.57 (C-5); 148.40 (C-7), 139.20 (C-2), 138.67 (C-3), 131.38 (C-9), 124.04 (C-6), 120.39 (C-8), and 112.86 (C-10). Further, the data obtained matching perfectly with the published data of the *7*-methyljuglone (Bonnet *et al.*, 1984). as indicated from the previous analysis, the 7-methyljuglone has been isolated and purified as the main active constituent of *Euclea. natalensis* (Sankaram *et al.*, 1986), the compound purified in enough quantity for further biological activity studies.

4.4.2. The glucosinolate fraction of *Salvadora persica.*

The purifications of glucosinolate main enriched fraction from *S. persica* were carried out as explained in section (**4.2.3.2**).

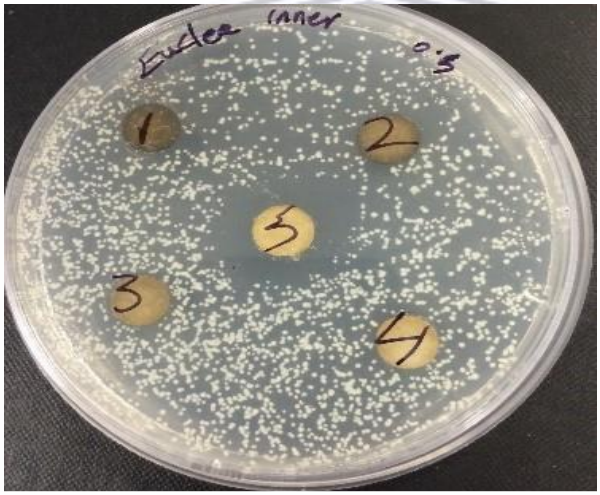
4.4.3. Screening of Total Extracts using Kirby-Bauer Disc-Diffusion Assay

4.4.3.1. *Euclea natalensis*

The inner and bark parts of *Euclea natalensis* were tested in the disc diffusion method to find out their biological activities against resistant clinical *Candida* isolates. Both of total extracts of bark (Figure 4.8A) and inner part of *Euclea natalensis* prepared with 5 serial dilution (300-500 µg/l), and each concentration was impregnated in the 9 mm disc and dried in the incubator for 2 hours. Impregnated disc was added to the YNBG media plated with a 0.5 MacFarland concentration of the species. Figure.4.8 B showed the positive and negative controls, which were Juglone and acetone respectively. With juglone the semi 7-methyljuglone chemical structure showed high effect in the screening trials while those dissolved in acetone showed no effect. The lower concentration of total inner extracts had higher biological effect than the higher concentrated disk (Figure.4.8 C).

(A) Screening of total extraction of bark parts of *E. natalensis* concentration *range was* between (300-500 up/ml

(B) Screening of total extraction of Juglone.2 mg/ml



(C) Screening of total extraction of inner parts of *E. natalensis* concentration range was between (300-500 ug/ml

Figure 4.8: Disk diffusion screening profiles of total extracts active against *C. albicans* in YNBG media in the presence of a fluconazole disk as a positive control.

4.4.3.2. *Salvadora persica*

Preliminary screening was done using total extraction of *Salvadora persica* to assess biological activity at the concentration of 0.5 mg/ml

4.4.4. Antimicrobial susceptibility results using Vitek 2 compact system

As mentioned earlier in chapter 3 section (3.2.), 18 of 32 isolates of *Candida* species resistant to fluconazole, namely *C. albicans, C. glabrata, C. guilliermondii* and *C. krusei* have been tested as well with total extractions of selected plants with their compound and fractions.

4.4.5. Broth microdilution assay using total extraction of *Euclea natalensis,* compounds 7-methyljuglone, and Juglone (5-Hydroxy-1, 4-naphthoquinone)

Tetrazolium microplate assays were performed on different *Candida* species which showed resistance to fluconazole using fluconazole disc diffusion and confirmed by Vitek 2 compact system

In this study, the RPMI 1640 medium with L-glutamine as a negative control showed no colour change indicating (Pinkish in colour), no growth. The DMSO control of 10% showed a colour change showing that the highest DMSO concentration used in the assay had no effect on the growth of *Candida species*. This proved that any growth inhibition seen in the treated cells was a result of the activity of the fractions and compounds extracts themselves. The d H_20 well also showed a change in colour indicating that the diluent used in this assay had no effect on *Candida* species which were resistant to fluconazole. Fluconazole (positive control) showed a colour change confirming that the *Candida* species used were fluconazole-resistant. A colour change was also seen in the *Candida* control which indicated that cells were actively growing whilst carrying out the assay.

Tetrazolium Microplate Assay results of clinical *Candida glabrata* from oral mucosa of T2DM patients which showed resistance to Fluconazole using DD and Vitek 2. *Candida glabrata* exposed to total extraction of *Euclea natalensis* row B1-3 diluted to H1-3, &*7-methylojug*lone row B5-7 diluted to H5-7, and Juglone *(5-Hydroxy-1, 4-naphthoquinone) for* a 24-hour incubation period with INT chloride reagent. Row A1-3 consisted of controls including a 200 µl of RPMI-1640 broth medium as control, negative DMSO control at 6% (A10-12), a positive 25 µg fluconazole powder control (A7-9), and finally a yeast broth control (A4-6). Treated cells ranged from row C-H where a two-fold serial dilution was carried out in the respective wells to obtain extract concentrations 18.75 µg/ml E1-3.

The MIC of total extraction of 7-methylojuglone against *C. glabrata* is shown (arrow) in rows F5-7 at 1.56 µg/ml. The tetrazolium microplate assay MIC test results for Juglone (5-Hydroxy-1, 4-naphthoquinone) against *C. glabrata* is shown (arrow) in rows F10-12 at 1.56 µg/ml. The Tetrazolium Microplate Assay MIC test results were available after a 24-hour incubation period. All control wells except the RPMI-1640 broth medium control (A1-3) indicated a change in color due to INT chloride reagent being reduced by biologically active *C. glabrata* cells. The yeast control (A4-6), 6% DMSO negative control (A10-12), and the positive control (A7-9) 25 µg fluconazole powder positive control all showed a color change to pink indicating viable *C. glabrata* cells. No change in color was observed from the total extraction of *Euclea natalensis* concentrations of 18.75 µg/ml, 7-methylojuglone compound at 1.56 µg/ml, and Juglone at1.56 µg/ml. All of them showed the lowest concentration capable of preventing growth.

The MIC was defined as the lowest concentration of the extract showing no color change which was at 18.75 µg/ml, 1.56 µg/ml and 1.56 µg/ml respectively (Figure.9). After a 48- hour's incubation, there was no change in the result (Figure.4.9)

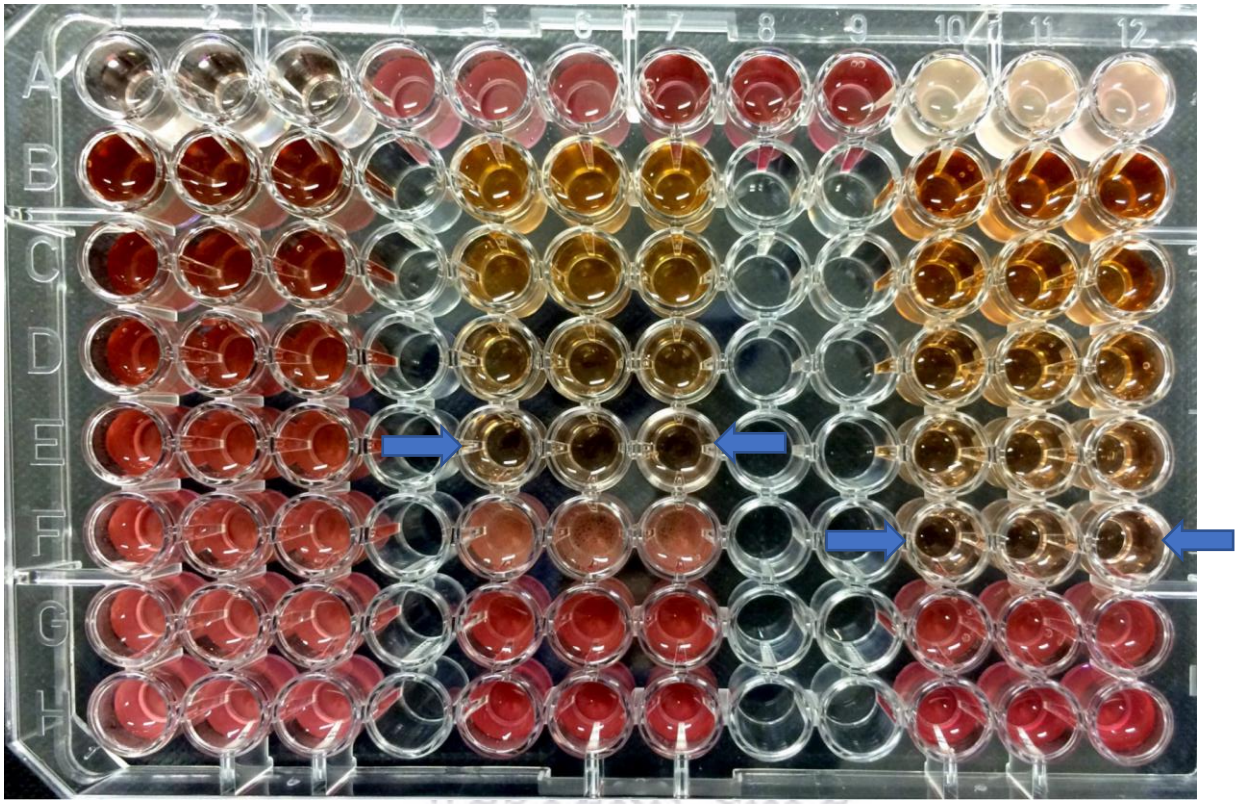


Figure 4.9 Microdilution screening colorimetric profiles of total extracts of *Euclea natalensis (B 1-3), 7-*methyljuglone compound *(B 5-7),* and Juglone *(B 10-12)* against *C. glabrata*

4.4.6. Minimum Inhibitory (MIC) of using purified compound and fraction

The purified compound from *Euclea natalensis* 7-methyljuglone and the commercial compound Juglone (5-Hydroxy-1, 4-naphthoquinone) showed susceptibilities against *Candida* resistant species to fluconazole (Table 4.2), but the total extraction was not as active as its compound *7*-methyljuglone and to commercially prepared Juglone.

Antifungal susceptibility of *Euclea n.* (7-methyljuglone compound), against *Candida* species were available within 24 hours using microdilution assay. Results revealed an anticipated susceptibility of 7-methyljuglone, which showed high susceptibility with MIC's ranging between 25 µg/ml - 0.390 µg/ml and the commercial Juglone ranging between 12.5 µg/ml - 0.390 µg/ml (Table 4.2), using the most sensitive technique microdilution method.

Table 4.2: MIC ranges and their Average of *Candida* species resistant to fluconazole using Total Extracts of Euclea n, 7-methyljuglone.

	Candida specie	No. of tested isolates	MIC_{90} (µg/ml)	MIC_{50} (µg/ml)	MIC range (µg/ml)	No. of serial Dilutions	MICs Average
E. natalensis	***C. krusei***	5	150	150	75- 150.	5	135
	C. glabrata	11	150	75	9.37 - 150.	5	67.33
	C. guilliermondii	1	-	-	-	1	37.50
	C. albicans	1	-	-	-	1	150
7-methyljuglone	***C. krusei***	5	1.56	0.39	0.39 - 1.56	3	0.70
	C. glabrata	11	12.50	12.50	1.56 - 25.0	3	12.64
	C. guilliermondii	1	-	-	-	1	3.13
	C. albicans	1	-	-	-	1	0.78
Juglone	***C. krusei***	5	0.781	0.781	0.39 - 0.781	2	0.63
	C. glabrata	11	12.5	6.25	1.56 - 12.5	3	8.10
	C. guilliermondii	1	-	-	-	1	1.56
	C. albicans	1	-	-	-	1	1.56

Total extraction of *E. natalensis* against the 5 *C. krusei* isolates tested, showed that 4 demonstrated susceptibility at 150 µg/ml, while 1 demonstrated activity at 75 µg /ml. Of the 11 *C. glabrata* isolates tasted, 4 demonstrated susceptibility at 75 µg/ml, 3 demonstrated susceptibility at 37.5 µg /ml, 2 demonstrated susceptibility at 150 µg/ml, while 1 demonstrated susceptibility at 18.75 µg/ml and another at 9.375 µg/ml. Additionally, one each of *C. albicans* and *C. guilliermondii* demonstrated susceptibility at 150 µg/ml and 37.50 µg/ml respectively (Table 4.3).

Testing of 7-methyljuglone compound against of the 5 *C. krusei* isolates showed that 3 demonstrated susceptibility at 0.390 µg/ml, while 1 each demonstrated susceptibility at 0.781 µg / ml and 1.56 µg /ml. Of the 11 *C. glabrata* isolates tasted, 9 demonstrated susceptibility at 12.5 µg/ml, 1 each demonstrated susceptibility at 1.56 µg /ml and 25 µg /ml. Additionally, one each of *C. albicans* and *C. guilliermondii* demonstrated susceptibility at 0.781 µg/ml and 3.125 µg/ml respectively (Table 4.2).

Juglone compound against of the 5 *C. krusei* isolates tested, 4 demonstrated susceptibility at 0.390 µg/ml, while 1 each demonstrated susceptibility at 0.781 µg / ml. Of the 11 *C. glabrata* isolates tasted, 6 demonstrated susceptibility at 12.5 µg/ml, 4 demonstrated susceptibility at 12.25 µg /ml and only one demonstrated susceptibility at 1.56 µg /ml. Additionally, one each of *C. albicans* and *C. guilliermondii* demonstrated susceptibility at 0.156 µg/ml (Table 4.2).

4.4.7. Broth Microdilution Assays for *S. persica*

The tetrazolium microplate assay MIC test results for total extraction of Miswak and its fraction Glucosinolate against fluconazole-resistant *Candida* spp. with a MIC's ranging between 25 mg/ml - 0.781 mg/ml (Table 4.3).

Table 4.3: Minimum Inhibitory Concentration (MIC) total extracts of *S. persica* and its total extracts of glucosinolate fraction.

	Candida species	MIC (mg/ml) of total extracts S. *persica*	Total extracts of glucosinolate. fraction*:* (mg/ml)
	C. albicans	3.125	3.125
	C. glabrata	3.125	6.25
	C. glabrata	3.125	3.125
	C. glabrata	3.125	3.125
	C. glabrata	3.125	3.125
	C. glabrata	3.125	3.125
	C. glabrata	3.125	3.125
	C. glabrata	3.125	3.125
	C. glabrata	3.125	3.125
	C. glabrata	0.781	1.562
	C. glabrata	1.562	12.5
	C. glabrata	3.125	3.125
	C. guilliermondii	25	6.25
	C. krusei	1.562	12.5
	C. krusei	3.125	3.125
	C. krusei	3.125	3.125
	C. krusei	6.25	25
	C. krusei	3.125	3.125
+ve control	*Flu 25ug/ml*	R	R
-ve control	*Sterile RPMI broth medium*	C	C

[R] Resistance, [C] Clear.

