CHAPTER ONE

1.0 INTRODUCTION

Fungi are a group of eukaryotic microorganisms that can occur as yeasts, molds or combination of both forms. They are widespread in the environment. Some are associated with animals and humans as commensals, but turn pathogenic or opportunistic pathogens after alteration of the host immune system. The incidence and prevalence of invasive fungal infections have increased since the 1980s, especially in the large population of immunocompromised patients and/or those hospitalized with serious underlying diseases (Arendrup *et al*., 2005; Espinel-Ingroff *et al*., 2009). Candidiasis is a fungal infection caused by yeasts in the genus *Candida* and species in this genus can cause yeast infections in many areas of the body.

In healthy individuals, *Candida* species are harmless members of the normal gastrointestinal tract (GI), oral, and vaginal microbial flora. It is assumed that everyone carries *Candida* in their GI tract (MacCallum, 2010), with *C. albicans* the species most frequently identified in faecal sampling, representing 40–70% of isolates (Scanlan and Marchesi, 2008). The fungus *Candida* lives in small numbers in a healthy vagina, rectum and mouth. About 75% of women generally harbour this fungus without it causing harm to them (Dam *et al*., 2002). However, sometimes it becomes pathogenic, invades the mucous membrane and causes candidiasis (opportunistic infection) in immunocompromised individuals (Jha *et al*., 2006; Shivanand and Saldanha, 2011).

The clinical spectrum of candidiasis ranges from mucocutaneous overgrowth to disseminated infections like candidemia (Eggimann *et al*., 2003). Candidemia has been estimated as the fourth most common nosocomial infection with an attributed mortality of about 50% (Gudlaugsson *et al*., 2003).

Vulvovaginal candidiasis (VVC) is a *Candida* infection of the lower genital tract affecting approximately 75% of women of child-bearing age and it has remained a common problem worldwide despite therapeutic advances (Sobel, 2007). *C. albicans* is most commonly isolated species, with *C. glabrata* also found, but at a lower frequency (Sobel, 2007; Kennedy and Sobel, 2010), reflecting the species normally in the vulvovaginal area.

Some factors which predispose women to vaginal candidiasis are change in pH, use of oral contraceptives, tight clothing, and personal hygiene (Enweani *et al*., 2001). The increased number of AIDS patients, organ transplantations and other immunocompromised conditios and neoplastic diseases involving broad-spectrum antibiotics, and

immunosuppressive drugs has markedly increased the incidence of disease caused by *Candida* species (La Valle *et al*., 2000; Berzaghi *et al*., 2009). *C. albicans* can transform from yeast cells to hyphal form, a virulence trait that enables it to invade host tissues (Gow *et al*., 2011). Expression of virulence factors like adhesins, phenotypic switching, thigmotropism, and the production of hydrolytic enzymes (mainly proteinases, phospholipases and haemolysins), as well as evasion of host immune cells contribute to the pathogenesis of candidiasis (Silva *et al*., 2011; Mayer *et al*., 2013; Sardi *et al*., 2013).

The prevalence of vaginal colonization of *Candida* species in pregnant women is higher when compared to that of their non-pregnant counterparts (Hay and Czeizel. 2007). Vaginal candidiasis in pregnant women has been reported to cause blood stream infections particularly in low birth weight and premature infants and vertical transmission of *C. albicans, C. parapsilosis*, and *C. glabrata* has been documented (Chong *et al*., 2003).

Although most infections are attributed to *C. albicans*, the shift towards treatment resistant non-albicans *Candida* (NAC) species is evident in recent years (Richter *et al*., 2005; Deorukhkar and Saini, 2013). There has been an increase in the frequency of *C. glabrata*, and *C. krusei* and also to a lesser extent, *C. parapsilosis* and *C. tropicalis*. In addition, non-albicans *Candida* species resistant to many antifungal drugs have been identified. Thus, accurate identification of *Candida* species is crucial to determine appropriate antifungal therapy (Pappas *et al*., 2016).

C. tropicalis is one of the most common NAC species isolated from various clinical candidiasis (Pahwa *et al*., 2014). In India, *C. tropicalis* is the most common cause of health care associated candidemia (Giri and Kindo, 2012). The increased isolation of *C. tropicalis* from various clinical candidiasis is of concern because of its ability to develop rapid resistance to fluconazole (Yang *et al*., 2004). Also in Malaysia, there have been reports of the emergence of fluconazole resistance among *C. albicans*, *C. tropicalis*, and *C. parapsilosis* isolated from candidemic patients (Amran *et al*., 2011). Up to 33% of NAC species are responsible for recurrent infections (Onifade and Olorunfemi, 2005) and this can be a result of a relatively high antifungal resistance rate of non-albicans *Candida* species. This increase in NAC candidiasis has been attributed to overuse of antifungal therapy, which has resulted in the elimination of the more sensitive *C. albicans* and selection of other, more resistant species.

Apart from the limited number of antifungal drugs used to treat vulvovaginal candidiasis, oral azoles have a potential of systemic toxicity. Thus, the need of an alternative source of antifungal agents for treatment cannot be overemphasized. Plants have been used over the

years for the treatment of diseases both in animals and humans. Thus, there is no doubt that plants represent an alternative source of new antibiotics (Afolayan, 2003) especially for those diseases caused by microorganisms that have developed resistance to commonly used drugs. Antibacterial as well as antifungal activities of extracts of *Moringa oleifera, Vernonia amygdalina* and *Ocimum gratissimum* have been documented by some researchers (Alo *et al*., 2012; Moyo *et al*., 2012; Mabekoje *et al*., 2013). However, there is not much literature of the antifungal activities of the extracts of *Vernonia amygdalina* and *Ocimum gratissimum*.

Ghamba *et al*. (2014), showed the *in vitro* antimicrobial activities of *Vernonia amygdalina* on selected clinical isolates including *Candida albicans*. Also, in India, Pinal *et al*. (2014) carried out a research on the antifungal activity of aqueous and ethanolic extracts of *Moringa oleifera* against *Saccharomyces cerevisiae* (MTCC No.170), *Candida albicans* (MTCC No.183), *and Candida tropicalis* (MTCC No.1000). Lika *et al*. (2014) investigated the effect of the ethanolic 70% extract and flavenoids extract of *Moringa oleifera* Lam. on *Staphylococcus aureus*, *Streptococcus pyogenes* and *Candida albicans* isolated from the oral cavity of patients suffering from dentured stomatitis in Bagdad city. Mbakwem *et al*. (2012) evaluated the antifungal effects of the ethanolic extract of *Ocimum gratissimum* leaves on common dermatophytes and causative agent of *Pityriasis versicolor* in Rivers State, Nigeria. The antifungal activity of ethanolic crude extract of *O. gratissimum* leaves against *Cryptococcus neoformans* in Goiânia city, Goiás, Brazil has been investigated (Janine de Aquino *et al*., 2005). Vroumsia *et al*. (2013) worked on the sensitivity of *Candida albicans* to six extracts of locally used antifungal plants which included *Moringa oleifera* and *Ocimum gratissimum*.

The concurrent use of any of these plants with any of the antifungal drugs like the azoles or polyenes against clinical isolates of *Candida* species could yield greater potency against *Candida* species, especially resistant strains. However, there is less work carried out on the combined effects of these plants with any of the antifungal drugs like the azoles or polyenes against clinical isolates of *Candida* species. In one study, the effect of interaction between the ethanolic extracts of *Ocimum gratissimum* leaves and the antifungal drugss (Ketoconazole and Nystatin) was synergistic on clinical *Candida albicans* (Nweze and Eze, 2009). The essential oil fraction of *Pelargonium graveolens* and its main components, geraniol and citronellol, have been reported to exhibit strong synergism with ketoconazole against *Trichophyton schoenleinii* and *Trichophyton soudanense*, with fractional inhibitory concentration (FIC) indices in the range of 0.18–0.38 (Shin and Lim, 2004).