Table 4.4: MIC ranges of total extracts tested against fluconazole resistant *Candida* species

	***Candida* specie**	**No. of tested isolates**	**MIC_{90} (mg/ml)**	**MIC_{50} (mg/ml)**	**MIC range (mg/ml)**	**No. of Dilutions**	**Average**
Total Extracts of *S. persica*	***C. krusei***	5	6.25	3.125	1.56 - 6.25	3	3.44
	C. glabrata	11	3.125	3.125	0.781 - 3.13	3	2.56
	C. guilliermondii	1	-	-	-	1	25.00
	C. albicans	1	-	-	-	1	3.13.
Total Extracts of Glucosinolate fraction	***C. krusei***	5	25	3.125	3.13 - 25.00	5	9.38
	C. glabrata	11	3.125	3.125	1.57 - 12.50	4	4.12
	C. guilliermondii	1	-	-	-	1	6.25
	C. albicans	1	-	-	-	1	3.13.

Total Extracts of *S. persica* against the 5 *C. krusei* isolates tested, showed that 3 demonstrated susceptibility at 3.125 mg/ml, while 1 demonstrated susceptibility at 6.25 mg / ml and another at 1.562 mg /ml. Of the 11 *C. glabrata* isolates tasted, 8 demonstrated susceptibility at 3.125 mg/ml, 2 demonstrated susceptibility at 0.781 mg /ml, and 1 demonstrated activity at 1.562 mg /ml. One each of *C. albicans* and *C. guilliermondii* demonstrated susceptibility at 3.125 mg/ml and 25 mg/ml respectively (Table 4.4).

Total extracts of glucosinolate fraction against of the 5 *C. krusei* isolates tested showed that 3 demonstrated susceptibility at 3.125 mg/ml, while 1 demonstrated susceptibility at 12. 5 mg / ml and another at 25 mg /ml. Of the 11 *C. glabrata* isolates tasted, 8 demonstrated susceptibility at 3.125 mg/ml, 3 demonstrated susceptibility at 3 concentrations namely 1.562 mg /ml 6.25 mg /ml and 12.5 mg /ml respectively. Additionally, one each of *C. albicans* and *C. guilliermondii* demonstrated susceptibility at 3.125 mg/ml and 6.25 mg/ml respectively (Table 4.4).

MIC_{90} and MIC_{50} of juglone against *C. krusei* were observed as 0.781 µg/ml equally demonstrating that this treatment was quite effective MIC ranges of 5 isolates of *C. krusei* were 0.39 - 0.781 µg/ml within an average 0.63 µg/ml (Table 4.2). On the other hand, *C. glabrata* MIC_{90} was 12.5 µg/ml, and half of it (6.25 µg/ml) was its $MIC_{50.}$ MIC ranges of 11 isolates of *C. glabrata* 1.56 - 12.5 µg/ml with an average of 8.10 µg/ml (Table 4.4).

Figure 4.10: Screening of total extraction of Miswak

DISCUSSION

4.1. Phytochemical activity of *Euclea natalensis* extracts and *Juglone*

Compounds were subjected to an evaluation of their level of activity using the broth microdilution method to estimate the minimal inhibitory concentration (MIC) (CLSI 2008; PA, 2009). From the results, it can be observed that hexane fraction was found to be very efficient against *Candida* species resistant to fluconazole (*C. glabrata, C. krusei, C. albicans, and C. guilliermondii*). The compound, *7-methyljuglone* seemingly the main active component of the plant extraction, demonstrated promising antifungal effect, being more powerful than fluconazole. MIC values of compounds 7-*methyljuglone* against eleven isolates of *C. glabrata* and 5 isolates of *C. krusei* ranged from 1.56 – 25 μg/ml and 0.39 - 1.56 μg/ml, respectively. While a similar chemical structure compound, *Juglone* was also very active against these *Candida* species, comparable to that of fluconazole. Its MIC values for *C. glabrata* and *C. krusei* were within range of 1.56 - 12.5 μg/ml and 0.39 - 0.781 μg/ml, respectively. It is the first time that this activity has been shown with these compounds against *Candida* isolates resistant to fluconazole.

The first report of the anti-mycobacterial activity of 7-methyljuglone *neodiospyrin*, *mamegakinone* and *isodiospyrin* of the *Euclea natalensis* plant was published in 2006. The MIC values of *7-methyljuglone* (0.5 μg/ml, diospyrin (8.0 μg/ml), isodiospyrin (10.0 μg/ml),) and neodiospyrin (10.0 μg/ml) compared well to those of the known anti-mycobacterial drugs, isoniazid, ethambutol and rifampicin (Van Der Kooy *et al.*, 2006).

Bapela *et al.* (2008) reported anti-tuberculosis activity of 7-methyljuglone compound at concentrations ranging from 0 g/kg to 3.77 g/kg (Bapela *et al.*, 2008). Rauf *et al.* (2017)

demonstrated the use of 7-methyljuglone as sedative in traditional remedy and recommended it for new and innovative pharmaceutical and therapeutic applications (Rauf *et al.*, 2017). In Pakistan, 7-methyljuglone compound (which was extracted from Diospyros lotus) was identified as a useful new therapeutic agent with anti-hyperalgesia activity (Rauf *et al.*, 2015), and later reported as also being traditionally used in the treatment of infectious diseases (Rauf *et al.*, 2016). This compound, 7-methyljuglone (which was extracted from polygonum cuspidatum), also showed powerful antibacterial activity against *Helicobacter pylori* with MIC50 value of 0.30 µM and MIC90 value of 0.39 µM (Khalil *et al.*, 2016).

A similar study was conducted by (Bapela *et al.*, 2008) to investigate the anti-mycobacterial effect of 7- methyljuglone on mycobacteria species ranging between 0.78 mg/ml to 10 µg/ml . However, the MIC ranges of 7- methyljuglone against mycobacteria were higher than the MIC values obtained against *Candida* species in this study.

The results of these natural products' compounds showed high activity of 7-methyljuglolne with MIC's ranging between 25 µg/ml – 0.390 µg/ml. the *Juglone* activity effect was almost similar with MIC's ranging between 12.5 µg/ml – 0.390 µg/ml, which might be due to the similar chemical structure, the only difference being the methyl group location in their chemical structures.

A phytochemistry study done in Spain (Sánchez-Calvo *et al.*, 2016) used 1,4-naphthoquinone (Juglone) as an antifungal agent against *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019, with an MIC of 2 µg/mL for both species.

4.2. The biological activities of Miswak (*Salvadora persica*) against *Candida* isolates resistant to fluconazole

S. persica, is active against several types of cariogenic bacteria frequently found in the human oral cavity (Ababneh, 1995, Almas, 1999). Additionally, numerous *in vitro* studies have showed that total extraction of *S. persica* constitutes substances that possess dental plaque inhibiting properties against oral microbes (Almas, 1999, Abdelrahman and Skaug, 2002, Bonjar, 2004). Almas (1999) showed amazing antimicrobial effects of Miswak on *Streptococcuss mutance* and *S. fecalis,* which might result in dental plaque formation (Almas, 1999). Furthermore, *S. persica* has positive therapeutic properties, and also has the potential to be an effective adaptogenic herbal therapy (Ahmad and Rajagopal, 2013).

Ababneh (1995) found that the derivatives of *S. persica* used in three different laboratory methods, demonstrated strong antibacterial effects on the growth of *Streptococcus sp*. and *Streptococcus aureus* (Ababneh, 1995). Another study revealed that *Enterococcus faecalis* is susceptible to *S. persica*, with no significant difference in the antimicrobial effects of freshly cut and 1-month-old miswak (Almas *et al.*, 1997). A comparison of alcoholic and aqueous extracts of *S. persica* miswak showed that the alcoholic extract had more potential antimicrobial activity than did the aqueous extract (Almas *et al.*, 1997).

Mirkamandar *et al*., (2012) measured an *in vitro* antimicrobial activity of a metabolic extract of *S. persica* solution on *Helicobacter pylori* isolated from a duodenal ulcer. The results showed that at a concentration of 750 µg / ml of *S. persica* allowed for a few *H. pylori* cells to form colonies on Brucella agar supplemented with sheep blood cells and antibiotics, and thus concluded that higher concentrations of *S. persica* would be needed to inhibit the growth of *H. pylori* (Mirkamandar *et al.*, 2012).

Also, the anthelmintic activity of *S. persica* was studied where the alcoholic extract showed more significant effect on paralyzing earth worms, in terms of paralysis time, at every concentration to that of aqueous root extract when compared with standard Piperazine citrate at the same concentration (Majeed, 2011). He confirmed that the aqueous and alcoholic root extracts *S. persica* anthelmintic activity is dose dependent when compared with standard drugs, which are effective against parasitic infections of humans (Majeed, 2011).

Last year, the *in vitro* scolicidal effect of root extracts of *S. persica* plant against protoscolices from hydatid cysts of *Echinococcus granulosus*, was investigated using 10, 20, 30, and 50 mg/ml concentration for 10, 20, and 30 min and suggested that ethanolic extract of *S. persica* has high scolicidal power *in vitro* (Abdel-Baki *et al*., 2016).

To the best of our knowledge, so far, there was not antifungal activity of glucosinolate fraction from Miswak extracts tested or studied against *C. albicans* or non-*albicans Candida.* An *in vitro* study showed the antifungal activity of the aqueous extract of *S. persica* miswak where the inhibitory effect on the growth of *C. albicans* could be attributed to its high sulphate content (Al-Bagieh *et al.*, 1994).

In another study, only two *C. albicans* strains presented a high MIC value (3.125 mg/ml), with the author indicating that total extracts of *Salvadora persica* may comprise of compounds which might result in potential antifungal activity against *Candida* strains (Noumi *et al.*, 2010). Our study was the first study to report and characterise *Candida* isolates from T2DM patients in Libya and to screen *Candida* isolates for susceptibility. It is also the first to examine the use of natural compounds and fractions against azole resistant isolates.

The total number of resistant *Candida* isolates was 18 isolates namely *C. albicans* (1 isolate), *C. guilliermondii* (1 isolate), *C. krusei* (5 isolates), and *C. glabrata* (11 isolates).

The results of Al-Bagieh and Almas (1997) proposed that aqueous extracts of miswak can diminish the growth of *C. albicans* to 36 hours and at a concentration of 15%. Also, the continued use of *S. persica* has an effective positive change on salivary and subgingival plaque bacteria (Al-Bagieh and Almas, 1997, Al Sadhan and Almas, 1999, Okemo *et al.*, 2001, Al-Bayati and Sulaiman, 2008), while also inhibiting the growth of other different microorganisms (Okemo *et al.*, 2001, Wu *et al.*, 2001, Haberland-Carrodeguas *et al.*, 2002, Runyoro *et al.*, 2006, Chong *et al.*, 2007, Pereira *et al.*, 2007, Hajlaoui *et al.*, 2008). Our results showed total extracts of *Salvadora persica* and its fraction glucosinolate have reduced the growth of *Candida* species for up to 48 h.

A phytochemical analysis of *S. persica* revealed that the plant contained specific amounts of salvadorine; terpenes; trimethyamine, gypsum, organic compounds, such as pyrrolidine, pyrrole, and piperidine derivatives; chlorides; sulphur; vitamin C; carbohydrates; glycosides; large amounts of fluoride and silica; and trace amounts of tannins, saponins, flavonoids and sterols (Khatak *et al.*, 2010, Arora and Gupta, 2011), which may demonstrate antidiabetic activity.

Hence it can be concluded that *S. persica* extract has revealed a significant beneficial effect, including its ability to reduce blood glucose levels, total cholesterol, and triglyceride and increase high density lipoprotein (HDL) level (Ramadan and Alshamrani, 2015).

Our data showed that, MIC_{50} of total extracts of *Salvadora persica* and its fraction (*glucosinolate*) against *C. krusei* and *C. glabrata* were observed as 3.125 mg/ml equally thereby demonstrating that it was quite effective, while MIC_{90} total extracts of *Salvadora persica* against 5 isolates of *C. krusei* ranged between 1.56 - 6.25 mg/ml. Glucosinolate MIC_{90} of 5 isolates of *C. krusei* ranged

between 3.13 - 25.00 mg/ml within an average 9.38 mg/ml. On the other hand, *C. krusei* MIC_{90} using *glucosinolate* was quarter MIC_{90} using *Salvadora persica* total extracts of 6.25 mg/ml in same species, and *C. glabrata* MIC_{90} (3.125 mg/ml) was same as *Salvadora persica* total extracts as in its fraction. MIC ranges of 11 isolates of *C. glabrata* ranges 1.56 - 12.5 µg/ml within an average 8.10 µg/ml

S. persica plant and its fraction (glucosinolate) proposed beneficial effects on the oral antifungal therapy which could promote good oral hygiene. Therefore, *S. persica* extracts and its fraction could be advised for everyday use, due to their effects on oral health, costly reliable, and effortlessness of use.

To our knowledge, no study has described the use of natural products in treating *Candida* species resistant to fluconazole in Libya. The results described here will therefore make a good contribution to the literature when published.

CHAPTER 5

SUMMARY AND CONCLUSION

Candida are commensal organisms, not normally causing disease, but as opportunistic organisms, are a cause of concern in immunocompromised patients including those with T2DM, (Brown et al., 2006). (Belazi *et al.*, 2005, Khosravi *et al.*, 2008, Elhwuegi *et al.*, 2012).

Metformin is a commonly prescribed drug for treating T2DM (Bailey and Turner, 1996),targeting improved insulin signalling (Musi *et al.*, 2002) and inhibiting glucose uptake in the small intestine (Hundal and Inzucchi, 2003).

Both *in vitro* and *in vivo* studies related to metabolic syndrome pathophysiology and T2DM, suggest that metformin may down regulate inflammatory responses (Saisho, 2015). It may this be used as a positive control in order to investigate the potential of natural plant extracts on the modulation of metabolic syndrome and T2DM pathophysiology.

Candida infections can be both superficial and systemic. The treatment of diseases caused by this fungus is through the use of fungicides, which are sometimes problematic due to the potential toxicity to cells (Cao *et al.*, 2005, Williams *et al.*, 2011). In addition, bacterial infection has been shown to affect the secretion of insulin (Nikolić, 2011), thus establishing an association between the use of antibiotics and both diabetes and *Candida*. Each condition is thought to support the existence of the other. However, *Candida* can create diabetes on its own through direct and indirect means. Secreted Aspartyl Proteases (SAPs) are a recognised virulence mechanism of *Candida* and may destroy tissues and obtain nutrients in the body leading to increased blood sugar levels. Sugars are an excellent source of fuel that can rapidly increase the growth of *Candida* (Nikolic). The

incidence and prevalence of *Candida* carriage in diabetic patients have dramatically raised with the increased use of antibiotics and thus the link between *Candida*, diabetes, and antibiotics is become a reality (Khosravi et al., 2008). Of even greater concern is the emergence of antifungal drug resistance in DM patients. Fluconazole is the most common antifungal drug available in African countries and the emerging resistance renders it ineffective against *Candida* infections.

The objectives of this study were to:

i. Investigate *Candida* species prevalence in Libyan T2DM patients.
ii. examine the *Candida* isolates for antifungal susceptibility
iii. investigate the antifungal activity of natural products from plants such as *S. persica* and *E. natalensis* against fluconazole resistant *Candida* species.

As demonstrated in the proceeding chapters, these objectives have been achieved.

This is the first comprehensive study of the prevalence and antifungal profiles of *Candida* species from Libyan T2DM patients against whom the incidence rate is considered to be quite high, with one in five Libyans having T2DM *Candida* carriage was observed in 170 of 330 patients examined, with a total of 182 isolates.