Zhang *et al*. (2017) investigated the antifungal activity of a hydroalcoholic extract from *Flos Rosae Chinensis* combined with Fluconazole against clinical isolates of *Candida albicans* resistant to Fluconazole. In a study carried out by Anejionu *et al*. (2011) there was neither synergism nor antagonism in all the combinations of *O. gratissimum* oil and ibuprofen against *Candida albicans*.

It is clear that literature on the antifungal activities of these plants (especially *Ocimum gratissimum* and *Vernonia Amygdalina*) against clinical isolates of *Candida* species is not sufficient. Also, not much has been reported vis-à-vis the resistance pattern of clinical isolates of non-albicans *Candida* species in the Southeastern part of Nigeria. This study was therefore undertaken to evaluate the antifungal resistance pattern among clinical isolates of *Candida* species and their susceptibility to some medicinal plants.

1.1. Statement of Problem

Between 20 – 50% of healthy asymptomatic women have *Candida* species as part of the lower genital tract flora. Although *Candida* species colonize mucosal surfaces in an asymptomatic manner, they can become one of the most significant causes of a disabling and lethal infection (Wisplinghoff *et al*., 2006; Vincent *et al*., 2009).

Reports show that about 75% of all women, experience at least one episode of which physician approved to be candidiasis in their lifetime (Akah *et al*., 2010).

Vaginal candidiasis is a frequent companion of pregnancy, which greatly complicates the course of the pregnancy and threatens the health of both mother and child (Giraldo *et al*., 2012). Vaginal candidiasis in pregnant women was reported to cause blood stream infections particularly in low birth weight and premature infants (Bliss *et al*., 2008).

The second most common cause of abnormal discharge after bacterial vaginosis in healthy women of reproductive age is vulvovaginal candidiasis (Udayalaxmi *et al*., 2014).

Over 80% patients, referred by physicians with a putative diagnosis of Vulvovaginal candidiasis (VVC) were found to have some other cause of vaginitis, and therefore most patients fail to respond to antifungal therapy because of incorrect diagnosis (Weissenbacher *et al*., 2009).

There is an increase in non-albicans *Candida* (NAC) vulvovaginal candidiasis which is attributed to overuse of antifungal therapy and this has led to antifungal resistance. Apart from the limited number of antifungal drugs used to treat vulvovaginal candidiasis, oral azoles have a potential of systemic toxicity. Thus, there is the need for an alternative source of antifungal agents for treatment.

1.2. Aim of this research:

The aim of this research was to evaluate the antifungal resistance pattern among clinical isolates of *Candida* species and their susceptibility to some medicinal plants.

1.3. Objectives of this research:

The objectives of this research were to;

1. Isolate and identify *Candida* species from high vaginal swabs (HVS) of women attending the Obstetrics and Gynecology Unit of the University of Nigeria Teaching Hospital, Ituku/Ozalla, Enugu State.
2. Determine the prevalence of vaginal *Candida* colonization among the women based on age, marital status, pregnancy, trimester of pregnancy, symptoms and antibiotics intake.
3. Evaluate the *in vitro* antifungal resistance pattern of the *Candida* isolates to some antifungal drugs (Fluconazole, Ketoconazole, Voriconazole, Clotrimazole, Itraconazole, Flucytosine, Nystatin and Amphotericin B).
4. Evaluate the *in vitro* effect of the ethanolic extracts of 3 plants (*Moringa oleifera*, *Ocimum gratissimum* and *Vernonia amygdalina*) against some strains of the *Candida* species resistant to some of the above antifungal drugs.
5. Evaluate the combined effect of Fluconazole with each of the plant extract against the selected resistant strains by Checkerboard method.
6. Determine the nucleotide sequence of resistant gene responsible for the resistance in the selected resistant strains.
7. Identify mutations present in the resistant gene that contribute to amino acid substitutions in the target enzyme

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Kingdom Fungi:

This is made of organisms that include microorganisms such as yeasts and molds, as well as the mushrooms. These organisms are classified as a kingdom (fungi) which is separate from the other eukaryotic life kingdoms of plants and animals. The Kingdom Fungi has been estimated to have 2.2 million to 3.8 million species (Hawksworth and Lücking, 2017). Of these, only about 120,000 have been described, with over 8,000 species known to be detrimental to plants and at least 300 that can be pathogenic to humans. Based on their macroscopic appearance, fungi can exist as molds or yeasts or a combination of both (that is dimorphic fungi) depending on the temperature. The molds are multicellular while the yeasts are unicellular. *Candida* species are examples of yeasts.

2.2. *Candida* species

This genus contains over 150 heterogeneous species (Calderone, 2002) and more than 17 different *Candida* species are known to be causative agents of human infections (Pfaller *et al*., 2007). *Candida* species are members of the normal flora of the skin, mucous membranes and gastrointestinal tract. Several species are capable of causing candidiasis. However, the most common aetiologic agents of candidiasis are *Candida albicans*, *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis*, *Candida guilliermondii* and *Candida dubliniensis*. There has also been the emergence of more azole-resistant species (*Candida krusei* and *Candida lusitaniae*)(Brooks *et al*., 2013).

Candida albicans has been reported to be the species most often isolated especially from clinical specimens (Akortha *et al*., 2009; Abruquah, 2012; Amar *et al*., 2014; Madhumati *et al*., 2015; Efunshile *et al*., 2016). In their study involving pregnant women attending antenatal clinic in a tertiary health center in North-west Nigeria, Nnadi and Sing (2017) reported *Candida albicans* was the most frequent species isolated in 73.8% of the pregnant women while the rest were NAC species constituting 26.3%. In another study by Alizadeh *et al*. (2017) in Mashhad, Iran, *C. albicans* was the most frequently isolated species constituting 58.5%, followed by *C. tropicalis* (16.9%), *C. glabrata* (7.7%), *C. parapsilosis* (7.7%), and *C. guilliermondii* (3.1%). Prevalence rate of *Candida albicans* in 60% of high vaginal smears has been reported from a study in Jos, Northen Nigeria (Ibrahim *et al*., 2013) while in India, studies indicated that *C. albicans* was present in 74.4% while 25.6% were NAC species

(Jindal *et al*., 2007). One reason why *Candida albicans* predominates NAC species especially from vaginal specimens may be due to the fact that *C. albicans* adheres to vaginal epithelial cells in significantly higher numbers than do other *Candida* species (Dias *et al*., 2011; Payne *et al*., 2016). This could explain the relative infrequency of the later in vaginal candidiasis.

A rising trend in the isolation of NAC vaginitis has been reported by some studies from India and this was attributed to the indiscriminate use of anti-fungal agents which eliminates the more sensitive *C. albicans* and selects resistant NAC species (Pirotta *et al*., 2003). Amutaigwe *et al*. (2017) working with isolates from high vagina swab (HVS) specimens in Nnewi reported *Candida tropicalis* was the most predominant species constituting 38.2%. The results of Almeida *et al*. (2013) also indicated *Candida tropicalis* as the most commonly isolated yeast species from patients being treated at the University Hospital of the Federal University at Grande Dourados, Central-western Brazil. A similar study by Okungbowa *et al*. (2003), in some seven cities in Nigeria, revealed that *Candida glabrata* had the highest frequency of occurrence (33.7%) while *Candida parapsilosis* had the least (5%).

2.3. Identification Methods of *Candida* Species

There are several identification methods for *Candida* species. These include Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry and growth on chromogenic medium (CHROMagar *Candida*).

2.3.1 Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry

Most commonly isolated yeast species can be identified by the commercially available biochemical test systems but the not so common species may remain unidentified or misidentified by these tests (Sanguinetti *et al*., 2007). Molecular methods have been developed to overcome the inaccuracies of biochemical identification methods. The assays involved in molecular methods are highly accurate. However, they require considerable processing time and expensive reagents. Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) can be used as an alternative to biochemical and molecular identification schemes for the reliable identification of *Candida* species. The use of MALDI-TOF MS for the routine identification of microorganisms (including yeasts) has been documented (Bizzini *et al*., 2010; van Veen *et al*., 2010; Bessède *et al*., 2011). In the MALDI-TOF MS technique, spectra are compared to a reference database, and recognition is

acquired by matching the unknown spectrum to the most similar spectrum in the database to identify the unknown microorganism (Lagacé-Wiens *et al*., 2012). This implies that microorganisms are identified through their unique protein profiles (mass spectrum) which are generated by the instrument and then matched to a reference database of known microbial spectra.

Mechanism of Matrix Assisted Laser Desorption/Ionization: In the MALDI-TOF MS pure cells prepared from culture materials (e.g agar plates) serve as the analyte. Basically, a small amount of intact cells is directly streaked onto the MALDI target plate and overlaid with a small amount of matrix solution (usually a UV-absorbing weak organic acid) lysing the cells. Drying embeds the components of the cells (that have been released) into a crystalline matrix. The matrix serves both as the chemical ionizing agent and for energy transfer from the laser to the analyte. The analyte-matrix mixture is bombarded with a laser. The matrix stimulates the process of transforming laser energy into excitation energy (Morris *et al*., 2005). The analyte is desorbed from the target plate by laser fire producing ions. The resulting ions are accelerated in an electric field and focused to fly along the flight tube to heat the detector. The mass of ions is determined on the basis of time particular ions take to drift through the spectrometer. Time-resolved impact on the detector yields a characteristic spectrum where the observed peaks represent mass-to-charge (*m*/*z*) ratio and abundance of the underlying analytes, with small molecules flying more than large ones. The list of peak masses above the S/N threshold is classified in a central database by comparison to those lists obtained from reference spectra. These spectra are highly reproducible within a species but sufficiently different even between highly related species to allow a precise species determination.

The MALDI Biotyper (Bruker Daltonics, Bremen, Germany), the AXIMA@SARAMIS database (AnagnosTec, Potsdam, Germany and Shimadzu, Duisburg, Germany), the Andromas (Andromas, Paris, France) and VITEK MS systems (bioM´erieux, Marcy l'Etoile, France) are four commercial systems in use (Bader, 2012). In some cases where identification was not possible at the first attempt, this could be achieved by simply repeating the analysis (Bader *et al*., 2011). Three preparation procedures exist: extraction, on-target-lysis and direct smear methods (Bader, 2012). The MALDI Biotyper reference spectra have been made by extraction and the measure for quality of the database hit is the 'log scores'. In clinical routine, log-scores ≥2.000 are classified as "species level" identifications, log-scores between 1.700 and 1.999 are initially classified as "genus level only" (Bader, 2012; Cameron *et al*.,

2017) and a score of < 1.700 is considered not reliable identification (Cameron *et al*., 2017) All spectra scoring ≥2.000 must be of the same species, all spectra scoring ≥1.700 of the same genus.

2.3.2 Chromogenic medium - CHROMagar *Candida:* The chromogenic medium CHROMagar *Candida* (CHROMagar *Candida*, France) is used for the isolation and presumptive identification of *C. albicans* based on the pigmentation of the developing colonies, which is due to different enzyme activities from *Candida* species. This medium shows different color colonies for *C. albicans* (green), *C. tropicalis* (metallic blue, with a pink halo), and *C. krusei* (pink with velvety appearance) (Eraso *et al*., 2006; Ghelardi *et al*., 2008).

2.4 The Pathogenicity of *Candida* species: *Candida* is a genus that is the most common cause of candidal infections worldwide (Manolakaki *et al*., 2010). Many species are harmless commensals of humans. However, when mucosal barriers are disrupted or the immune system is compromised they can invade and cause disease (Kourkoumpetis *et al*., 2011). Overgrowth of several species including *Candida albicans* can cause infections ranging from superficial (such as oropharyngeal candidiasis and vulvovaginal candidiasis to systemic (such as fungemia and invasive candidiasis). There are virulence factors that aid in the colonization and pathogenicity of *Candida* species. These virulence factors include the ability to evade host defences, expression of adhesins and invasins, biofilm formation (on host tissue and on medical devices) and the production of tissue-damaging hydrolytic enzymes (such as proteases, phospholipases and haemolysin), morphogenetic transformation and phenotypic switching (Silva *et al*., 2011). Infection models of candidosis in animals suggest that *Candida albicans* is the most pathogenic species (Samaranayake and Samaranayake, 2001), and it also expresses higher levels of putative virulence factors compared with other species (Jayatilake *et al*., 2006).

2.4.1 Adherence to host surfaces: This is the primary event in *Candida* colonization and is required for initial colonization. Adherence is considered very essential in the establishment of disease as it contributes to persistence of the *Candida* species within the host. The profile of cell wall proteins (Chaffin, 2008) and the physicochemical properties of the cell surface (Henriques *et al*., 2002) are important factors that influence adhesion. Previous studies of the cell wall of *Candida* have suggested a relationship between cell surface hydrophobicity and

adherence (Panagoda *et al*., 2001). *Candida* species can also adhere to the surfaces of medical devices and form biofilms.

2.4.2 Proliferation and biofilm formation: Once attached to host or medical devices, cell division takes place followed by proliferation and biofilm formation (Ramage *et al*., 2006). Biofilms are described as surface-associated communities of microorganisms embedded within an extracellular matrix. Biofilms are now considered to represent the most prevalent growth form of microorganisms (Silva *et al*., 2009a). The species most frequently associated with formation of biofilms affecting different types of immunocompromised patients is *Candida albicans* (Ramage *et al*., 2005). The biofilm matrix is composed mainly of carbohydrates, proteins, phosphorus and hexosamines but the amount of each component is highly dependent on the strain (Silva *et al*., 2009a). An important virulence factor for a number of *Candida* species is biofilm formation, as it confers significant resistance to antifungal therapy. This is because the penetration of substances through the matrix is limited and also the cells are protected from host immune responses (Donlan and Costerton, 2002; Mukherjee and Chandra, 2004). The formation of biofilms by *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis* and *Candida glabrata* isolates have been associated with higher morbidity and mortality rates compared with isolates that are unable to form biofilms (Kumamoto, 2002).

4.2.3 Virulence Enzymes: After successful adhesion to host cells, the *Candida* species can only penetrate the host further by the aid of some virulence enzymes. These hydrolytic enzymes released by the *Candida* species facilitate the destruction of host tissues. They play a major role in the overgrowth of *Candida* species as they pave the way for adhesion, penetration and tissue invasion (Schaller *et al*., 2005). Secreted aspatyl proteinases (SAPs) disrupt the host mucosal membranes thus, facilitating invasion and colonization of host tissues. Both vaginal and skin isolates of *Candida parapsilosis* have been documented to exhibit higher *in vitro* SAP activity than blood isolates, thus, establishing a trend relating SAP production and site of strain isolation (Dagdeviren *et al*., 2005). Phospholipases hydrolyze phospholipids into fatty acids and are suggested to contribute to host cell damage which could also expose receptors to facilitate adherence (Kantarcioˇ lu and Y¨ucel, 2002). Also, proteolytic enzymes secreted by *Candida* isolates destroy host proteins such as lactoferrin and immuno-globulin. The destruction of these host proteins attenuates the host immune system thereby contributing to infection development (Meiller *et al*., 2009). *Candida*

species use haemolysins to degrade haemoglobin and facilitate recovery of the elemental iron from host cells.