The study examined the prevalence of *Candida* according to age, gender, weight, height, BMI, and whether patients were smokers or denture wearers. Methods used for the detection of *Candida* included chromogenic media, API ID 32C biochemical tests, Vitek 2 compact system, and phenotype microarrays. Nine type strains were included as controls to ensure the accuracy of the detection and identification methods used.

The predominant species were *C. albicans* followed by *C. dubliniensis, C. glabrata, C. krusei, C. tropicalis, C. kefyr, C. parapsilosis. Candida* carriage was significantly associated with gender (p

= 0.02), denture wearing (p = 0.020) and smoking (p = 0.047). No significant association was observed with age or BMI even though *Candida* prevalence increased with increasing obesity classification (i.e. class I-III).

Most of the *Candida* isolates showed antifungal susceptibility (63%) with 17% showing intermediate resistance and 20 % demonstrating total resistance.

Using Vitek 2 compact system, susceptibility to 6 antifungal drugs was tested and these included amphotericin B, caspofungin, micafungin, fluconazole, voriconazole and flucytosine. *Candida* demonstrating resistance and intermediate resistance were used to evaluate the antifungal activity of *S. persica* and *E. natalensis* which have previously demonstrated antibacterial activity. To ensure that there were antifungal activity 15 susceptible strains were included as controls.

Fractionation of selected bioactive extracts from both plants in this study was done using state of the art techniques such as TLC, HLPC and NMR for the biological evaluation of total extracts and fractions against different strains of *Candida in vitro*

Medicinal plants have been shown to have promising antifungal biological activities. Yet, currently there has been little attention in developing these medicinal plants as alternative medicine against communicable diseases.

In this study, the *E. natalensis* extraction was found to be very effective against fluconazole resistant *Candida* species with 7-methyljuglone demonstrating a better antifungal effect than fluconazole.

Juglone, a commercial compound, demonstrated antifungal activity comparable to fluconazole. This has not previously been reported. The antifungal effect of *S. persica* (in aqueous extract) has

previously been demonstrated (Al-Bagieh *et al.*, 1994), but to our knowledge, this is the first study to demonstrate the antifungal activity of the glucosinolate fraction from *S. persica* extracts.

Further studies on the prevalence and drug sensitivity of *Candida* species in T2DM subjects across North African countries is needed, in order to improve patient care. As suggested by Mbaveng and Kuete (2014) studies such as this can serve as the starting point for evaluating the phytochemical activity of 7-methyljuglone with a view to drug development to address the emerging fluconazole resistance of *Candida.*

Future studies include molecular studies of possible mechanisms of drug resistance which could include restricted penetration of drugs through the *Candida* colonies, phenotypic changes, and the expression of resistance genes

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Appendix 1A

CONSENT FORM FOR PARTICIPATION IN RESEARCH PROJECT

Title of Project: The antifungal effect of *Salvadora persica* and *Euclea natalensis* on *Candida* isolates from Libyan patients with type II Diabetes mellitus.

Names of Researchers: Mustafa H. Esmaio, Dr Ahmed Mohammed, Prof Charlene WJ Africa

If you would like to participate in this study, please tick the relevant boxes:

1. Have you read the attached information sheet and has the purpose of the research project been explained to you?
2. Do you understand the method of sample collection and any risks involved?
3. Do you grant permission for information from your medical records to be disclosed to the research team as and when necessary?

YES	NO
YES	NO
YES	NO

4. Do you agree that samples collected for research or diagnostic testing can be stored for possible use in future research projects conducted by the above-named researchers and /or other research collaborators?

YES	NO

I declare that my participation in this research project is voluntary and that I am free to withdraw my approval for use of the sample(s) at any time without giving a reason and without my medical treatment or legal rights being affected. I understand that any information contained in my file will remain confidential and that I (or my doctor) will be informed if any of the results of the medical tests done (as part of the research) have implications for my health. I know how to contact members of the research team should I change my mind about participating in this study.

…….	…….	…….
Name of patient	**Date**	**Signature**
(BLOCK CAPITALS)		
……..	**……**	**……**
Name of person taking consent	**Date**	**Signature**
……	**……**	**……**
Name of researcher	**Date**	**Signature**

THANK YOU FOR AGREEING TO PARTICIPATE IN THIS RESEARCH

Appendix 1B

INFORMATION SHEET

Prospective participants are requested to read this information sheet carefully and to ask questions where necessary, before signing the attached consent form. This sheet must be detached and retained by the participant and the consent form filed for record.

Candida is a yeast found as part of the normal flora of the gut and mouth of some individuals. Normally, it does not cause a problem but in the case of persons with Diabetes Mellitus (DM), due to the emerging resistance of *Candida* to antifungal drugs, alternatives need to be found. This study will investigate the use of *Salvadora persica* (miswak) and *Euclea natalensis* as alternatives to the use of azoles for the treatment of resistant oral *Candida* species in DM patients.

The clinical procedure will entail the collection of samples from the mouth using cotton swabs. The sample collection procedure is non-invasive and safe and will be carried out with the utmost care to ensure the comfort of the patient.

Patients will be required to sign the attached form granting consent for the collection of swab samples and for the use of the samples donated and clinical parameters recorded. The patient will also be required to grant permission for his/her MD status and other medical history to be disclosed if necessary. Participants will not be recorded by name, but samples and information will be coded to protect the identity of the individual. However, the coding will be used by the clinic to trace the individual if relevant information (as a result of the study) should be passed to him/her or his/her doctor. Permission will also be sought for the use of additional biological material collected in the clinic, which is usually discarded but which the researchers may find useful for future research.

Participation in this study is voluntary and refusal to participate will not prejudice the treatment of the patient in any way. Consent to participate will be recorded by completing the attached form. Should individuals agree to participate and later change their minds, they may withdraw by calling the following persons:
Prof C. Africa, University of the Western Cape, Department of Medical Biosciences, Tel: 021 9592341, or Dr Ahmed Mohammed, University of the Western Cape, cell phone: 0860751751.

Appendix 2

University of the Western Cape

Department of Medical Biosciences

Patient Questionnaire

Patient number: ☐ **Date:** …../……/……..

Please tick the appropriate box.

Gender M ☐ F ☐

Marital Status ☐

Occupation ☐

Partner's gender M ☐ F ☐

Age: ☐

Weight: ☐

Height ☐

Calculated BMI ☐

Race: Black ☐ Coloured ☐ White ☐ Other ☐

Dentures wearing ☐ non-wearing ☐

Duration of wearing Dentures ☐

Date diagnosed with diabetes:

Do you smoke? **Yes** ☐ **No** ☐

If yes, how many cigarettes do you smoke a day? ☐

Clinical presentation: clinical candidiasis ☐ sub-clinical oral candidiasis. ☐

Are you currently receiving treatment for candidiasis? Yes ☐ No ☐

If yes, what is the duration of treatment? ☐

If no, have you received treatment for candidiasis in the past 2 months?

Name the antifungal used. ☐

Person assisting with the questionnaire:

Location of Diabetes clinic:

Appendix 3

Tables 2 show the results obtained using Sabouraud's agar, which allows for *Candida* growth on Fluka and Oxoid chromogenic agar, identification using API ID 32 C and confirmed by Vitek 2 compact system

Table 2: Results obtained from the different selective media as well as identification using API ID 32 C and Vitek 2 compact system**.**

Patient No.	Sabouraud agar	Fluka chromogenic agar	Oxoiod chromogenic agar	ID API 32C	VIETK ID
1	*Candida*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*
3	*Candida*	*C.albicans*	*C.albicans*	*C. dubliniesis*	*C. dubliniesis*
5	*Candida*	*C.unidentified*	*C.unidentified*	*C. sake*	N/A
8	*Candida*	*C.albicans*	*C.albicans*	*C. sake*	N/A
9	*Candida*	*C.dubliniesis*	*C.tropicalis*	*C. dubliniesis*	*C. dubliniesis*
11	*Candida*	*C.unidentified*	*C.unidentified*	*C. humicola*	*Stephanoascus ciferrii*
13	*Candida*	*C.albicans*	*C.albicans*	*C. dubliniesis*	*C. dubliniesis*
14	*Candida*	*C.albicans*	*C.albicans*	*C. albicans*	*C.albicans*
15	*Candida*	*C.tropicalis*	*C.dubliniesis*	*C. dubliniesis*	*C. dubliniesis*
16	*Candida*	*C.albicans*	*C.dubliniesis*	*C. dubliniesis*	*C.dubliniesis*
17A	*Candida*	*C.unidentified*	*C.kefyr*	*C.kefyr*	*C.kefyr*
17B	*Candida*	*C.albicans*	*C.albicans*	*C. humicola*	*Stephanoascus ciferrii*
18	*Candida*	*C.unidentified*	*C.unidentified*	*C. humicola*	*Stephanoascus ciferrii*
20A	*Candida*	*C.albicans*	*C.albicans*	*C. humicola*	*Stephanoascus ciferrii*
20B	*Candida*	*C.unidentified*	*C.krusei*	*C.krusei*	*C.krusei*
22	*Candida*	*C.albicans*	*C.albicans*	*C. humicola*	*Stephanoascus ciferrii*
23	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
24A	*Candida*	*C.albicans*	*C.albicans*	*C.membranifaciens*	N/A
24B	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
25	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
28	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
29A	*Candida*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*
29B	*Candida*	*C.albicans*	*C.albicans*	*C. humicola*	*Stephanoascus ciferrii*
32	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*

33	*Candida*	*C.albicans*	*C.albicans*	*C. humicola*	*Stephanoascus ciferrii*
37	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
39B	*Candida*	*C.unidentified*	*C.glabrata*	*C.glabrata*	*C.glabrata*
40A	*Candida*	*C.unidentified*	*C.unidentified*	*C. humicola*	*Stephanoascus ciferrii*
40B	*Candida*	*C.unidentified*	*C.unidentified*	*C.tropicalis*	*C.tropicalis*
41	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
42	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
43	*Candida*	*C.unidentified*	*C.unidentified*	*Saprochaete capitata*	*Saprochaete capitata*
49	*Candida*	*C.unidentified*	*C.unidentified*	*Saprochaete capitata*	*Saprochaete capitata*
52A	*Candida*	*C.unidentified*	*C.glabrata*	*C.glabrata*	*C.glabrata*
52B	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
57	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
59A	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
59B	*Candida*	*C.albicans*	*C.albicans*	*C.globosa*	N/A
60	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
61	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
63	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
64	*Candida*	*C.krusei*	*C.krusei*	*C.krusei*	*C.krusei 97%*
65	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
66	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
67	*Candida*	*C.dubliniesis*	*C.tropicalis*	*C.dubliniesis*	*C.dubliniesis*
69A	*Candida*	*C.unidentified*	*C.unidentified*	*Saprochaete capitata*	*Saprochaete capitata*
69B	*Candida*	*C.unidentified*	*C.unidentified*	*C.albicans*	*C.albicans*
71	*Candida*	*C.unidentified*	*C.glabrata*	*C.glabrata*	*C.glabrata*
73	*Candida*	*C.unidentified*	*C.glabrata*	*C.glabrata*	*C.glabrata*
74A	*Candida*	*C.glabrata*	*C.glabrata*	*C.parapsilosis*	*C.parapsilosis*
74B	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
76	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
77	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
78	*Candida*	*C.others*	*C.glabrata*	*C.glabrata*	*C.glabrata*
80	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
81	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
82	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
83	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
85	*Candida*	*C.albicans*	*C.albicans*	*C. humicola*	*Stephanoascus ciferrii*
87	*Candida*	*C.albicans*	*C.albicans*	*C.humicola*	*Stephanoascus ciferrii*
88	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*

91	*Candida*	*C.albicans*	*C.albicans*	*C. humicola*	*Stephanoascus ciferrii*
92	*Candida*	*C.unidentified*	*C.glabrata*	*C.glabrata*	*C.glabrata*
95	*Candida*	*C.albicans*	*C.albicans*	*C.humicola*	*Stephanoascus ciferrii*
97	*Candida*	*C.dubliniesis*	*C.albicans*	*C.albicans*	*C.albicans*
98	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
99	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
100	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
101	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
103	*Candida*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*
106	*Candida*	*C.unidentified*	*C.unidentified*	*C. humicola*	*Stephanoascus ciferrii*
109A	*Candida*	*C.unidentified*	*C.unidentified*	*C.kefyr*	*C.kefyr*
109B	*Candida*	*C.dubliniesis*	*C.albicans*	*C. humicola*	*Stephanoascus ciferrii*
112	*Candida*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*
113	*Candida*	*C.unidentified*	*C.unidentified*	*C.guilliermondii*	*C.guilliermondii*
116	*Candida*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*
120	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
121	*Candida*	*C.tropicalis*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*
123	*Candida*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*
125	*Candida*	*C.unidentified*	*C.unidentified*	*C. humicola*	*Stephanoascus ciferrii*
126	*Candida*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*
129	*Candida*	*C.unidentified*	*C.tropicalis*	*C.tropicalis*	*C.tropicalis*
131	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
132	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
134	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
135	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
137A	*Candida*	*C.albicans*	*C.albicans*	*C.humicola*	*Stephanoascus ciferrii*
137B	*Candida*	*C.unidentified*	*C.unidentified*	*Kloeckera japonica*	N/A
138	*Candida*	*C.parapsilosis*	*C.parapsilosis*	*C.parapsilosis*	*C.parapsilosis*
139A	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
139B	*Candida*	*C.albicans*	*C.albicans*	*Trichosporon mucoides*	N/A
140	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
141	*Candida*	*C.albicans*	*C.albicans*	*C. humicola*	*Stephanoascus ciferrii88%*
142A	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
142B	*Candida*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*
143	*Candida*	*C.dubliniesis*	*C.dubliniesis*	*C.albicans*	*C.albicans*
144	*Candida*	*C.glabrata*	*C.glabrata*	*C.humicola*	*Stephanoascus ciferrii*

145	*Candida*	*C.lusitaneae*	*C.krusei*	*C.krusei*	*C.krusei*
146A	*Candida*	*C.albicans*	*C.albicans*	*C. humicola*	*Stephanoascus ciferrii*
146B	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
149	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
150	*Candida*	*C.dubliniesis*	*C.albicans*	*C. humicola*	*Stephanoascus ciferrii*
150A	*Candida*	*C.unidentified*	*C.unidentified*	*C.magnoliae*	*C.magnoliae*
151A	*Candida*	*C.albicans*	*C.albicans*	*C.magnoliae*	*C.magnoliae*
151B	*Candida*	*C.unidentified*	*C.unidentified*	*C.kefyr*	*C.kefyr*
152	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
154	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
156	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
158	*Candida*	*C.dubliniesis*	*C.dubliniesis*	*Cryptococcus humicola*	*Stephanoascus ciferrii*
159	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
162	*Candida*	*C.albicans*	*C.albicans*	*Kloeckera apis/apiculata*	*Kloeckera apis/apiculata*
163	*Candida*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*
164	*Candida*	*C.unidentified*	*C.unidentified*	*C. tropicalis*	*C. tropicalis*
166	*Candida*	*C.dubliniesis*	*C.dubliniesis*	*C.albicans*	*C.albicans*
167	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
168	*Candida*	*C.albicans*	*C.albicans*	*Cryptococcus humicola*	*Stephanoascus ciferrii*
171	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
172	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
177	*Candida*	*C.unidentified*	*C.kefyr*	*C.kefyr*	*C.kefyr*
179	*Candida*	*C.unidentified*	*C.unidentified*	*Kloeckera apis/apiculata*	*Kloeckera apis/apiculata*
181	*Candida*	*C.dubliniesis*	*C.albicans*	*C.dubliniesis*	*C.duliniesis*
184	*Candida*	*C.dubliniesis*	*C.albicans*	*C.albicans*	*C.albicans*
186	*Candida*	*C.others*	*C.glabrata*	*C.glabrata*	*C.glabrata*
187	*Candida*	*C.lusitaneae*	*C.krusei*	*C.krusei*	*C.krusei*
188	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
192	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
194	*Candida*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*
200	*Candida*	*C.dubliniesis*	*C.dubliniesis*	*C.albicans*	*C.albicans*
201	*Candida*	*C.unidentified*	*C.tropicalis*	*C.tropicalis*	*C.tropicalis*
204	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
205	*Candida*	*C.albicans*	*C.albicans*	*C.sake*	*Stephanoascus ciferrii*
209	*Candida*	*C.albicans*	*C.albicans*	*C. humicola*	*Stephanoascus ciferrii*
213	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
214	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*

217	*Candida*	*C.albicans*	*C.albicans*	*C. humicola*	*Stephanoascus ciferrii*
235	*Candida*	*C.unidentified*	*C.unidentified*	*C.glabrata*	*C.glabrata*
236	*Candida*	*C.unidentified*	*C.glabrata*	*C.glabrata*	*C.glabrata*
238	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
242	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
243	*Candida*	*C.unidentified*	*C.unidentified*	*C.sake*	*N/A*
245	*Candida*	*C.albicans*	*C.albicans*	*C. humicola*	*C.albicans*
246	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
247	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
249	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
254	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
258	*Candida*	*C.unidentified*	*C.glabrata*	*C.glabrata*	*C.glabrata*
259	*Candida*	*C.unidentified*	*C.glabrata*	*C.glabrata*	*C.glabrata*
263	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
264	*Candida*	*C.dubliniesis*	*C.dubliniesis*	*C.albicans*	*C.albicans*
266	*Candida*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*
267	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
268	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
271	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
272	*Candida*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*
274	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
275	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
279	*Candida*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*	*C.dubliniesis*
281	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
282	*Candida*	*C.albicans*	*C.albicans*	*C. humicola*	*Cryptococcus laurentii*
283A	*Candida*	*C.tropicalis*	*C.tropicalis*	*C.tropicalis*	*C.tropicalis*
283B	*Candida*	*C.unidentified*	*C.unidentified*	*Saprochaete capitata*	*Saprochaete capitata*
285	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
287	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
288	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
289	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
290	*Candida*	*C.unidentified*	*C.unidentified*	*C.glabrata*	*C.glabrata*
291	*Candida*	*C.others*	*C.glabrata*	*C.glabrata*	*C.glabrata*
292	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
293	*Candida*	*C.unidentified*	*C.glabrata*	*C.glabrata*	*C.glabrata*
297	*Candida*	*C.unidentified*	*C.unidentified*	*C.glabrata*	*C.glabrata*
298	*Candida*	*C.unidentified*	*C.unidentified*	*C.glabrata*	*C.glabrata*
301	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
302	*Candida*	*C.glabrata*	*C.glabrata*	*C.glabrata*	*C.glabrata*
303	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*

304	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
306	*Candida*	*C.dubliniesis*	*C.tropicalis*	*C.dubliniesis*	*C.dubliniesis*
308	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
309	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
310	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
312	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
314	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
315	*Candida*	*C.krusei*	*C.krusei*	*C.krusei*	*C.krusei*
316	*Candida*	*C.unidentified*	*C.unidentified*	*C.glabrata*	*C.glabrata*
318	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
319	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
322	*Candida*	*C.unidentified*	*C.glabrata*	*C.glabrata*	*C.glabrata*
323	*Candida*	*C.unidentified*	*C.glabrata*	*C.glabrata*	*C.glabrata*
325	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
329	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
330	*Candida*	*C.albicans*	*C.albicans*	*C.albicans*	*C.albicans*
332	*Candida*	*C.albicans*	*C.albicans*	*C.dubliniesis*	*C.dubliniesis*
333	*Candida*	*C.kefyr*	*C.kefyr*	*C.kefyr*	*C.kefyr*

Appendix 4

Table 3 shows the cumulative results obtained from T2DM Libyan patients. Data from the questionnaire and patient's folder are included.

Table 3: Cumulative results from all T2DM patients.

Patient No.	Gender	Marital Status	Occupation	Partner's gender	Age	Weight	Height	Calculated BMI	International classification (Who)	Denture wearing	Duration of wearing dentures	Date diagnosed	smoking	clinical presentation	Antifungal treatment	*Candida* Carriage
1	Female	Married	House Wife	Male	60	66	150	29.33	Pre-obese	Non	N/A	20	No	Sub-Clinical Candidiasis	No	Positive
2	Female	Married	House Wife	Male	60	97	165	35.63	Obese class II	Non	N/A	8	No	Sub-Clinical Candidiasis	No	Negative
3	Female	Divorsed	House Wife	No Partner	60	77	147	35.63	Obese class II	Non	N/A	16	No	Sub-Clinical Candidiasis	No	Positive
4	Female	Married	House Wife	Male	50	96	160	37.5	Obese class II	Non	N/A	25	No	Sub-Clinical Candidiasis	No	Negative
5	Female	Married	House Wife	Male	70	54	131	31.47	Obese class I	Non	N/A	50	No	Clinical Candidiasis	No	Positive
6	Female	Married	House Wife	Male	58	70	151	30.7	Obese class I	Non	N/A	10	Yes	Sub-Clinical Candidiasis	Yes	Negative
7	Female	Married	House Wife	Male	60	77	150	34.22	Obese class I	Non	N/A	22	No	Sub-Clinical Candidiasis	No	Negative
8	Female	Married	House Wife	Male	95	65	143	31.79	Obese class I	Non	N/A	5	No	Sub-Clinical Candidiasis	No	Positive
9	Female	Married	House Wife	Male	55	95	163	36.89	Obese class II	Non	N/A	14	No	Sub-Clinical Candidiasis	No	Positive
10	Female	Married	House Wife	Male	55	115	152	49.77	Obese class III	Yes	15	23	No	Sub-Clinical Candidiasis	No	Negative
11	Female	Married	House Wife	Male	59	72	146	33.78	Obese class I	Yes	16	7	No	Sub-Clinical Candidiasis	No	Positive
12	Female	Married	House Wife	Male	62	114	165	41.87	Obese class III	Non	N/A	16	No	Sub-Clinical Candidiasis	No	Negative
13	Female	Married	House Wife	Male	60	108	166	39.19	Obese class II	Non	N/A	6	No	Sub-Clinical Candidiasis	No	Positive
14	Female	Married	House Wife	Male	68	82	152	35.49	Obese class III	Non	N/A	13	No	Sub-Clinical Candidiasis	No	Positive
15	Female	Married	House Wife	Male	66	66	147	30.54	Obese class I	Non	N/A	2	No	Sub-Clinical Candidiasis	No	Positive
16	Female	Married	House Wife	Male	62	96	154	40.48	Obese class III	Non	N/A	9	No	Sub-Clinical Candidiasis	No	Positive
17	Female	Married	House Wife	Male	65	73	164	27.14	Pre-obese	Non	N/A	20	No	Sub-Clinical Candidiasis	No	Positive
18	Female	Married	House Wife	Male	70	83	147	38.41	Obese classIII	Yes	15	12	No	Clinical Candidiasis	No	Positive
19	Female	Married	House Wife	Male	65	81	146	38	Obese classIII	Non	N/A	26	No	Clinical Candidiasis	No	Negative
20	Female	Married	House Wife	Male	75	82	154	34.58	Obese class I	Non	N/A	15	No	Clinical Candidiasis	Yes	Positive
21	Female	Married	House Wife	Male	65	85	163	31.99	Obese class I	Yes	N/A	5	No	Sub-Clinical Candidiasis	No	Negative
22	Female	Married	House Wife	Male	70	83	151	36.4	Obese class II	Non	N/A	33	No	Sub-Clinical Candidiasis	No	Positive
23	Female	Married	House Wife	Male	55	50	140	25.51	Pre-obese	Non	N/A	4	No	Sub-Clinical Candidiasis	No	Positive
24	Male	Married	Business Man	Female	66	96	170	33.22	Obese class I	Yes	N/A	15	No	Sub-Clinical Candidiasis	No	Positive
25	Male	Married	Business Man	Female	61	105	169	36.76	Obese class II	Yes	14	16	No	Sub-Clinical Candidiasis	No	Positive
26	Male	Married	Employer	Female	61	72	172	23.66	Normal range	Yes	N/A	6	Yes	Sub-Clinical Candidiasis	No	Negative

27	Male	Married	Employer	Female	49	94	184	27.76	Pre-obese	Non	N/A	13	Yes	Sub-Clinical Candidiasis	No	Negative
28	Male	Married	Business Man	Female	71	103	167	36.93	Obese class II	Non	N/A	30	Yes	Sub-Clinical Candidiasis	No	Positive
29	Male	Married	Teacher	Female	62	83	182	25.06	Pre-obese	Non	N/A	10	No	Sub-Clinical Candidiasis	Yes	Positive
30	Male	Married	Teacher	Female	51	105	170	36.33	Obese class II	Non	N/A	12	No	Sub-Clinical Candidiasis	No	Negative
31	Male	Married	Military	Female	56	80	174	26.42	Pre-obese	Non	N/A	10	Yes	Sub-Clinical Candidiasis	No	Negative
32	Male	Married	Employer	Female	45	65	177	20.75	Normal range	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Positive
33	Male	Married	Business Man	Female	51	107	168	37.91	Obese class II	Non	N/A	26	Yes	Sub-Clinical Candidiasis	No	Positive
34	Male	Married	Business Man	Female	60	85	186	24.57	Normal range	Non	N/A	30	No	Sub-Clinical Candidiasis	No	Negative
35	Male	Married	Employer	Female	60	110	176	35.51	Obese class II	Non	N/A	17	No	Sub-Clinical Candidiasis	No	Negative
36	Male	Married	Business Man	Female	70	85	174	28.08	Pre-obese	Non	N/A	33	No	Sub-Clinical Candidiasis	Yes	Negative
37	Male	Married	Business Man	Female	76	83	169	29.06	Pre-obese	Yes	5	13	Yes	Sub-Clinical Candidiasis	No	Positive
38	Male	Married	Retired	Female	73	77	168	27.28	Pre-obese	Non	N/A	12	Yes	Sub-Clinical Candidiasis	No	Negative
39	Male	Married	Business Man	Female	75	93	172	31.44	Obese class I	Yes	10	27	Yes	Sub-Clinical Candidiasis	No	Positive
40	Male	Married	Employer	Female	70	71	160	27.73	Pre-obese	Yes	3	4	Yes	Clinical Candidiasis	No	Positive
41	Male	Single	Student	No Partner	18	70	172	23.66	Normal range	Non	N/A	Less 6 M	No	Sub-Clinical Candidiasis	No	Positive
42	Male	Married	Employer	Female	56	70	168	24.8	Normal range	Non	N/A	9	Yes	Sub-Clinical Candidiasis	No	Positive
43	Male	Married	Retired	Female	73	92	176	29.7	Pre-obese	Yes	10	20	Yes	Sub-Clinical Candidiasis	No	Positive
44	Male	Married	Retired	Female	85	70	163	26.35	Pre-obese	Non	N/A	N/A	Yes	Sub-Clinical Candidiasis	No	Negative
45	Male	Married	Business Man	Female	65	84	171	28.73	Pre-obese	Non	N/A	27	No	Sub-Clinical Candidiasis	No	Negative
46	Male	Married	Business Man	Female	67	90	160	35.16	Obese class II	Yes	7	9	Yes	Sub-Clinical Candidiasis	No	Negative
47	Male	Married	Truck Driver	Female	62	110	178	34.72	Obese class I	Non	N/A	30	No	Sub-Clinical Candidiasis	No	Negative
48	Male	Married	Business Man	Female	65	57	160	22.27	Normal range	Non	N/A	7	Yes	Sub-Clinical Candidiasis	No	Negative
49	Male	Married	Retired	Female	65	79	160	30.86	Obese class I	Yes	3	14	No	Sub-Clinical Candidiasis	No	Positive
50	Male	Married	Teacher	Female	51	66	160	25.78	Pre-obese	Non	N/A	6	No	Sub-Clinical Candidiasis	No	Negative

51	Male	Married	Business Man	Female	67	102	178	32.19	Obese class I	Non	N/A	18	Yes	Sub-Clinical Candidiasis	No	Negative
52	Male	Married	Employer	Female	54	86	170	29.76	Pre-obese	Yes	N/A	17	Yes	Clinical Candidiasis	No	Positive
53	Male	Married	Business Man	Female	70	85	164	31.6	Obese class I	Non	N/A	16	No	Sub-Clinical Candidiasis	No	Negative
54	Male	Married	Business Man	Female	68	81	167	29.04	Pre-obese	Yes	5	12	Yes	Sub-Clinical Candidiasis	No	Negative
55	Male	Married	Carpenter	Female	70	93	165	34.16	Obese class I	Non	N/A	4	No	Sub-Clinical Candidiasis	No	Negative
56	Male	Married	Soldier	Female	64	80	168	28.34	Obese class I	Non	N/A	20	Yes	Sub-Clinical Candidiasis	No	Negative
57	Female	Married	House Wife	Male	70	94	153	40.16	Obese class III	Yes	10	25	No	Clinical Candidiasis	No	Positive
58	Female	Married	House Wife	Male	60	125	164	46.48	Obese class III	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Negative
59	Female	Married	House Wife	Male	62	80	150	35.56	Obese class II	Yes	6	8	No	Sub-Clinical Candidiasis	No	Positive
60	Female	Married	House Wife	Male	75	75	153	32.04	Obese class I	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Positive
61	Female	Married	House Wife	Male	68	89	163	33.5	Obese class I	Non	N/A	19	No	Clinical Candidiasis	No	Positive
62	Female	Married	House Wife	Male	65	84	146	39.41	Obese class II	Non	N/A	30	No	Clinical Candidiasis	No	Negative
63	Female	Married	House Wife	Male	65	78	153	33.32	Obese class I	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Positive
64	Female	Married	House Wife	Male	60	66	147	30.54	Obese class I	Yes	5M	7M	No	Clinical Candidiasis	No	Positive
65	Female	Married	House Wife	Male	75	93	155	38.71	Obese class II	Non	N/A	2	No	Clinical Candidiasis	No	Positive
66	Female	Married	House Wife	Male	70	75	148	34.24	Obese class I	Yes	14	16	No	Clinical Candidiasis	No	Positive
67	Female	Married	House Wife	Male	67	77	156	31.64	Obese class I	Non	N/A	13	No	Clinical Candidiasis	No	Positive
68	Female	Married	House Wife	Male	70	92	154	38.79	Obese class II	Yes	2	6	No	Clinical Candidiasis	No	Negative
69	Female	Married	House Wife	Male	63	77	145	36.62	Obese class II	Yes	4	25	No	Sub-Clinical Candidiasis	No	Positive
70	Female	Married	House Wife	Male	60	84	158	33.65	Obese class I	Yes	2	17	No	Sub-Clinical Candidiasis	No	Negative
71	Female	Married	House Wife	Male	52	75	154	31.62	Obese class I	Yes	1	6	No	Sub-Clinical Candidiasis	No	Positive
72	Female	Married	House Wife	Male	65	92	149	41.44	Obese class III	Non	N/A	5	No	Sub-Clinical Candidiasis	No	Negative
73	Female	Married	House Wife	Male	56	97	159	38.37	Obese class II	Yes	8	15	No	Clinical Candidiasis	No	Positive
74	Female	Married	House Wife	Male	58	108	158	43.26	Obese class III	Non	N/A	25	No	Sub-Clinical Candidiasis	No	Positive