Haemolysins are considered one of the key virulence factors that enable pathogen survival and persistence in the host (Luo *et al*., 2004). The ability of *Candida albicans* to produce haemolysins enables it to acquire elemental iron from host tissues which it uses for metabolism, growth and establishment of infection in humans (Almeida *et al*., 2009). The first step of *Candida albicans* infections *in vivo* involves binding to erythrocytes through the complement system. *Candida parapsilosis*, *Candida tropicalis* and *Candida glabrata* have the ability to produce haemolysins *in vitro* which can induce partial or total lyses of erythrocytes. However, the production of haemolysins is species dependent (Luo *et al*., 2004).

2.4.4 Dimorphic transition: In *Candida*, the dimorphic transition (dimorphic switching) from the yeast to the hyphal phase is a crucial step in the formation of biofilms. Mutant strains that lack the ability to germinate are unable to form dense and homogeneous biofilms (Kruppa *et al*., 2004; Jabra-Rizk *et al*., 2006). The fungal invasion is facilitated more by transition between yeast cells and filamentous growth than yeast growth (Cullen and Sprague, 2012). In an *in vitro* research, *Candida albicans* that lack hyphal formation was shown to exhibit lower ability to invade tissue compared with wild-type *Candida albicans* strains (Jayatilake *et al*., 2006). Also, it has been documented that filamentous forms (hyphae and/or pseudohyphae) of *Candida* species demonstrate increased resistance to phagocytosis compared with yeast (Gow *et al*., 2002).

2.5 Epidemiology of Vaginal Candidiasis

Candida species are opportunistic pathogens that reside on the mucosa of the gastrointestinal tract as well as the mouth, oesophagus and vagina (Kim and Sudbery, 2011; Lim *et al*., 2012). Although *Candida* species colonize mucosal surfaces in an asymptomatic manner, they can become one of the most significant causes of a disabling and lethal infection (Wisplinghoff *et al*., 2006; Vincent *et al*., 2009). The second most common cause of abnormal discharge after bacterial vaginosis in healthy women of reproductive age is vulvovaginal candidiasis (Udayalaxmi *et al*., 2014). Between 20 – 50% of healthy asymptomatic women have *Candida* species as part of the lower genital tract flora. Vulvovaginal candidiasis (VVC) is a *Candida* infection of the lower genital tract affecting

approximately 75% of women of child-bearing age and it has remained a common problem worldwide despite therapeutic advances (Sobel, 2007).

Vulvovaginal candidosis (VVC) is usually classified as either acute or recurrent on the basis of episodic frequency (Sobel, 2007). Acute VVC is reported in three out of four women, while nearly one in two women have two or more episodes during their lifetimes. However, 5 – 8% of women have recurrent VVC which is defined as four or more episodes every year (Sobel, 2007). The prevalence of vaginal candidiasis is highest among women within the age group 16-30 years. The highest prevalence of vaginal *Candida* species has been recorded in age groups 26 – 30 (35%), 15 – 20 (25.6%), 16 – 20 (15.6%) and 21 – 30 (46.8%) by Okungbowa *et al*. (2003), Abruquah (2012), Maikenti *et al*. (2016) and Okolo *et al*. (2017) respectively. This can be attributed to the fact that women aged 16 to 30 years are very much sexually active and more often engage in sexual promiscuity, drug abuse and the use of contraceptives making them more vulnerable. The least prevalence of vaginal *Candida* isolates has been documented to be found among women aged 41 years and above (Okungbowa *et al*., 2003; Abruquah, 2012; Uzoh *et al*., 2016; Okolo *et al*., 2017)

There are factors that predispose women to vaginal candidiasis. These factors include change in pH, use of oral contraceptives, tight clothing, and personal hygiene. Pregnancy, diabetes and prolonged intake of antibacterial drugs are also predisposing factors of vaginal candidiasis. The increased number of AIDS patients and other immunocompromised patients has markedly increased the incidence of disease caused by *Candida* species (Berzaghi *et al*., 2009).

2.5.1. Pregnancy: A higher prevalence of vaginal colonization and symptomatic vaginitis is more often seen in pregnant women than in those who are not pregnant. This can be attributed to high concentrations of reproductive hormones during pregnancy. These increase the glycogen content in the vaginal tissue thus, providing a carbon source for *Candida* organisms (Dennerstein and Ellis, 2001). This causes the organism to grow faster and stick more easily with the walls of vagina (Parveen *et al*., 2008). Thus, there is almost a twofold incidence of candidiasis in pregnant women (particularly in the third trimester) when compared to their non-pregnant counterparts (Fernández *et al*., 2004). Uzoh *et al*. (2016) and Amutaigwe *et al*. (2017) demonstrated a higher prevalence of vaginal *Candida* colonization in the pregnant women (40% and 36.8%) than in their non-pregnant counterparts (17% and 21.7%) respectively in Asaba (South-South, Nigeria) and Nnewi (South-East, Nigeria). Vroumsia *et al*. (2013) also reported a higher prevalence rate of vulvovaginal candidiasis

amongst pregnant women (55.4%) than amongst non-pregnant women (35.4%) in Maroua, Far-North, Cameroon.

2.5.2. Douching and the Use of Contraceptives: Some women engaged in various forms of practices including douching and the use of contraceptives to prevent pregnancy. Frequent douching with antiseptics can alter the microbial flora of the vagina thus exposing it to *Candida* infection (Vroumsia *et al*., 2013). Oviasogie and Okungbowa (2009) showed there was a significant difference in the rate of colonization by *Candida* between women who used contraceptives (58.3%) and those who never used contraceptives (35.3%). Also, Eweani *et al*. (2001) showed that women who used contraceptive had a prevalence of 51.5% of vaginal colonization compared to 40.6% for non-contraceptive users in Edo State, Nigeria.

2.5.3. Diabetes Mellitus: Diabetes mellitus predisposes individuals to fungal infections, including those caused by *Candida* species especially if blood sugar is not well controlled. Diabetes mellitus patients are at increased risk of vulvovaginal candidiasis (Goswami *et al*., 2006). Elevated blood sugar appears in the mucus of the vagina and vulva, so they serve as an excellent culture medium for yeast. Amar *et al*. (2014) reported that diabetes mellitus was the major predisposing factor causing candidiasis constituting 33% followed by pregnancy (22.3%).

2.5.4. Prolonged Use of Antibacterial Drugs: Yeasts of the genus *Candida* are normal commensals colonizing the vagina but their overgrowth is checked by the lactobacilli. *Lactobacillus acidophilus* which produces hydrogen peroxide (H_2O_2) as a by-product. This H_2O_2 is toxic to pathogens and this helps to keep the healthy vagina pH acidic. A dynamic equilibrium is maintained between the yeasts and bacterial population. Administration of antimicrobial drugs (antibacterial) suppresses the friendly bacterial population (especially lactobacilli) in the vagina, altering the vaginal microflora and thus, leading to the increase in number of various bacteria and yeasts (Brooks *et al*., 2013) including *Candida* species.

2.6 Classes of Antifungal Drugs

There are several classes of compounds that comprise the arsenal used to treat *Candida* infections (Whaley *et al*., 2017) and other mycoses. The major classes of antifungal drugs are the azoles, polyenes, nucleoside analog, echinochandins and allylamines. These drugs are used with varying efficacy depending on the type and site of infection and the sensitivity of the *Candida* species (Pfaller *et al*., 2010; Pfaller *et al*., 2013; Pappas *et al*., 2016).

2.6.1 The Azoles

The azoles comprise the largest family of antifungal drugs. The azole family includes imidazoles (Miconazole, Econazole, Clotrimazole and Ketoconazole) and triazoles (Fluconazole, Itraconazole, Voriconazole and Posacconazole. Voriconazole is a second-generation, synthetic triazole derivative of Fluconazole while Posaconazole is a hydroxylated analogue of Itraconazole. Fluconazole is the most widely used drug for treating candidiasis (Sheikh *et al*., 2013) generally, and is the most commonly prescribed antifungal used for most *Candida albicans* infections (Pfaller *et al*., 2010). Fluconazole and Itraconazole have been used extensively for chemoprophylaxis and treatment of systemic fungal infections owing to their favorable oral bioavailability and safety profiles (Livermore, 2004). A major problem in antifungal therapy is the wide spread and prolonged use of azoles which promote rapid development of the phenomenon of multidrug resistance (Isabelle *et al*., 2006; Sheikh *et al*., 2013).