75	Female	Married	House Wife	Male	62	63	152	27.27	Pre-obese	Yes	2	8	No	Clinical Candidiasis	No	Negative
76	Female	Married	House Wife	Male	65	70	142	34.72	Obese class I	Yes	9	10	No	Sub-Clinical Candidiasis	No	Positive
77	Female	Married	House Wife	Male	67	77	152	33.33	Obese class I	Non	N/A	5	No	Sub-Clinical Candidiasis	No	Positive
78	Female	Married	House Wife	Male	65	65	154	27.41	Pre-obese	Yes	15	19	No	Sub-Clinical Candidiasis	No	Positive
79	Female	Married	House Wife	Male	68	75	161	28.93	Pre-obese	Yes	4	4	No	Sub-Clinical Candidiasis	No	Negative
80	Female	Married	House Wife	Male	60	70	167	25.1	Pre-obese	Non	N/A	20	No	Clinical Candidiasis	No	Positive
81	Female	Married	House Wife	Male	64	90	146	42.22	Obese class III	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Positive
82	Male	Married	Retired	Female	80	88	171	30.09	Obese class I	Non	N/A	17	No	Sub-Clinical Candidiasis	No	Positive
83	Male	Married	Employer	Female	72	67	164	24.91	Normal range	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Positive
84	Male	Married	Naval architect	Female	52	70	160	27.34	Pre-obese	Non	N/A	17	Yes	Sub-Clinical Candidiasis	No	Negative
85	Male	Married	Employer	Female	64	98	167	35.14	Obese class II	Non	N/A	9	No	Sub-Clinical Candidiasis	No	Positive
86	Male	Married	Business Man	Female	44	103	176	33.25	Obese class I	Non	N/A	30	Yes	Sub-Clinical Candidiasis	No	Negative
87	Male	Married	Retired	Female	80	83	163	31.24	Obese class I	Yes	2	12	No	Sub-Clinical Candidiasis	No	Positive
88	Male	Married	Employer	Female	55	89	179	27.78	Pre-obese	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Positive
89	Male	Married	Teacher	Female	53	74	164	27.51	Pre-obese	Non	N/A	2	No	Sub-Clinical Candidiasis	No	Negative
90	Male	Married	Taxi Driver	Female	72	101	165	37.1	Obese class II	Non	N/A	3	Yes	Sub-Clinical Candidiasis	No	Negative
91	Male	Married	Employer	Female	54	103	178	32.51	Obese class I	Non	N/A	8	Yes	Sub-Clinical Candidiasis	No	Positive
92	Male	Married	Employer	Female	65	98	176	31.64	Obese class I	Yes	2	11	No	Sub-Clinical Candidiasis	No	Positive
93	Male	Married	Employer	Female	60	70	177	22.34	Normal range	Non	N/A	25	No	Sub-Clinical Candidiasis	No	Negative
94	Male	Married	Employer	Female	53	92	182	27.77	Pre-obese	Non	N/A	16	No	Sub-Clinical Candidiasis	No	Negative
95	Male	Married	Teacher	Female	63	95	170	32.87	Obese class I	Non	N/A	15	Yes	Sub-Clinical Candidiasis	No	Positive
96	Male	Married	Employer	Female	52	88	167	31.55	Obese class I	Non	N/A	8	No	Sub-Clinical Candidiasis	No	Negative
97	Male	Married	Soldier	Female	60	64	171	21.89	Normal range	Non	N/A	11	Yes	Sub-Clinical Candidiasis	No	Positive
98	Male	Married	Employer	Female	47	85	160	33.2	Obese class I	Yes	3	16	No	Sub-Clinical Candidiasis	No	Positive

99	Male	Married	Retired	Female	65	59	158	23.63	Normal range	Non	N/A	3	No	Sub-Clinical Candidiasis	No	Positive
100	Male	Married	Truck Driver	Female	65	75	165	27.55	Pre-obese	Non	N/A	12	No	Sub-Clinical Candidiasis	No	Positive
101	Male	Married	Electrician	Female	49	105	110	86.78	Obese class III	Non	N/A	1	Yes	Sub-Clinical Candidiasis	No	Positive
102	Male	Married	Retired	Female	69	72	159	28.48	Obese class I	Yes	1	1	Yes	Sub-Clinical Candidiasis	No	Negative
103	Male	Married	Retired	Female	67	80	168	28.34	Pre-obese	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Negative
104	Male	Married	Employer	Female	57	95	172	32.11	Obese class I	Yes	10	12	Yes	Sub-Clinical Candidiasis	No	Negative
105	Male	Married	Employer	Female	80	81	175	26.45	Pre-obese	Non	N/A	27	Yes	Sub-Clinical Candidiasis	No	Negative
106	Male	Married	Employer	Female	65	71	174	23.45	Normal range	Non	N/A	9	Yes	Sub-Clinical Candidiasis	No	Positive
107	Male	Married	Business Man	Female	60	82	170	28.37	Pre-obese	Non	N/A	21	No	Sub-Clinical Candidiasis	No	Negative
108	Male	Married	Employer	Female	70	79	167	28.33	Pre-obese	Non	N/A	19	No	Sub-Clinical Candidiasis	No	Negative
109	Male	Married	Driver	Female	76	85	160	33.2	Obese class I	Non	N/A	27	No	Sub-Clinical Candidiasis	No	Positive
110	Male	Married	Employer	Female	65	80	162	30.48	Obese class I	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Negative
111	Male	Married	Employer	Female	60	79	165	29.02	Pre-obese	Yes	2	5	No	Sub-Clinical Candidiasis	No	Negative
112	Male	Married	Business Man	Female	57	71	160	27.73	Pre-obese	Non	N/A	2	Yes	Sub-Clinical Candidiasis	No	Positive
113	Male	Married	Business Man	Female	70	70	175	22.86	Normal range	Non	N/A	2	No	Sub-Clinical Candidiasis	No	Positive
114	Male	Married	Employer	Female	55	85	175	27.76	Pre-obese	Non	N/A	17	Yes	Sub-Clinical Candidiasis	No	Negative
115	Male	Married	Business Man	Female	65	81	167	29.04	Pre-obese	Non	N/A	23	No	Sub-Clinical Candidiasis	No	Negative
116	Male	Married	Employer	Female	60	100	170	34.6	Obese class I	Non	N/A	3	No	Sub-Clinical Candidiasis	No	Positive
117	Male	Married	Business Man	Female	54	90	176	29.05	Pre-obese	Non	N/A	14	No	Sub-Clinical Candidiasis	No	Negative
118	Male	Married	Business Man	Female	75	90	178	28.41	Pre-obese	Non	N/A	23	No	Sub-Clinical Candidiasis	No	Negative
119	Male	Married	Employer	Female	40	67	159	26.5	Pre-obese	Non	N/A	3	No	Sub-Clinical Candidiasis	No	Negative
120	Male	Married	Employer	Female	42	87	175	28.41	Pre-obese	Non	N/A	3	Yes	Sub-Clinical Candidiasis	No	Positive
121	Male	Married	Retired	Female	60	120	176	28.41	Pre-obese	Non	N/A	6	Yes	Sub-Clinical Candidiasis	No	Positive
122	Male	Married	Employer	Female	52	85	171	29.07	Pre-obese	Yes	10	1	No	Sub-Clinical Candidiasis	No	Negative

123	Male	Married	Employer	Female	58	79	169	27.66	Pre-obese	Non	N/A	12	No	Sub-Clinical Candidiasis	No	Positive
124	Male	Married	Business Man	Female	59	111	176	35.83	Obese class II	Non	N/A	1	No	Sub-Clinical Candidiasis	No	Negative
125	Male	Married	Employer	Female	61	95	172	32.11	Obese class I	Yes	1	3	Yes	Sub-Clinical Candidiasis	No	Positive
126	Male	Married	Employer	Female	60	105	165	38.57	Obese class II	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Positive
127	Male	Married	Police Officer	Female	46	96	172	32.45	Obese class I	Non	N/A	2	No	Sub-Clinical Candidiasis	No	Negative
128	Male	Married	Employer	Female	51	66	176	21.31	Normal range	Yes	20	6M	Yes	Sub-Clinical Candidiasis	No	Negative
129	Male	Married	Employer	Female	77	120	170	41.52	Obese class III	Yes	5M	15	No	Sub-Clinical Candidiasis	No	Positive
130	Male	Married	Employer	Female	90	98	168	34.68	Obese class I	Non	N/A	32	No	Sub-Clinical Candidiasis	No	Negative
131	Male	Married	Business Man	Female	66	122	170	42.21	Obese class III	Yes	13	15	No	Sub-Clinical Candidiasis	No	Positive
132	Male	Married	Teacher	Female	65	70	160	27.34	Pre-obese	Yes	5M	16	No	Sub-Clinical Candidiasis	No	Positive
133	Female	Married	House Wife	Male	55	67	160	26.17	Pre-obese	Non	N/A	4	No	Sub-Clinical Candidiasis	No	Negative
134	Female	Married	House Wife	Male	63	72	160	28.12	Pre-obese	Yes	8	11	No	Sub-Clinical Candidiasis	No	Positive
135	Female	Married	House Wife	Male	70	97	160	37.89	Obese class II	Yes	20	20	No	Sub-Clinical Candidiasis	No	Positive
136	Female	Married	House Wife	Male	62	75	160	29.3	Pre-obese	Non	N/A	17	No	Sub-Clinical Candidiasis	No	Negative
137	Female	Married	House Wife	Male	60	92	160	35.94	Obese class II	Yes	10	22	No	Sub-Clinical Candidiasis	No	Positive
138	Female	Married	House Wife	Male	70	67	150	29.78	Pre-obese	Yes	9	11	No	Sub-Clinical Candidiasis	No	Positive
139	Female	Married	House Wife	Male	60	78	160	30.47	Obese class I	Non	N/A	1	No	Sub-Clinical Candidiasis	No	Positive
140	Female	Married	House Wife	Male	60	75	160	29.3	Pre-obese	Yes	7	20	No	Sub-Clinical Candidiasis	No	Positive
141	Female	Married	House Wife	Male	53	82	150	36.44	Obese class II	Non	N/A	3	No	Sub-Clinical Candidiasis	No	Positive
142	Female	Married	House Wife	Male	78	60	150	26.67	Pre-obese	Non	N/A	7	No	Sub-Clinical Candidiasis	No	Positive
143	Female	Married	House Wife	Male	68	86	150	38.22	Obese class II	Yes	5	14	No	Sub-Clinical Candidiasis	No	Positive
144	Female	Married	House Wife	Male	75	95	160	37.11	Obese class II	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Positive
145	Female	Married	House Wife	Male	85	95	160	37.11	Obese class II	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Positive
146	Female	Married	House Wife	Male	60	83	165	30.49	Obese class I	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Positive

147	Female	Married	House Wife	Male	57	93	165	34.16	Obese class I	Non	N/A	20	No	Sub-Clinical Candidiasis	No	Negative
148	Female	Married	House Wife	Male	48	108	150	48	Obese class III	Non	N/A	2	No	Sub-Clinical Candidiasis	No	Negative
149	Female	Married	House Wife	Male	60	96	164	35.69	Obese class II	Non	N/A	9	No	Sub-Clinical Candidiasis	No	Positive
150	Female	Married	House Wife	Male	58	67	152	29	Pre-obese	Non	N/A	2	No	Sub-Clinical Candidiasis	No	Positive
151	Female	Married	House Wife	Male	71	57	150	25.33	Pre-obese	Yes	4	14	No	Sub-Clinical Candidiasis	No	Positive
152	Female	Married	House Wife	Male	58	108	150	48	Obese class III	Yes	10	23	No	Sub-Clinical Candidiasis	No	Positive
153	Female	Married	House Wife	Male	70	90	150	40	Obese class III	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Negative
154	Female	Married	House Wife	Male	75	90	160	35.16	Obese class II	Non	N/A	7	No	Sub-Clinical Candidiasis	No	Positive
155	Female	Married	House Wife	Male	70	60	150	26.67	Pre-obese	Non	N/A	30	No	Sub-Clinical Candidiasis	No	Negative
156	Female	Married	House Wife	Male	52	80	150	35.56	Obese class II	Non	N/A	1	No	Sub-Clinical Candidiasis	No	Positive
157	Female	Married	House Wife	Male	52	68	160	26.56	Pre-obese	Non	N/A	8	No	Sub-Clinical Candidiasis	No	Negative
158	Female	Married	House Wife	Male	48	75	160	29.3	Pre-obese	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Positive
159	Female	Married	House Wife	Male	52	60	180	18.52	Normal range	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Positive
160	Female	Married	House Wife	Male	62	105	160	41.02	Obese class III	Non	N/A	30	No	Sub-Clinical Candidiasis	No	Negative
161	Female	Married	House Wife	Male	66	70	150	31.11	Obese class I	Non	N/A	8	No	Sub-Clinical Candidiasis	No	Negative
162	Female	Married	House Wife	Male	67	70	150	31.11	Obese class I	Yes	12	1	No	Sub-Clinical Candidiasis	No	Positive
163	Female	Married	House Wife	Male	62	70	150	31.11	Obese class I	Yes	13	19	No	Sub-Clinical Candidiasis	No	Positive
164	Female	Married	House Wife	Male	55	80	150	35.56	Obese class II	Non	N/A	13	No	Sub-Clinical Candidiasis	No	Positive
165	Female	Married	House Wife	Male	60	60	150	26.67	Pre-obese	Yes	3	1	No	Sub-Clinical Candidiasis	No	Negative
166	Female	Married	House Wife	Male	60	83	150	36.89	Obese class II	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Positive
167	Male	Married	Business Man	Female	58	94	165	34.53	Obese class I	Yes	10	3	No	Sub-Clinical Candidiasis	No	Positive
168	Male	Married	Retired	Female	80	70	150	31.11	Obese class I	Non	N/A	30	No	Sub-Clinical Candidiasis	No	Positive
169	Female	Married	House Wife	Male	66	80	150	35.56	Obese class II	Non	N/A	14	No	Sub-Clinical Candidiasis	No	Negative
170	Male	Married	Retired	Female	59	70	160	27.34	Pre-obese	Yes	2	7	No	Sub-Clinical Candidiasis	No	Negative

171	Female	Married	House Wife	Male	54	90	160	35.16	Obese class II	Non	N/A	13	No	Sub-Clinical Candidiasis	No	Positive
172	Female	Married	House Wife	Male	68	81	150	36	Obese class II	Non	N/A	20	No	Sub-Clinical Candidiasis	No	Positive
173	Female	Married	House Wife	Male	80	70	150	31.11	Obese class I	Non	N/A	30	No	Sub-Clinical Candidiasis	No	Negative
174	Female	Married	House Wife	Male	65	74	160	28.91	Pre-obese	Yes	7	10	No	Sub-Clinical Candidiasis	No	Negative
175	Female	Married	House Wife	Male	55	98	150	43.56	Obese class III	Yes	27	31	No	Sub-Clinical Candidiasis	No	Negative
176	Female	Married	House Wife	Male	52	90	150	40	Obese class III	Non	N/A	12	No	Sub-Clinical Candidiasis	No	Negative
177	Female	Married	House Wife	Male	52	70	160	27.34	Pre-obese	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Positive
178	Female	Married	House Wife	Male	60	68	160	26.56	Pre-obese	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Negative
179	Female	Married	House Wife	Male	65	88	150	39.11	Obese class II	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Positive
180	Female	Married	House Wife	Male	60	80	150	35.56	Obese class II	Non	N/A	25	No	Sub-Clinical Candidiasis	No	Negative
181	Male	Married	Business Man	Female	68	108	185	31.56	Obese class I	Non	N/A	25	No	Sub-Clinical Candidiasis	No	Positive
182	Male	Married	Retired	Female	67	90	168	31.89	Obese class I	Non	N/A	8	Yes	Sub-Clinical Candidiasis	No	Negative
183	Male	Married	Employer	Female	52	100	170	34.6	Obese class I	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Negative
184	Male	Married	Employer	Female	50	80	175	26.12	Pre-obese	Non	N/A	7	Yes	Sub-Clinical Candidiasis	No	Positive
185	Male	Married	Retired	Female	69	80	165	29.38	Pre-obese	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Negative
186	Male	Married	Business Man	Female	66	77	178	24.3	Normal range	Yes	8	6	No	Sub-Clinical Candidiasis	No	Positive
187	Male	Married	Retired	Female	71	100	170	34.6	Obese class I	Yes	1	10	No	Sub-Clinical Candidiasis	No	Positive
188	Male	Married	Employer	Female	62	79	170	27.34	Pre-obese	Yes	7	7	No	Sub-Clinical Candidiasis	No	Positive
189	Male	Married	Retired	Female	68	95	170	32.87	Obese class I	Yes	2	10	Yes	Sub-Clinical Candidiasis	No	Negative
190	Male	Married	Employer	Female	52	67	168	23.74	Normal range	Non	N/A	5	Yes	Sub-Clinical Candidiasis	No	Negative
191	Female	Married	House Wife	Male	58	84	150	37.33	Obese class II	Non	N/A	14	No	Sub-Clinical Candidiasis	No	Negative
192	Female	Married	House Wife	Male	67	70	160	31.11	Obese class I	Non	N/A	20	No	Sub-Clinical Candidiasis	No	Positive
193	Male	Married	Employer	Female	41	115	179	35.89	Obese class II	Non	N/A	6	Yes	Sub-Clinical Candidiasis	No	Negative
194	Male	Married	Business Man	Female	66	76	170	26.3	Pre-obese	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Positive

195	Male	Married	Business Man	Female	53	100	160	39.06	Pre-obese	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Negative
196	Male	Married	Retired	Female	74	107	175	34.94	Obese class I	Non	N/A	19	No	Sub-Clinical Candidiasis	No	Negative
197	Male	Married	Soldier	Female	56	108	165	39.67	Obese class II	Yes	1	6	No	Sub-Clinical Candidiasis	No	Negative
198	Female	Married	House Wife	Male	55	70	150	31.11	Obese class I	Non	N/A	7	No	Sub-Clinical Candidiasis	No	Negative
199	Male	Married	Business Man	Female	65	95	170	32.87	Obese class I	Non	N/A	20	No	Sub-Clinical Candidiasis	No	Negative
200	Female	Married	House Wife	Male	59	79	150	35.11	Obese class II	Yes	10	10	No	Sub-Clinical Candidiasis	No	Positive
201	Female	Married	House Wife	Male	62	73	150	36.89	Obese class II	Yes	10	5	No	Sub-Clinical Candidiasis	No	Positive
202	Female	Married	House Wife	Male	58	75	150	33.33	Obese class I	Yes	38	1	No	Sub-Clinical Candidiasis	No	Negative
203	Female	Married	House Wife	Male	56	65	160	25.39	Obese class I	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Negative
204	Female	Married	House Wife	Male	63	63	160	24.61	Normal range	Non	N/A	21	No	Sub-Clinical Candidiasis	No	Positive
205	Female	Married	House Wife	Male	65	70	160	27.34	Pre-obese	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Positive
206	Female	Married	House Wife	Male	58	170	150	75.56	Obese class III	Non	N/A	18	No	Sub-Clinical Candidiasis	No	Negative
207	Male	Married	Retired	Female	56	120	178	37.87	Obese class II	Non	N/A	4	No	Sub-Clinical Candidiasis	No	Negative
208	Male	Married	Business Man	Female	54	89	165	32.69	Obese class I	Yes	5M	6	No	Sub-Clinical Candidiasis	No	Negative
209	Male	Married	Employer	Female	41	120	165	44.08	Obese class III	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Positive
210	Male	Married	Employer	Female	67	90	175	29.39	Pre-obese	Non	N/A	14	No	Sub-Clinical Candidiasis	No	Negative
211	Male	Married	Teacher	Female	53	95	175	31.02	Obese class I	Non	N/A	8	No	Sub-Clinical Candidiasis	No	Negative
212	Male	Married	Employer	Female	54	63	170	21.8	Normal range	Yes	6	7	No	Sub-Clinical Candidiasis	No	Negative
213	Male	Married	Business Man	Female	55	85	175	27.76	Pre-obese	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Positive
214	Male	Married	Employer	Female	58	100	180	30.86	Obese class I	Non	N/A	16	No	Sub-Clinical Candidiasis	No	Positive
215	Male	Married	Business Man	Female	54	70	160	27.34	Pre-obese	Non	N/A	2	No	Sub-Clinical Candidiasis	No	Negative
216	Male	Married	Business Man	Female	65	85	165	31.22	Obese class I	Yes	12	15	Yes	Sub-Clinical Candidiasis	No	Negative
217	Male	Married	Teacher	Female	86	80	170	27.68	Pre-obese	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Positive
218	Male	Married	Employer	Female	49	65	160	25.39	Pre-obese	Non	N/A	5	Yes	Sub-Clinical Candidiasis	No	Negative

219	Female	Married	House Wife	Male	55	100	170	34.6	Obese class I	Yes	18	25	No	Sub-Clinical Candidiasis	No	Negative
220	Male	Married	Business Man	Female	45	88	180	27.16	Pre-obese	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Negative
221	Male	Married	Retired	Female	66	85	160	33.2	Obese class I	Non	N/A	2	Yes	Sub-Clinical Candidiasis	No	Negative
222	Male	Married	Business Man	Female	54	90	170	31.14	Obese class I	Non	N/A	4	Yes	Sub-Clinical Candidiasis	No	Negative
223	Male	Married	Employer	Female	53	74	176	23.89	Normal range	Non	N/A	13	No	Sub-Clinical Candidiasis	No	Negative
224	Male	Married	Retired	Female	67	75	175	24.49	Normal range	Yes	15D	7	No	Sub-Clinical Candidiasis	No	Negative
225	Female	Married	House Wife	Female	53	82	170	28.37	Pre-obese	Non	N/A	1	No	Sub-Clinical Candidiasis	No	Negative
226	Female	Married	House Wife	Male	60	65	150	28.89	Pre-obese	Yes	1	20	No	Sub-Clinical Candidiasis	No	Negative
227	Female	Married	House Wife	Male	80	90	166	32.66	Obese class I	Non	N/A	5	No	Sub-Clinical Candidiasis	No	Negative
228	Female	Married	House Wife	Male	67	70	160	27.34	Pre-obese	Yes	13	10	No	Sub-Clinical Candidiasis	No	Negative
229	Male	Married	Retired	Female	75	80	160	31.25	Obese class I	Non	N/A	30	No	Sub-Clinical Candidiasis	No	Negative
230	Female	Married	House Wife	Male	60	77	155	32.05	Obese class I	Yes	21	20	No	Sub-Clinical Candidiasis	No	Negative
231	Female	Divorced	House Wife	No Partner	59	95	168	33.66	Obese class I	Yes	6M	5	No	Sub-Clinical Candidiasis	No	Negative
232	Female	Married	House Wife	Male	65	98	154	41.32	Obese class III	Yes	1	5	No	Sub-Clinical Candidiasis	No	Negative
233	Female	Married	House Wife	Male	58	63	160	24.61	Normal range	Yes	10	10	No	Sub-Clinical Candidiasis	No	Negative
234	Male	Married	Employer	Female	58	95	168	33.66	Obese class I	Non	N/A	5	No	Sub-Clinical Candidiasis	No	Negative
235	Female	Married	House Wife	Male	64	60	140	30.61	Obese class I	Non	N/A	2	No	Sub-Clinical Candidiasis	No	Positive
236	Female	Married	House Wife	Male	53	90	150	40	Obese class III	Non	N/A	1	No	Sub-Clinical Candidiasis	No	Positive
237	Female	Married	House Wife	Male	63	85	160	24.61	Normal range	Yes	1	4	No	Sub-Clinical Candidiasis	No	Negative
238	Female	Married	House Wife	Male	60	75	165	27.55	Pre-obese	Non	N/A	29	No	Sub-Clinical Candidiasis	No	Positive
239	Female	Married	House Wife	Male	54	95	170	32.87	Obese class I	Yes	2	14	No	Sub-Clinical Candidiasis	No	Negative
240	Female	Married	Teacher	Male	60	68	160	26.56	Pre-obese	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Negative
241	Female	Married	House Wife	Male	64	74	150	32.89	Obese class I	Yes	12	25	No	Sub-Clinical Candidiasis	No	Negative
242	Female	Married	House Wife	Male	52	80	160	31.25	Obese class I	Non	N/A	4	No	Sub-Clinical Candidiasis	No	Positive

243	Female	Married	House Wife	Male	46	80	160	31.25	Obese class I	Non	N/A	3	No	Sub-Clinical Candidiasis	No	Positive
244	Female	Married	House Wife	Male	50	80	160	31.25	Obese class I	Non	N/A	27	No	Sub-Clinical Candidiasis	No	Negative
245	Female	Married	House Wife	Male	63	73	160	28.52	Pre-obese	Yes	3	6	No	Sub-Clinical Candidiasis	No	Positive
246	Female	Married	House Wife	Male	62	95	160	37.11	Obese class II	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Positive
247	Female	Married	House Wife	Male	70	60	150	26.67	Pre-obese	Non	N/A	14	No	Sub-Clinical Candidiasis	No	Positive
248	Female	Married	House Wife	Male	66	75	165	27.55	Pre-obese	Non	N/A	2	No	Sub-Clinical Candidiasis	No	Negative
249	Female	Married	House Wife	Male	60	70	150	31.11	Obese class I	Yes	1	6	No	Sub-Clinical Candidiasis	No	Positive
250	Female	Married	House Wife	Male	60	87	160	33.98	Obese class I	Non	N/A	2	No	Sub-Clinical Candidiasis	No	Negative
251	Female	Married	House Wife	Male	55	95	160	37.11	Obese class II	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Negative
252	Female	Married	House Wife	Male	50	95	155	39.54	Obese class II	Non	N/A	1	No	Sub-Clinical Candidiasis	No	Negative
253	Female	Married	House Wife	Male	62	65	150	28.89	Pre-obese	Non	N/A	12	No	Sub-Clinical Candidiasis	No	Negative
254	Female	Married	Employer	Male	45	109	170	37.72	Obese class II	Non	N/A	12	No	Sub-Clinical Candidiasis	No	Positive
255	Female	Married	House Wife	Male	69	80	165	29.38	Pre-obese	Non	N/A	17	No	Sub-Clinical Candidiasis	No	Negative
256	Female	Married	House Wife	Male	50	90	150	40	Obese class III	Non	N/A	24	No	Sub-Clinical Candidiasis	No	Negative
257	Female	Married	House Wife	Male	50	90	160	35.16	Obese class II	Non	N/A	1	No	Sub-Clinical Candidiasis	No	Negative
258	Female	Married	House Wife	Male	63	90	150	40	Obese class III	Non	N/A	5	No	Sub-Clinical Candidiasis	No	Positive
259	Female	Married	House Wife	Male	60	70	150	31.11	Obese class I	Yes	5	11	No	Sub-Clinical Candidiasis	No	Positive
260	Female	Married	House Wife	Male	60	50	140	25.51	Pre-obese	Non	N/A	24	No	Sub-Clinical Candidiasis	No	Negative
261	Female	Married	House Wife	Male	70	80	150	35.56	Obese class II	Non	N/A	25	No	Sub-Clinical Candidiasis	No	Negative
262	Female	Married	House Wife	Male	75	60	150	26.67	Pre-obese	Non	N/A	20	No	Sub-Clinical Candidiasis	No	Negative
263	Female	Married	House Wife	Male	40	100	150	44.44	Obese class III	Non	N/A	4	No	Sub-Clinical Candidiasis	No	Positive
264	Female	Married	House Wife	Male	63	75	150	33.33	Obese class I	Yes	4	14	No	Sub-Clinical Candidiasis	No	Positive
265	Female	Married	House Wife	Male	50	65	150	28.89	Pre-obese	Yes	1	1	No	Sub-Clinical Candidiasis	No	Negative
266	Female	Married	House Wife	Male	58	75	150	33.33	Obese class I	Non	N/A	25	No	Sub-Clinical Candidiasis	No	Positive

267	Female	Married	House Wife	Male	63	80	165	28.34	Pre-obese	Non	N/A	14	No	Sub-Clinical Candidiasis	No	Positive
268	Female	Married	House Wife	Male	65	80	150	35.56	Obese class II	Yes	7	27	No	Sub-Clinical Candidiasis	No	Positive
269	Female	Married	House Wife	Male	50	70	170	24.22	Normal range	Non	N/A	9	No	Sub-Clinical Candidiasis	No	Negative
270	Female	Married	House Wife	Male	65	70	150	31.11	Obese class I	Non	N/A	24	No	Sub-Clinical Candidiasis	No	Negative
271	Female	Married	House Wife	Male	65	70	150	31.11	Obese class I	Yes	6	14	No	Sub-Clinical Candidiasis	No	Positive
272	Female	Married	House Wife	Male	60	70	140	35.71	Obese class II	Non	N/A	2	No	Sub-Clinical Candidiasis	No	Positive
273	Female	Married	House Wife	Male	55	80	165	28.34	Pre-obese	Non	N/A	19	No	Sub-Clinical Candidiasis	No	Negative
274	Female	Married	Teacher	Male	52	92	169	32.21	Obese class I	Yes	30	11	No	Sub-Clinical Candidiasis	No	Positive
275	Female	Married	House Wife	Male	49	80	150	35.56	Obese class II	Non	N/A	6M	No	Sub-Clinical Candidiasis	No	Positive
276	Female	Married	House Wife	Male	52	85	170	29.41	Pre-obese	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Negative
277	Female	Married	House Wife	Male	60	75	150	33.33	Obese class I	Yes	2	5	No	Sub-Clinical Candidiasis	No	Negative
278	Female	Married	House Wife	Male	53	114	170	39.45	Obese class II	Non	N/A	4	No	Sub-Clinical Candidiasis	No	Negative
279	Female	Married	House Wife	Male	65	70	140	35.71	Obese class II	Non	N/A	12	No	Sub-Clinical Candidiasis	No	Positive
280	Female	Married	House Wife	Male	68	90	150	40	Obese class III	Yes	10	24	No	Sub-Clinical Candidiasis	No	Negative
281	Female	Married	House Wife	Male	68	100	140	51.02	Obese class III	Yes	15	14	No	Sub-Clinical Candidiasis	No	Positive
282	Female	Married	House Wife	Male	65	70	150	31.11	Obese class I	Non	N/A	4	No	Sub-Clinical Candidiasis	No	Negative
283	Female	Married	House Wife	Male	60	80	155	33.3	Obese class I	Yes	5	5	No	Sub-Clinical Candidiasis	No	Positive
284	Female	Married	House Wife	Male	57	80	150	35.56	Obese class II	Non	N/A	5	No	Sub-Clinical Candidiasis	No	Negative
285	Female	Married	House Wife	Male	52	80	150	35.56	Obese class II	Non	N/A	25	No	Sub-Clinical Candidiasis	No	Positive
286	Female	Married	House Wife	Male	58	120	160	46.87	Obese class III	Non	N/A	24	No	Sub-Clinical Candidiasis	No	Negative
287	Female	Married	House Wife	Male	73	60	140	30.61	Obese class I	Yes	5	23	No	Sub-Clinical Candidiasis	No	Positive
288	Female	Married	House Wife	Male	27	80	165	29.38	Pre-obese	Non	N/A	6M	No	Sub-Clinical Candidiasis	No	Positive
289	Female	Married	House Wife	Male	75	80	160	31.25	Obese class I	Yes	25	10	No	Sub-Clinical Candidiasis	No	Positive
290	Female	Married	House Wife	Male	53	105	160	41.02	Obese class III	Non	N/A	11	No	Sub-Clinical Candidiasis	No	Positive