Mechanism of action: The azoles as a group possess similar mechanisms of action. However, they clearly have differing spectra of activity. For example, Fluconazole shows good activity against the yeasts but is not the drug of choice for most common mold infections, such as aspergillosis (Forthergill *et al*., 2006). The azoles act by targeting the biosynthesis of ergosterol which is the major sterol of the fungal plasma membranes (Morschhäuser, 2002; Sheikh *et al*., 2013). Ergosterol ensures the integrity and functionality of the fungal plasma membrane. The azoles target a cytochrome P-450 enzyme (lanosterol 14α-demethylase). This enzyme catalyses the conversion of lanosterol to ergosterol by the oxidative removal of the 14α methyl group from lanosterol. The azoles bind to the catalytic site (containing a hemecofactor) of the enzyme and as a result inhibit the demethylation of lanosterol (Sheikh *et al*., 2013). This inhibition makes ergosterol synthesis and cell membrane formation impossible. This results in ergosterol depletion and accumulation of

toxic ergosterol precursors into the plasma membrane, thus disrupting membrane's integrity and some of its functions such as nutrient transport. All these finally lead to the inhibition of fungal growth (Xu *et al*., 2008; Sheikh *et al*., 2013).

Mechanisms of Azole Resistance: In *Candida* species, the mechanism of resistance to azole antifungal agents may be as a result of (i) qualitative or quantitative changes in the target enzyme and (ii) reduction in the intracellular concentration of readily accessed azole to its target. In the qualitative change, there are alterations in the affinity of the target enzyme (lanosterol 14 α – demethylase), which results in a reduced binding affinity of the enzyme to azoles (Morschhauser, 2002). The quantitative change leads to increase cellular content of lanosterol 14 α – demethylase due to target site mutation that finally results into increased ergosterol synthesis. Increased intracellular production of the enzyme leads to an increase in the effective drug dose (Strzelczyk *et al*., 2013).

Reduction in the intracellular concentration of readily accessed azole is caused by changes in the cell wall or plasma membrane, which lead to impaired azole uptake. Alterations in sterol and/or phospholipid composition of the membrane and related reduced permeability of the cell wall or plasma membrane may be the cause of this poor penetration of azoles across the membrane. Another cause of reduction in the intracellular concentration of readily accessed azole may be due to pumping out by over expressed efflux systems (Morschhauser, 2002; Arikan and Rex, 2005).

Molecular Aspect of Azole Antifungal Resistance Mechanism: Azole antifungal resistance in *Candida* species has been most extensively studied for Fluconazole and *Candida albicans* (Perea and Patterson, 2002; Whaley *et al*., 2017). The molecular mechanisms include (i) Alterations in the gene (ERG 11) encoding the target enzyme and (ii) over expression of genes coding for membrane transport proteins. Several point mutations in resistant strains of *Candida albicans* have been identified in the ERG 11 gene sequence and so far, seven different point mutations with azole resistance have been defined. A point mutation leading to replacement of arginine with lysine at amino acid 467 has been found to be associated with azole resistance in a clinical strain of *Candida albicans* (White, 1997). In a study that was carried out, 63 Fluconazole-resistant clinical isolates of *Candida albicans* were examined for mutations within their ERG11 alleles, and 55 were found to carry at least one mutation that resulted in amino acid substitutions with nine such predicted amino acid substitutions being novel (Flowers *et al*., 2015). Point mutations in the ERG11 gene that encodes the target

enzyme (lanosterol 14 α –demethylase), lead to an altered target with decreased affinity for azoles.

Over expression of the ERG 11 gene has been observed in azole resistant clinical isolates of *Candida albicans*. However, the role of this phenomenon to the development of resistance is not exactly known. Over expression of ERG 11 gene results in the production of high concentrations of the target enzyme, creating the need for higher intracellular azole (Fluconazole) concentrations to inhibit all of the enzyme molecules in the cell. Molecular studies have revealed that there are two types of efflux pumps, which are responsible for the development of azole resistance in *Candida* species; the ATP- binding cassette (ABC) transporters (encoded by CDR1 and CDR2 genes) and major facilitators superfamily (MFS) proteins (encoded by MDR1 and FLU1 genes). Over expression of these genes in fungal cells account for the enhanced efflux of azoles and reduced intracellular accumulation of those drugs in fungal cells (Morschhäuser, 2002; Xu *et al*., 2008).

In a study carried out with 52 clinical isolates of *Candida tropicalis* from China, the average ERG11 expression level was found to be more than 4-fold higher among Fluconazole-resistant isolates than-susceptible isolates (Jiang *et al*., 2013). ERG11 does not appear to play an important role in clinical azole resistance in *Candida glabrata* (Sanguinetti *et al*., 2005). Over expression of ERG11 has been observed in only two clinical isolates of *Candida glabrata* (Redding *et al*., 2003). *Candida glabrata* is able to grow with altered cell membrane sterols, and this allows it to evade azole treatment. Also, *Candida glabrata* has the ability to take up exogenous sterols (Nakayama *et al*., 2000), both when the ergosterol biosynthesis pathway is blocked and under normal conditions in wild type strains (Tsai *et al*., 2004; Bard *et al*., 2005).

Candida krusei is intrinsically resistant to Fluconazole, though the precise mechanism is not completely understood. The innate azole resistance of *Candida krusei* have been attributed to efflux pump activity, namely through the ATP-binding cassette transporter Abc1p, and reduced drug accumulation (Katiyar and Edlind, 2001; Lamping *et al*., 2009) in combination with reduced azole affinity for Erg11p (Guinea *et al*., 2006; Lamping *et al*., 2009).

2.6.2 The Polyenes

Polyenes are poly-unsaturated organic compounds that contain at least three alternating double and single carbon-carbon bonds. The polyenes that are used as antibiotics for humans include Amphotericin B, nystatin, candicidin, pimaricin, methyl partricin,

and trichomycin (Zotchev, 2003). Two of the most important polyenes used for the treatment of candidiasis are Amphotericin B and Nystatin. However, Amphotericin B is the most important as far as development of resistance is concerned (Sheikh *et al*., 2013).

Mechanism of Action of Polyenes: The polyenes act by binding or interacting with ergosterol. Ergosterol is an important component of fungal cell membrane and is essential for maintaining fluidity and integrity of the membrane as well as for proper functioning of the membrane-bound enzymes. Binding of the drug with ergosterol disrupts the fungal cytosplasmic membrane creating channels and aqueous pores. As a result, the cellular permeability of the membrane is altered leading to leakage of many cellular components particularly potassium and magnesium ions. This leakage destroys the proton gradient of the cytoplasmic membrane and thus, causes death of the cell (Ellis, 2002; Sanglard and Odds, 2002).

Mechanism of Resistance of *Candida* species to Polyenes: The affinity of polyenes for ergosterol is very much higher than that for other sterols such as fecosterol and episterol. This low affinity of polyenes for sterols such as fecosterol and episterol play a very important role in the development of resistance to polyenes (Arikan and Rex, 2005). Thus, changes in ergosterol content may contribute to the development of resistance to polyenes, especially Amphotericin B. These changes can be quantitative or qualitative.

The quantitative changes in ergosterol content that contribute to development of resistance include: (i) decrease in the content of ergosterol which is a result of inhibition of its synthesis (ii) alteration of sterol content by replacement of ergosterol with sterols with reduced affinity and (iii) alterations in the ratio of sterol to phospholipids (Arikan and Rex, 2005).