291	Female	Married	House Wife	Male	75	60	150	26.67	Pre-obese	Non	N/A	12	No	Sub-Clinical Candidiasis	No	Positive
292	Female	Married	House Wife	Male	50	115	160	44.92	Obese class III	Non	N/A	7	No	Sub-Clinical Candidiasis	No	Positive
293	Female	Married	House Wife	Male	49	90	165	33.06	Obese class I	Yes	3M	10	No	Sub-Clinical Candidiasis	No	Positive
294	Female	Married	House Wife	Male	53	75	168	26.57	Pre-obese	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Negative
295	Female	Married	House Wife	Male	55	80	170	27.68	Pre-obese	Non	N/A	5	No	Sub-Clinical Candidiasis	No	Negative
296	Female	Married	House Wife	Male	73	85	160	33.2	Obese class I	Non	N/A	25	No	Sub-Clinical Candidiasis	No	Negative
297	Female	Married	House Wife	Male	58	70	150	31.11	Obese class I	Non	N/A	33	No	Sub-Clinical Candidiasis	No	Positive
298	Female	Married	House Wife	Male	70	80	170	27.68	Pre-obese	Yes	10	44	No	Sub-Clinical Candidiasis	No	Positive
299	Female	Single	House Wife	No Partner	39	101	165	37.1	Obese class II	Non	N/A	2	No	Sub-Clinical Candidiasis	No	Negative
300	Female	Married	House Wife	Male	50	130	165	47.75	Obese class III	Non	N/A	7	No	Sub-Clinical Candidiasis	No	Negative
301	Female	Married	House Wife	Male	67	80	160	31.25	Obese class I	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Positive
302	Female	Married	House Wife	Male	63	76	160	29.69	Pre-obese	Non	N/A	27	No	Sub-Clinical Candidiasis	No	Positive
303	Female	Married	House Wife	Male	65	75	160	29.3	Pre-obese	Yes	3M	24	No	Sub-Clinical Candidiasis	No	Positive
304	Female	Married	House Wife	Male	33	61	165	22.41	Normal range	Non	N/A	7	No	Sub-Clinical Candidiasis	No	Positive
305	Female	Married	House Wife	Male	65	80	160	31.25	Obese class I	Yes	2	15	No	Sub-Clinical Candidiasis	No	Negative
306	Female	Married	House Wife	Male	58	78	160	30.47	Obese class I	Yes	1	8	No	Sub-Clinical Candidiasis	No	Positive
307	Female	Single	House Wife	No Partner	37	94	170	32.53	Obese class I	Non	N/A	20	No	Sub-Clinical Candidiasis	No	Negative
308	Female	Married	House Wife	Male	53	121	165	44.44	Obese class III	Non	N/A	14	No	Sub-Clinical Candidiasis	No	Positive
309	Female	Married	House Wife	Male	65	73	150	32.44	Obese class I	Non	N/A	5	No	Sub-Clinical Candidiasis	No	Positive
310	Female	Married	House Wife	Male	55	85	150	37.78	Obese class II	Non	N/A	21	No	Sub-Clinical Candidiasis	No	Positive
311	Female	Married	House Wife	Male	54	80	150	35.56	Obese class II	Non	N/A	6	No	Sub-Clinical Candidiasis	No	Negative
312	Female	Married	House Wife	Male	73	80	160	31.25	Obese class I	Yes	4	15	No	Sub-Clinical Candidiasis	No	Positive
313	Female	Married	House Wife	Male	70	85	165	31.22	Obese class I	Non	N/A	10	No	Sub-Clinical Candidiasis	No	Negative
314	Female	Married	House Wife	Male	80	90	165	33.06	Obese class I	Yes	15	21	No	Sub-Clinical Candidiasis	No	Positive

315	Female	Married	House Wife	Male	60	70	165	25.71	Pre-obese	Non	N/A	30	No	Sub-Clinical Candidiasis	No	Positive
316	Female	Married	House Wife	Male	57	60	160	23.44	Normal range	Yes	10	14	No	Sub-Clinical Candidiasis	No	Positive
317	Female	Married	House Wife	Male	54	96	170	33.22	Obese class I	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Negative
318	Female	Married	House Wife	Male	53	110	165	38.97	Obese class II	Non	N/A	5	No	Sub-Clinical Candidiasis	No	Positive
319	Female	Married	House Wife	Male	65	57	170	19.72	Normal range	Non	N/A	5	No	Sub-Clinical Candidiasis	No	Positive
320	Female	Married	House Wife	Male	24	80	170	27.68	Pre-obese	Non	N/A	20	No	Sub-Clinical Candidiasis	No	Negative
321	Male	Married	Employer	Female	55	130	173	43.44	Obese class III	Non	N/A	8	No	Sub-Clinical Candidiasis	No	Negative
322	Male	Married	Business Man	Female	46	95	186	27.46	Pre-obese	Non	N/A	19	No	Sub-Clinical Candidiasis	No	Positive
323	Male	Married	Employer	Female	54	80	165	29.38	Pre-obese	Non	N/A	15	No	Sub-Clinical Candidiasis	No	Positive
324	Male	Married	Employer	Female	49	115	180	35.49	Obese class II	Non	N/A	7	No	Sub-Clinical Candidiasis	No	Negative
325	Male	Married	Employer	Female	63	83	178	26.2	Pre-obese	Yes	8	18	No	Sub-Clinical Candidiasis	No	Positive
326	Female	Married	House Wife	Male	52	75	165	27.55	Pre-obese	Non	N/A	2	No	Sub-Clinical Candidiasis	No	Negative
327	Female	Married	House Wife	Male	63	95	170	32.87	Obese class I	Non	N/A	30	No	Sub-Clinical Candidiasis	No	Negative
328	Female	Married	House Wife	Male	50	86	165	31.59	Obese class I	Non	N/A	12	No	Sub-Clinical Candidiasis	No	Negative
329	Female	Married	House Wife	Male	53	75	166	27.22	Pre-obese	Non	N/A	13	No	Sub-Clinical Candidiasis	No	Positive
330	Female	Married	House Wife	Male	48	86	170	29.76	Pre-obese	Yes	7	22	No	Sub-Clinical Candidiasis	No	Positive
331	Female	Married	House Wife	Male	60	80	168	28.34	Pre-obese	Non	N/A	34	No	Sub-Clinical Candidiasis	No	Negative
332	Female	Married	House Wife	Male	50	75	169	26.26	Pre-obese	Non	N/A	5	No	Sub-Clinical Candidiasis	No	Positive
333	Female	Married	House Wife	Male	61	84	170	29.07	Pre-obese	Yes	3M	2	No	Sub-Clinical Candidiasis	No	Positive
334	Female	Married	House Wife	Male	65	80	160	31.25	Obese class I	Non	N/A	35	No	Sub-Clinical Candidiasis	No	Negative

Appendix 5

Tables 4 shows the results obtained from the fluconazole disk diffusion susceptibility testing when samples were grown in YNBG media in triplicate. Score given as following (0 = a clear zone with no micro-colonies, 1 = a few micro-colonies present, 2 = moderate growth of micro-colonies and 3 = many micro-colonies in the susceptibility area.

Table 4: Fluconazole sensetifity testing results in YNBG media.

Patient No.	ID API 32C	MIC (DD)	Interpretation
1	*C.dubliniesis*	18	Susceptible
3	*C.dubliniesis*	12	Intermediate
5	*C.sake*	11	Resistance
8	*C.sake*	16	Susceptible
9	*C.dubliniesis*	14.5	Susceptible
11	*C. humicola*	8.5	Intermediate
13	*C.dubliniesis*	12.5	Susceptible
14B	*C.albicans*	12	Susceptible
15	*C.dubliniesis*	19	Susceptible
16B	*C.dubliniesis*	17.5	Susceptible
17A	*C.kefyr*	10	Intermediate
17B	*C. humicola*	14	Susceptible
18	*C. humicola*	13.5	Susceptible
20A	*C. humicola*	16	Susceptible
20B	*C.krusei*	R	Resistance
22	*C. humicola*	16.5	Susceptible
23	*C.dubliniesis*	15	Susceptible
24A	*C.membranifaciens*	10.5	Inermediate
24B	*C.dubliniesis*	11.5	Intermediate
25	*C.dubliniesis*	20	Susceptible
28	*C.dubliniesis*	15	Susceptible
29A	*C.dubliniesis*	7.5	Intermediate
29B	*C. humicola*	15	Susceptible
32	*C.albicans*	15.5	Susceptible
33	*C. humicola*	6.5	Resistance
37	*C.albicans*	7.5	Intermediate
39B	*C.glabrata*	R	Resistance
40A	*C. humicola*	6.5	Resistance
40B	*C.tropicalis*	7	Intermediate
42	*C.albicans*	9	Intermediate

52A	*C.glabrata*	R	Resistance
52B	*C.albicans*	15	Susceptible
57	*C.albicans*	22.5	Susceptible
59A	*C.dubliniesis*	14	Susceptible
59B	*C.globosa*	13.5	Susceptible
60	*C.albicans*	14	Susceptible
61	*C.dubliniesis*	16	Susceptible
63	*C.albicans*	6.5	Resistance
64	*C.krusei*	R	Resistance
65	*C.albicans*	7.5	Intermediate
66	*C.dubliniesis*	16.5	Susceptible
67	*C.dubliniesis*	27.5	Susceptible
69B	*C.albicans*	14.5	Susceptible
71	*C.glabrata*	R	Resistance
73	*C.glabrata*	R	Resistance
74A	*C.parapsilosis*	10	Intermediate
74B	*C.dubliniesis*	14	Susceptible
76	*C.albicans*	19	Susceptible
77	*C.albicans*	20	Susceptible
78	*C.glabrata*	R	Resistance
80	*C.dubliniesis*	16.5	Susceptible
81	*C.dubliniesis*	17	Susceptible
82	*C.albicans*	12.5	Susceptible
83	*C.dubliniesis*	11.5	Intermediate
85	*C. humicola*	14.5	Susceptible
87	*C. humicola*	18	Susceptible
88	*C.albicans*	13	Susceptible
91	*C. humicola*	15	Susceptible
92	*C.glabrata*	R	Resistance
95	*C. humicola*	17	Susceptible
97	*C.albicans*	R	Resistance
98	*C.dubliniesis*	14.5	Susceptible
99	*C.albicans*	16	Susceptible
100	*C.albicans*	10	Intermediate
101	*C.dubliniesis*	11.5	Intermediate
106	*C. humicola*	17.5	Susceptible
109A	*C.kefyr*	7	Intermediate
109B	*C. humicola*	12.5	Susceptible
112	*C.dubliniesis*	14	Susceptible
113	*C.guilliermondii*	R	Resistance
116	*C.dubliniesis*	17.5	Susceptible

120	*C.albicans*	15	Susceptible
121	*C.dubliniesis*	11.5	Intermediate
123	*C.dubliniesis*	14.5	Susceptible
125	*C. humicola*	7.5	Intermediate
126	*C.dubliniesis*	16	Susceptible
129	*C.tropicalis*	7.5	Intermediate
131	*C.dubliniesis*	17	Susceptible
132	*C.albicans*	12.5	Susceptible
134	*C.albicans*	13	Susceptible
135	*C.dubliniesis*	11.5	Intermediate
137A	*C. humicola*	12.5	Susceptible
138	*C.parapsilosis*	1	Resistance
139A	*C.albicans*	14.5	Susceptible
140	*C.albicans*	20.5	Susceptible
141	*C. humicola*	16	Susceptible
142A	*C.albicans*	22	Susceptible
142B	*C.dubliniesis*	14.5	Susceptible
143	*C.albicans*	13	Susceptible
144	*C. humicola*	20	Susceptible
145	*C.krusei*	R	Resistance
146A	*C. humicola*	15	Susceptible
146B	*C.albicans*	13	Susceptible
149	*C.albicans*	16	Susceptible
150	*C. humicola*	17	Susceptible
150A	*C.magnoliae*	15.5	Susceptible
151A	*C.magnoliae*	13.5	Susceptible
151B	*C.kefyr*	10.5	Intermediate
152	*C.albicans*	13	Susceptible
154	*C.albicans*	18	Susceptible
156	*C.dubliniesis*	16.5	Susceptible
158	*C. humicola*	11	Intermediate
159	*C.albicans*	17	Susceptible
163	*C.dubliniesis*	18.5	Susceptible
164	*C. tropicalis*	14	Susceptible
166	*C.albicans*	11	Intermediate
167	*C.albicans*	8	Intermediate
168	*C. humicola*	R	Resistance
171	*C.dubliniesis*	11.5	Intermediate
172	*C.albicans*	12	Susceptible
177	*C.kefyr*	12.5	Susceptible
181	*C.dubliniesis*	18	Resistance

184	*C.albicans*	R	Resistance
186A	*C.glabrata*	R	Resistance
187	*C.krusei*	R	Resistance
188	*C.albicans*	15	Susceptible
192	*C.albicans*	17	Susceptible
194	*C.dubliniesis*	16.5	Susceptible
200	*C.albicans*	18	Susceptible
201	*C.tropicalis*	12	Susceptible
204	*C.dubliniesis*	21	Susceptible
205	*C.sake*	20	Susceptible
209	*C. humicola*	16	Susceptible
213	*C.albicans*	11.5	Intermediate
214	*C.albicans*	11.5	Intermediate
217	*C. humicola*	17	Susceptible
235	*C.glabrata*	R	Resistance
236	*C.glabrata*	R	Resistance
238	*C.albicans*	14	Susceptible
242	*C.albicans*	13.5	Susceptible
243	*C.sake*	4	Resistance
245	*C. humicola*	17	Susceptible
246	*C.dubliniesis*	16	Susceptible
247	*C.albicans*	16.5	Susceptible
249	*C.albicans*	17	Susceptible
254	*C.albicans*	16	Susceptible
258	*C.glabrata*	R	Resistance
259	*C.glabrata*	R	Resistance
263	*C.albicans*	13	Susceptible
264	*C.albicans*	16	Susceptible
266	*C.dubliniesis*	17	Susceptible
267	*C.albicans*	17	Susceptible
268	*C.albicans*	16.5	Susceptible
271	*C.albicans*	10.5	Intermediate
272	*C.dubliniesis*	17.5	Susceptible
274	*C.albicans*	12.5	Susceptible
275	*C.albicans*	11.5	Intermediate
279	*C.dubliniesis*	15.5	Susceptible
281	*C.albicans*	12	Susceptible
282	*C. humicola*	12.5	Susceptible
283A	*C.tropicalis*	11	Intermediate
285	*C.albicans*	5.5	Resistance
287	*C.albicans*	10.5	Intermediate

288	*C.albicans*	13.5	Susceptible
289	*C.albicans*	12.5	Susceptible
290	*C.glabrata*	R	Resistance
291	*C.glabrata*	R	Resistance
292	*C.dubliniesis*	13.5	Susceptible
293	*C.glabrata*	R	Resistance
297	*C.glabrata*	R	Resistance
298	*C.glabrata*	R	Resistance
301	*C.albicans*	17	Susceptible
302	*C.glabrata*	R	Resistance
303	*C.albicans*	12	Susceptible
304	*C.albicans*	16	Susceptible
306	*C.dubliniesis*	17	Susceptible
308	*C.albicans*	15	Susceptible
309	*C.albicans*	17.5	Susceptible
310	*C.albicans*	13	Susceptible
312	*C.albicans*	16.5	Susceptible
314	*C.albicans*	13	Susceptible
315	*C.krusei*	R	Resistance
316	*C.glabrata*	R	Resistance
318	*C.albicans*	15.5	Susceptible
319	*C.albicans*	16.5	Susceptible
322	*C.glabrata*	R	Resistance
323	*C.glabrata*	R	Resistance
325	*C.albicans*	14.5	Susceptible
329	*C.albicans*	18.5	Susceptible
330	*C.albicans*	11	Intermediate
332	*C.dubliniesis*	18	Susceptible
333	*C.kefyr*	13	Susceptible

Appendix 6

Table 5: Fluconazole susceptibility testing of rare *Candida* species and other genuse of yeasts results in YNBG media

ID API 32C	ID API 32C *Candida*	Edge of the disc to the edge of S. zone/ mm	MIC (DD)	Interpertation of the results
Candida sake	*Candida species*	6	3	R
Candida sake	*Candida species*	16	0	S
Candida sake	*Candida species*	8	3	R
Candida sake	*Candida species*	10	3	R
Candida humicola	*Candida species*	8.5	0	I
Candida humicola	*Candida species*	14	1	S
Candida humicola	*Candida species*	13.5	0	S
Candida humicola	*Candida species*	16	0	S
Candida humicola	*Candida species*	16.5	0	S
Candida humicola	*Candida species*	15	2	S
Candida humicola	*Candida species*	6.5	0	R
Candida humicola	*Candida species*	6.5	0	R
Candida humicola	*Candida species*	14.5	0	S
Candida humicola	*Candida species*	18	2	S
Candida humicola	*Candida species*	12	3	R
Candida humicola	*Candida species*	11.5	I	I
Candida humicola	*Candida species*	17.5	0	S
Candida humicola	*Candida species*	12.5	0	S
Candida humicola	*Candida species*	7.5	0	I
Candida humicola	*Candida species*	12.5	0	S
Candida humicola	*Candida species*	13	3	R
Candida humicola	*Candida species*	10	0	I
Candida humicola	*Candida species*	15	0	S
Candida humicola	*Candida species*	17	0	S
Candida humicola	*Candida species*	11	0	I
Candida humicola	*Candida species*	R	R	R
Candida humicola	*Candida species*	11	3	R
Candida humicola	*Candida species*	17	0	S
Candida humicola	*Candida species*	12.5	0	S
Candida humicola	*Candida species*	12.5	0	**S**
Candida magnoliae	*Candida species*	15.5	0	S
Candida magnoliae	*Candida species*	13.5	2	S
Candida guilliermondii	*Candida species*	R	R	R

Candida globosa	*Candida species*	13.5	0	S
Candida membranifaciens	*Candida species*	10.5	0	I
Saprochaete capitata	*Non-Candida species*	1	0	R
Saprochaete capitata	*Non-Candida species*	0.5	0	R
Saprochaete capitata	*Non-Candida species*	5.5	0	R
Saprochaete capitata	*Non-Candida species*	0.5	0	R
Saprochaete capitata	*Non-Candida species*	R	R	R
Kloeckera apis/apiculata	*Non-Candida species*	11.5	0	
Kloeckera apis/apiculata	*Non-Candida species*	10.5	0	
Trichosporon mucoides	*Non-Candida species*	14.5	0	
Kloeckera japoica	*Non-Candida species*	14	0	

Appendix 7

Spiecment No	Spiecment ID by Vietk	Amphotericin B	Caspofungin	Fluconazole	5-Flucytosine	Micafungin	Voriconazole
97	*C.albicans*	1	<=0.25	4	<= 1	<=0.06	<= 0.12
184A	*C.albicans*	1	<=0.25	≥= 64	2	<=0.06	≥= 8
166	*C.albicans*	0.5	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
167	*C.albicans*	1	<=0.25	8	<= 1	<=0.06	<= 0.12
42	*C.albicans*	1	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
213	*C.albicans*	0.5	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
287	*C.albicans*	0.5	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
275	*C.albicans*	0.5	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
271	*C.albicans*	0.5	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
330	*C.albicans*	0.5	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
100	*C.albicans*	0.5	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
88B	*C.albicans*	0.5	<=0.25	4	<= 1	<=0.06	<= 0.12
63	*C.albicans*	0.5	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
16B	*C.dubliniesis*	<=0.25	<=0.25	16	≥=64	<=0.06	≥=8
24B	*C.dubliniesis*	1	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
116B	*C.dubliniesis*	<=0.25	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
83	*C.dubliniesis*	0.5	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
121B	*C.dubliniesis*	<=0.25	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
135	*C.dubliniesis*	<=0.25	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
101	*C.dubliniesis*	<=0.25	<=0.25	<= 1	<= 1	<=0.06	<= 0.12
171	*C.dubliniesis*	<=0.25	<=0.25	8	<= 1	<=0.06	4
74	*C.dubliniesis*	0.5	<=0.25	<= 1	<= 1	1	<= 0.12
181	*C.dubliniesis*	<=0.25	<=0.25	32	<= 1	<=0.06	<= 0.12
39B	*C. glabrata*	0.5	<=0.25	2	<= 1	<=0.06	<= 0.12
71	*C. glabrata*	0.5	<=0.25	2	<= 1	<=0.06	<= 0.12
73	*C. glabrata*	0.5	<=0.25	2	<= 1	<=0.06	<= 0.12
78	*C. glabrata*	0.5	<=0.25	4	<= 1	<=0.06	<= 0.12
92	*C. glabrata*	0.5	<=0.25	2	<= 1	<=0.06	<= 0.12
186A	*C. glabrata*	2	<=0.25	8	<= 1	<=0.06	0.25
290	*C. glabrata*	0.5	<=0.25	2	<= 1	<=0.06	<= 0.12
291	*C. glabrata*	0.5	<=0.25	2	<= 1	<=0.06	<= 0.12
293	*C. glabrata*	1	<=0.25	4	<= 1	<=0.06	0.25
297	*C. glabrata*	0.5	<=0.25	4	<= 1	<=0.06	<= 0.12
298	*C. glabrata*	0.5	<=0.25	2	<= 1	<=0.06	<= 0.12

302	*C. glabrata*	0.5	< =0.25	2	< = 1	< =0.06	< = 0.12
235	*C. glabrata*	0.5	< =0.25	2	< = 1	< =0.06	0.25
236	*C. glabrata*	2	< =0.25	4	< = 1	< =0.06	0.25
258	*C. glabrata*	0.5	< =0.25	4	< = 1	< =0.06	0.25
259A	*C. glabrata*	0.5	< =0.25	4	< = 1	< =0.06	0.25
316	*C. glabrata*	1	< =0.25	4	< = 1	0.12	0.25
322	*C. glabrata*	0.5	< =0.25	8	< = 1	< =0.06	0.25
323	*C. glabrata*	0.5	< =0.25	2	< = 1	< =0.06	< = 0.12
52A	*C. glabrata*	0.5	< =0.25	4	< = 1	< =0.06	0.25
20**B**	*C. krusei*	0.5	≤ = 0.25	8*	16*	0.12	≤ = 0.12
64	*C. krusei*	0.5	< =0.25	8*	8*	0.12	< = 0.12
187	*C. krusei*	0.5	< =0.25	16*	8*	0.12	< = 0.12
145	*C. krusei*	2	< =0.25	16*	4*	0.12	< = 0.12
315	*C. krusei*	0.5	< =0.25	16*	8*	0.12	< = 0.12
129	*C.tropicalis*	0.5	< =0.25	4	< = 1	< =0.06	< = 0.12
164	*C.tropicalis*	0.5	< =0.25	4	< = 1	< =0.06	< = 0.12
40B	*C.tropicalis*	0.5	< =0.25	< = 1	< = 1	< =0.06	< = 0.12
109A	*C.kefyr*	0.5	< =0.25	< = 1	< = 1	< =0.06	< = 0.12
17A	*C.kefyr*	0.5	< =0.25	2	< = 1	< =0.06	< = 0.12
113	*C. guilliermondii*	< =0.25	< =0.25	2	< = 1	0.5	< = 0.12
138	*C. parapsilosis*	0.5	1	< = 1	< = 1	2	< = 0.12

Spiecmen t No	*Spiecment ID by Vietk*	*Spiecment ID by API 32*	AB	CAS	FLU	5-FCT	MCF	VRC	FLUDD
ATCC-90028	*C.albicans*	*C.albicans*	0.5	<=0.25	<=1	<=1	<=0.06	<=0.12	12
ATCC	*C.glabrata*	*C.glabrata*	0.5	<=0.25	32	<=1	<=0.06	1	5
ATCC-2159	*C.krusei*	*C.krusei*	0.5	<=0.25	32*	8*	<=0.06	<=0.12	R
ATCC-950	*C.tropicalis*	*C.tropicalis*	0.5	<=0.25	<=1	<=1	<=0.06	<=0.12	11
ATCC-4135	*C.kefyr*	*C.kefyr*	0.5	<=0.25	<=1	<=1	<=0.06	<=0.12	15
ATCC-3449	*C.lusitaniae*	*C.lusitaniae*	0.5	<=0.25	<=1	<=1	<=0.06	<=0.12	16
ATCC-22019	*C.parapslosis*	*C.parapsilosis*	<=0.25	0.5	<=1	<=1	0.5	<=0.12	7
NCPF-3281	*C.albicans*	*C.albicans*	0.5	<=0.25	<=1	<=1	<=0.06	<=0.12	17
NCPF-3949	*C.dubliniesis*	*C.dubliniesis*	<=0.25	<=0.25	<=1	<=1	<=0.06	<=0.12	13

Appendix 8

The following tables show the statistical association between *Candida* species and gender, denture wearer, smokng, age group, body mass index, and obesity as determined by WHO criteria respectively, using the SPSS 24 statistics software.

Chi-Squared Tests for Species vs Gender

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	6.694[a]	1	.010		
Cntinuity Correction[b]	6.119	1	.013		
Likelihood Ratio	6.712	1	.010		
Fisher's Exact Test				.012	.007
Linear-by-Linear Association	6.673	1	.010		
McNemar Test				.004[c]	
N of Valid Cases	330				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 60.61.

b. Computed only for a 2x2 table

c. Binomial distribution used.

Chi-Squared Tests for Species vs Denture wearer

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.680[a]	1	.031		
Continuity Correction[b]	4.174	1	.041		
Likelihood Ratio	4.709	1	.030		
Fisher's Exact Test				.031	.020
Linear-by-Linear Association	4.665	1	.031		
McNemar Test				.000[c]	
N of Valid Cases	330				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 48.00.

b. Computed only for a 2x2 table

c. Binomial distribution used.

Chi-Squared Tests for Species vs Smoking

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	3.371[a]	1	.066		
Continuity Correction[b]	2.803	1	.094		
Likelihood Ratio	3.389	1	.066		
Fisher's Exact Test				.076	.047
Linear-by-Linear Association	3.361	1	.067		
McNemar Test				.000[c]	
N of Valid Cases	330				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 21.33.

b. Computed only for a 2x2 table

c. Binomial distribution used.

Chi-Squared Tests for Species vs Age group

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.482[a]	2	.289
Likelihood Ratio	2.530	2	.282
Linear-by-Linear Association	.845	1	.358
McNemar-Bowker Test			.[b]
N of Valid Cases	330		

a. 2 cells (33.3%) have expected count less than 5. The minimum expected count is 1.94.

b. Computed only for a PxP table, where P must be greater than 1.

Chi-Squared Tests for Species vs BMI

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.435[a]	2	.296
Likehood Ratio	2.436	2	.296
Linear-by-Linear Association	.855	1	.355
McNemar-Bowker Test			.[b]
N of Valid Cases	330		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 12.61.

b. Computed only for a PxP table, where P must be greater than 1.

Chi-Squared Tests for Species vs Obesity as determined by WHO criteria

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.658[a]	4	.454
Likelihood Ratio	3.674	4	.452
Linear-by-Linear Association	1.340	1	.247
McNemar-Bowker Test			.[b]
N of Valid Cases	330		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 12.61.

b. Computed only for a PxP table, where P must be greater than 1.

ID API 32C	ID API 32C Candida	dge of the disc to the edge of S. zone/ mn	MIC (DD)	Interpertation of the results
Candida sake	**Candida species**	6	3	R
Candida sake	**Candida species**	16	0	S
Candida sake	**Candida species**	8	3	R
Candida sake	**Candida species**	10	3	R
Candida humicola	**Candida species**	8.5	0	I
Candida humicola	**Candida species**	14	1	S
Candida humicola	**Candida species**	13.5	0	S
Candida humicola	**Candida species**	16	0	S
Candida humicola	**Candida species**	16.5	0	S
Candida humicola	**Candida species**	15	2	S
Candida humicola	**Candida species**	6.5	0	R
Candida humicola	**Candida species**	6.5	0	R
Candida humicola	**Candida species**	14.5	0	S
Candida humicola	**Candida species**	18	2	S
Candida humicola	**Candida species**	12	3	R
Candida humicola	**Candida species**	11.5	I	I
Candida humicola	**Candida species**	17.5	0	S
Candida humicola	**Candida species**	12.5	0	S
Candida humicola	**Candida species**	7.5	0	I
Candida humicola	**Candida species**	12.5	0	S
Candida humicola	**Candida species**	13	3	R
Candida humicola	**Candida species**	10	0	I
Candida humicola	**Candida species**	15	0	S
Candida humicola	**Candida species**	17	0	S
Candida humicola	**Candida species**	11	0	I
Candida humicola	**Candida species**	R	R	R
Candida humicola	**Candida species**	11	3	R
Candida humicola	**Candida species**	17	0	S
Candida humicola	**Candida species**	12.5	0	S
Candida humicola	**Candida species**	12.5	0	**S**
Candida magnoliae	**Candida species**	15.5	0	S
Candida magnoliae	**Candida species**	13.5	2	S
Candida guilliermondii	**Candida species**	R	R	R
Candida globosa	**Candida species**	13.5	0	S
Candida membranifaciens	**Candida species**	10.5	0	I
Saprochaete capitata	**Non-Candida species**	1	0	R
Saprochaete capitata	**Non-Candida species**	0.5	0	R
Saprochaete capitata	**Non-Candida species**	5.5	0	R
Saprochaete capitata	**Non-Candida species**	0.5	0	R
Saprochaete capitata	**Non-Candida species**	R	R	R
Kloeckera apis/apiculata	**Non-Candida species**	11.5	0	
Kloeckera apis/apiculata	**Non-Candida species**	10.5	0	
Trichosporon mucoides	**Non-Candida species**	14.5	0	
Kloeckera japoica	**Non-Candida species**	14	0	