The qualitative changes in ergosterol that may lead to development of resistance include reorientation or masking of ergosterol in the cell membrane as a result of which there is no binding with polyenes. There have been observations that most of the clinically isolated polyene-resistant *Candida* are the non-*Candida albicans* species notably *Candida tropicalis* and *Candida lusitaniae*. Also, the potential for polygene resistance is reported to be high in *Candida glabrata* and *Candida parapsilosis*. Owing to its haploid nature, *Candida glabrata* can mutate frequently, and develop resistance faster than *Candida albicans* and in *Candida parapsilosis.*

Molecular Aspect of Polyene (Amphotericin B) Resistance Mechanism: The polyenes (for example Amphotericin B) act by interacting with ergosterol. There are many enzymes that are involved in the synthesis of ergosterol of which C-8 sterol isomerase and C-5 sterol desaturase are among the most important. The enzyme, C-8 sterol isomerase catalyzes the production of episterol from fecosterol and its activity is regulated by ERG 2 gene while C- 5 sterol desaturase is responsible for the conversion of episterol into ergosterol and is encoded by ERG 3 gene. Mutations in ERG 2 and ERG 3 genes which encode these two important enzymes (involved in ergosterol synthesis) are responsible for Amphotericin B resistance. There have been reports of Amphotericin B resistant clinical strains of *Candida albicans* with defective ERG2 and ERG 3 genes and reduced ergosterol (Arikan and Rex, .2005)).

2.6.3 Nucleoside Analogs

A very important member in this group of antifungal drugs is Flucytosine (5-Fluorocytosine). Flucytosine (5-FC) was first synthesized in 1957 as an anticancer drug. However, it did not exhibit antineoplastic activity but was subsequently found to possess antifungal activity and was used in 1968 to treat human cryptococcosis and candidiasis (Vermes *et al*., 2000).

Mechanism of Action: Nucleoside analogs (such as Flucytosine) inhibit DNA/RNA and protein synthesis in fungi. Flucytosine is actively transported into the cell by cytosine permeases. After entry into the cell, it is deaminated (catalysed by cytosine deaminase) to 5-fluorouracil and then phosphorylated to 5-fluorodeoxyuridine monophosphate. Further phosphorylation produces 5-fluorouracil triphosphate. This reaction is catalysed by uracil phosphoribosyl transferase. The 5-fluorodeoxyuridine monophosphate interferes with DNA synthesis by inhibiting thymidine synthetase. Meanwhile, the 5-fluorouracil triphosphate gets incorporated into RNA and inhibits protein synthesis (Vermes *et al*., 2000; Arikan and Rex, 2005). Cytosine deaminase is lacking in mammalian cells and as a result, the mammalian cells are not directly subject to the toxic effects of Flucytosine.

Mechanism of Resistance Against Flucytosine: Primary resistance to Flucytosine remains low (<2%) while secondary resistance depends on inactivation of different enzymes of the pyrimidine pathway (Spampinato and Leonardi, 2013). Primary resistance has been observed in around 3% of isolates (Pfaller *et al*., 2002). One mechanism is a reduction in intracellular accumulation of the drug. This occurs when there are point mutations in the FCY2 gene that

encodes for cytosine permease (Espinel-Ingroff, 2008) which is involved in the active transport of the drug into the cell.

Another mechanism is by counteraction of the drug effect. This is acquired resistance which results from point mutations in the FCY1 gene which encodes for the cytosine deaminase or FUR1 gene which encodes for the uracil phosphoribosyl transferase. Cytosine deaminase and uracil phosphoribosyl transferase respectively catalyze the conversion of 5-fluorocytosine to 5-fluorouracil and 5-fluorouracil to 5- fluorouridine monophosphate. Point mutations in the FUR1 gene have been documented to be the basis of the most frequently acquired resistance to Flucytosine. Several point mutations have been described in *Candida albicans, Candida glabrata* and *Candida lusitaniae* (Chapeland-Leclerc *et al*., 2005; Espinel-Ingroff, 2008; Pem´an *et al*., 2009). Resistance to Flucytosine has been documented to emerge rapidly if used as monotherapy (Charlier *et al*., 2015).

2.6.4 Echinocandins

Echinocandins are lipopeptidic antifungal agents and include caspofungin, micafungin, and anidulafungin. These echinocandins exhibit concentration-dependent fungicidal activity against most species of *Candida* (Cappelletty and Eiselstein-McKitrick, 2009).

Mechanism of Action: Echinocandins inhibit the synthesis of fungal wall by noncompetitive blockage of the (1, 3)-β-D glucan synthase. Inhibition of this enzyme leads to the formation of fungal cell walls with impaired structural integrity and as a result the cell becomes vulnerable to osmotic lysis (Grover, 2010).

Mechanism of Resistance Against Echinocandins: Resistance is attributed to point mutations in the *FKS1* and/or *FKS2* genes which encode the (1,3)-β-D-glucan synthase complex (Kahn *et al*., 2007). In the vast majority of cases only a single mutation is responsible, although in rare cases several alterations are found (Lackner *et al*., 2014).

2.6.5 Allylamines

The allylamines are employed in the treatment of fungal infections of the skin and nails and are effective mostly against dermatophytes. Examples include naftifine and terbinafine which is an analog of naftifine (Bimbaum, 1990). While naftifine possesses only topical activity, terbinafine is active both topically and orally. Terbinafine is given orally to treat dermatophyte infections (Brooks *et al*., 2013).

Mechanism of Action: Allylamines inhibit ergosterol synthesis at the level of squalene epoxidase. They inhibit the enzyme, squalene epoxidase which catalyzes the first step in ergosterol biosynthesis, that is the conversion of squalene to squalene-2,3- epoxide. The buildup of squalene in the cell membrane is toxic to the cell causing pH imbalances and malfunction of membrane bound proteins. Also, this inhibition results in a decrease in the amounts of sterols causing cell death.

2.7 Antifungal Activities of some Medicinal Plants

Apart from the limited number of antifungal drugs used to treat vulvovaginal candidiasis, oral azoles have a potential of systemic toxicity. Thus, the need of an alternative source of antifungal agents for treatment cannot be overemphasized. Plants have been used over the years for the treatment of diseases both in animals and humans. Thus, there is no doubt that plants represent an alternative source of new antibiotics (Afolayan, 2003) especially for those diseases caused by microorganisms that have developed resistance to commonly used drugs. Antibacterial as well as antifungal activities of extracts of *Moringa oleifera*, *Vernonia amygdalina* and *ocimum gratissimum* have been documented by some researchers (Alo *et al*., 2012; Moyo *et al*., 2012; Mabekoje *et al*., 2013). However, there is not much literature of the antifungal activities of the extracts of the plants.

2.7.1 Moringa oleifera

Moringa oleifera, commonly known as 'Drumstick' has a number of other names by which it is referred to; horseradish tree, radish tree, drumstick tree, ben oil tree, miracle tree, West Indian ben, never die and 'Mothers best friend' (Ozumba, 2008). In Nigeria, it is called *Gawara, Kunamarade and Rini maka* (in Fulani); *Zagal, Zogallagandi, Barambo* and *Shipka hali* (in Haua); *Odudu oyibo, Okwe oyibo, Okochi egbu* and *Ikwe beke* (in Igbo); *Ewe ile, Ewe igbale and Adagba maloye* (in Yoruba) (Ozumba, 2008). It is native to Northern India and widely cultivated in tropical and subtropical areas (Uma *et al*., 2017). It is a small or medium sized tree, fast-growing and reaches up to 10 to 12m in its height. The leaves are outstanding as a source of vitamins A when raw and also as good sources of vitamin C and vitamin B and the leaves are among the best plant sources of minerals (Talhaliani and Kar, 2000). *Moringa oleifera* Lam (Drumstick) is one of such plants that have been reported to possess several medicinal properties. The leaves, stem bark, root bark, flowers, fruits and seeds are used in the indigenous systems of medicine for treating a variety of human ailments and some parts

are also eaten as vegetable (Rahman *et al.,* 2009). *Moringa oleifera* leaves are reported to possess various biological activities, which include hypocholesterolemic, antidiabetic and hypertensive agent (Mehta *et al*., 2003). Phytochemical analysis of the ethanolic extracts of the leaves of *M. oleifera* carried out by some researchers showed the presence of alkaloids, flavonoids, saponnins, glycosides, tannins and reducing sugars (Patel *et al*., 2014; Aisha *et al*., 2016).

In India, Pinal *et al*. (2014) reported the antifungal activity of aqueous and ethanolic extracts of *Moringa oleifera* against *Saccharomyces cerevisiae* (MTCC No.170), *Candida albicans* (MTCC No.183), *and Candida tropicalis* (MTCC No.1000). Both aqueous and ethanolic extracts were very active against *Saccharomyces cerevisiae*, had little activity against *Candida tropicalis* while *Candida albicans* was resistant to both extracts. In a study carried out by Moyo *et al*. (2012), both the acetone and aqueous extracts of *Moringa oleifera* did not exhibit any antifungal activity against the fungal species of *Candida albicans*, *Pennicillium notatum*, *Aspergillus flavus* and *Aspergillus niger* even at the highest concentration of 10 mg/ml. Rocha *et al*. (2014) demonstrated the antifungal activity of chloroform and ethanolic extracts (especially from leaves and flowers) of *Moringa oleifera* against some strains of *Candida* species (*Candida ciferrii*, *Candida famata*, *Candida guilliermondii*, *Candida parapsilosis* and *Candida tropicalis*) and *Hortae werneckii* isolated from cultivation water and prawns. Rocha *et al*. (2011) also showed the antifungal activity of flower extracts of *M. oleifera* against strains of *Candida albicans* and *Microsporum canis* strains isolated from dogs. In a study carried out by Aisha *et al*, (2016) in Dutse, Jigawa State, the ethanol extract of *M. oleifera* was found to possess antifungal activity against *Candida albicans*, *Aspergillus niger* and *Rhizopus stolonifer* with the inhibition zone diameter being up to 22mm against *Candida albicans* at a concentration of 5000µg/ml.

In a study carried out by Bassey *et al*. (2016), the ethanolic leaf extracts of *Moringa oleifera* was shown to exhibit antifungal activity against *Aspergillus fumigatus*, *Aspergillus niger* and *Candida albicans* giving inhibition zone diameters of 15.5, 14.5 and 11mm respectively at 100mg/ml concentration. However, *Candida albicans* was resistant at concentrations of 50, 25 and 12.5mg/ml.

Chuang *et al*. (2007) observed the antifungal activity of crude extracts and essential oil of *Moringa oleifera* Lam and reported that ethanol extracts showed antifungal activities *in vitro* against dermatophytes such as *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, and *Microsporum canis*.

2.7.2 *Vernonia amygdalina*

Vernonia amygdalina is a small shrub that grows in tropical Africa. Its bark is rough and it typically grows to a height of 2-5 m, with elliptical leaves that are up to 20 cm in length *(*Ijeh and Ejike, 2011). The leaves are dark green in colour with a characteristic odour and a bitter taste. Thus, *V. amygdalina* is commonly called bitter leaf in English because of its bitter taste. It is called "oriwo" in Edo, "ewuro" in Yoruba, "shikawa" in Hausa, and "olubu" in Igbo (Oboh and Masodje, 2009). The leaves are used as green leafy vegetable and may be consumed either as a vegetable (leaves are macerated in soups) or aqueous extracts used as tonics for the treatment of various illnesses (Igile *et al*., 1995). The leaves of *V. amygdalina* have been documented to contain phlobatannins, cardiac glycosides, tannins, terpenoids, flavonoids and alkanoids (Erute and Egboduku, 2013). The organic fraction extracts of *V. amygdalina* plant has been shown to possess antimicrobial and antiparasitic activities (2005).

Erute and Egboduka (2013) showed that ethanol and methanol extracts of *V. amygdalina* had antifungal activity against *Candida albicans* with inhibition zone diameters (IZD) of 10.67±1.15mm and 10.00 ±2.00 mm respectively at 100mg/ml. However, these extracts had no activity against *Candida krusei*. Oshim *et al*. (2016), observed clinical wound isolates of *Candida albicans* from patients in the surgical wards at Nnamdi Azikiwe Teaching Hospital (NAUTH), Nnewi were resistant to both the ethanol and methanol extracts of *V. amygdalina* at all concentrations (6.25mg/ml to 100mg/ml). Okigbo and Mmeka (2008) showed ethanol extract of *V. amygdalina* was active against *Candida albicans* with inhibition zone diameter (IZD) of 7±0 mm.

Ghamba *et al*. (2014) in Maiduguri, Borno State, observed that ethanol and aqueous leaves extracts of *V. amygdalina* displayed zones of inhibition of 12.4mm and 11.4mm respectively against *Candida albicans* isolated from urine specimens

2.7.3 *Ocimum gratissimum*

Ocimum gratissimum belongs to the group of plants known as spices. It is an erect small plumb with many barnacles usually not more than 1 m high (Vierra and Simon, 2000). The leaves, stems and flowers have been documented to contain phlobatannins, cardiac glycosides, terpenoids, flavonoids and alkanoids (Erute and Egboduku, 2013). In Nigeria, *Ocimum gratissimum* is called "Ncho-anwu" or "Ahuji" (in Igbo), "Efinrin" (in Yoruba), "Aramogbo" (in Edo) and "Daidoya" (in Hausa) (Koche *et al*., 2012).

The ethanol and methanol extracts of the leaves of *Ocimum gratissimum* showed antifungal effect against *Candida albicans* respectively producing inhibition zone diameters

of 10.67±1.15mm and 8.67±1.15. These extracts were more effective against *Candida krusei* producing inhibition zone diameters of 13.00±2.00mm and 14.67±1.53mm respectively (Erute and Egboduku, 2013). Mbakwem *et al*. (2012) studied the antifungal effects of the ethanolic extracts of *O. gratissimum* on *Malassezia furfur* and some major dermatophytes (*Microsporum*, *Trichophyton* and *Epidermophyton*) in Rivers State. Their results showed that the extract was effective against all the fungi tested at all concentrations (50, 100, 150, 200 and 250mg/ml) with inhibition zone diameters ranging from 12.50mm at 50mg/ml to 22.40 at 250mg/ml against *Microsporum* and *Epidermophyton* respectively. In a study carried out by Janine de Aquino *et al*. (2005) in Goiânia city, state of Goiás, Brazil, all extracts of *O. gratissimum*, including ethanolic crude extracts of the leaves showed activity against *Cryptococcus neoformans*.

Nweze and Eze (2009) carried out a study (in Nsukka, Enugu State) to justify the use of *Ocimum gratissimum* L in herbal medicine. Their results showed that ethanolic extract of the leaves was active against both the clinical isolate of *Candida albicans* and the control strain (*Candida albicans* ATCC 90028) giving inhibition zone diameters of 16mm and 13mm at 100mg/ml and 13mm and 10mm at 50mg/ml respectively. Both the clinical isolate and control strains were resistant to concentrations of 25, 12.5 and 6.25mg/ml of the extracts. The minimum inhibitory concentration (MIC) for both the clinical and control strains was 50mg/ml. In their study on the essential oils from the leaves of *O. gratissimum* from 13 populations of Kenya, Matasyoh *et al*. (2008) reported that the essential oils showed a marked antifungal activity against *Candida albicans*.

In vitro minimum inhibitory concentration (MIC) values and time-kill curves demonstrated by Nakamura *et al*. (2004) showed that the *Ocimum gratissimum* essential oil had fungicidal activity against *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis* and *Candida albicans*. *Candida parapsilosis* was the most susceptible and *Candida tropicalis* was the least. Anejionu *et al*. (2011) reported that *Ocimum gratissimum* oil showed good activity against all *Candida albicans* isolates used with MIC ranging from 0.16 to 0.8mg/ml.

2.8 Combined Activities of Antifungal Agents with Extracts of some Medicinal Plants

Indiscriminate and wide use of antimicrobial agents has led to the spread and emergence of resistant strains of pathogens. It has been suggested that combined antibiotic therapy may produce synergistic effects in the treatment of some infections and this combined therapy has been shown by some studies to delay the emergence of antimicrobial resistance (Adwan and Mhanna, 2009; Aiyegoro and Okoh, 2008). The usage of combination therapy has some

potential advantages one of which is the increased likelihood that the infecting pathogen will be susceptible to at least one of the components of the regimen. Also, combination therapy helps to prevent the emergence of resistance (Wade, 1989). Combination therapy can also reduce mortality probably as a result of an additive or even synergistic effect of the combination (Giamarellou, 1986). In combination studies, varying proportions of one drug and another drug ranging from 0:10 to 10:0 are mixed according to the continuous variation checkerboard method (Okore, 2005). The agar overlay inoculum susceptibility disc method is another method used in combination studies to test for the interaction of plant extracts with disc antibiotics.

In the Checkerboard method, the MICs of the various combinations are determined and interactions between the antimicrobial agents are assessed by determining their Fractional Inhibitory Concentration (FIC) index. The effects of the combinations are classified as synergistic if the FIC index value < 1 (Afunwa *et al*., 2014; Okore, 2009), additive, indifference and antagonistic, if the FIC Index = 1, > 1 ≤ 2 and > 2 respectively (Okore, 2009). In the agar overlay inoculum susceptibility disc method, a percentage change in the inhibition zone diameter is used to draw inference on the effects of the interaction on the test organisms. For a synergistic effect, it has been suggested that the inhibition zone diameter (IZD) in the plate containing the plant extract and the antibiotics (the test plate) should be greater than that in the plate containing extract-free base agar layer (the control plate) by at least 19%. A percentage increase in the IZD that is lower than 19% indicates additivity. If the IZDs in the test and the control are equal, it indicates indifference and if the IZD in the test is less than the one in the control, then there is antagonism (Ofokansi *et al*., 2013).

In one study, the effect of interaction between the ethanolic extracts of *Ocimum gratissimum* leaves and the antibiotics (Ketoconazole and Nystatin) was synergistic on clinical *Candida albicans*. However, against the control strain (*Candida albicans* ATCC 90028), Ketoconazole showed antagonism while Nystatin was additive (Nweze and Eze, 2009). The essential oil fraction of *Pelargonium graveolens* and its main components, geraniol and citronellol, have been reported to exhibit strong synergism with ketoconazole against *Trichophyton schoenleinii* and *Trichophyton soudanense*, with FIC indices in the range of 0.18–0.38 (Shin and Lim, 2004).

Zhang *et al*. (2017) investigated the antifungal activity of a hydroalcoholic extract from *Flos Rosae Chinensis* combined with Fluconazole against clinical isolates of *Candida albicans* resistant to Fluconazole. *Flos Rosae Chinensis* alone exerted efficient antifungal activities against *Candida albicans* within a MIC_{80} ranging from 20 μg/ml to 40 μg/ml. *Flos*

Rosae Chinensis failed to enhance the effects of Fluconazole against sensitive *Candida albicans* strains, although it rendered Fluconazole-resistant *Candida albicans* more sensitive. In a study carried out by Anejionu *et al*. (2011) there was no synergism nor antagonism in all the combinations (5:0, 1:4. 4:1, 5:5 and 0:5) of *O. gratissimum* oil and ibuprofen against *Candida albicans*. However, combinations 5:0 and 0:5 produced additivity while combinations 1:4, 4:1 and 5:5 produced indifference. The combinations 1:4, 4:1 and 5:5 were synergistic against *Trichophyton* species (*T. soudanense* and *T. mentagrophytes*).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was conducted in the University of Nigeria Teaching Hospital (UNTH), Ituku/Ozalla, Enugu State, located 6.44° N latitude and 7.50° E longitude and it is situated at elevation 192 meters above sea level in the South-East geopolitical zone of Nigeria (Appendix A).

3.2 Study Design

A descriptive cross-sectional study was conducted from April to December, 2015 and a random sampling method was used.

3.3 Determination of Sample Size

To figure out what sample size is needed, the formula $n = Z^2P(1-P) / d^2$ (Daniel, 1999) was used (Appendix A).

3.4 Inclusion Criteria

Women of child-bearing age (18-45 years) and above attending the Obstetrics and Gynecology Unit of UNTH and who also gave their consent were included in the study. Also, the women may present with symptoms or not but must not be menstruating.

3.5 Exclusion Criteria

Women attending the Obstetrics and Gynecology Unit of UNTH but did not give their consent or were menstruating were excluded from the study. Those below 18 years were also excluded from the study.

3.6 Ethical Consideration

An ethical clearance (see appendix C) was obtained from the Ethical Committee of UNTH before the specimens were collected. Participation in the study was voluntary and the informed consent of each of the participants was obtained from them.

3.7 Specimen Collection

A total of 340 (calculated as shown in Appendix A) high vaginal swab (HVS) specimens were collected from the women attending the Obstetrics and Gynecology Unit of the University of Nigeria Teaching Hospital (UNTH) Ituku/Ozalla, Enugu State, who were qualified for the study. Each HVS specimen was collected by a medical personnel using a sterile swab stick with the aid of a sterile speculum. Structured questionnaires (a copy in

Appendix B) were used to obtain some relevant information from the participants after obtaining their consent. The collected specimens were transported to the Department of Applied Microbiology and Brewing Laboratory, Nnamdi Azikiwe University, Awka for processing immediately.

The plant materials were all collected in November, 2015. Fresh leaves of *Moringa oleifera* were collected from around the Faculty of Biosciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria while *Vernonia amygdalina* leaves were collected from Umukwa village in Awka, Anambra State, Nigeria. The fresh leaves of *Ocimum gratissimum* were bought from "ogige" market (a local market) in Nsukka, Enugu state, Nigeria. The plants were identified by Dr (Mrs). Aziagba Bibian (a plant taxonomist), in the Department of Botany, Nnamdi Azikiwe University, Awka. The herbarium numbers of the plants were as follows: *Moringa oleifera* (NAU H No 01^{A}), *Vernonia amygdalina* (NAU H No 47^{A}) and *Ocimum gratissimum* (NAU H No 35^{A}).

The leaves were dried under shade for seven days, and milled into powder with the aid of an electric Qlink blender (Model QBL-20L40P) as described by Nweze *et al*. (2004). The pulverized leaves of each of the plants were weighed, appropriately labelled, covered tightly and kept at room temperature for further use.

3.8 Processing of the High Vaginal Swab Specimens and Culture

Each swab was inoculated directly unto already prepared plates of Sabouraud dextrose agar (SDA)(HiMedia Laboratory, Pvt. Ltd, India) supplemented with Chloramphenicol (0.05mg/ml). The plates were incubated aerobically at room temperature for 24-48 hours. Yeast colonies were subcultured for purification and pure cultures were then stored in Bijoux bottle slants of SDA supplemented with Chloramphenicol (0.05mg/ml.).

3.9 Identification of the yeast isolates

All the isolates were identified based on their macroscopic, microscopic, physiological as well as biochemical characteristics and also in comparison with photomacrographs and photomicrographs in Medical Mycology (Glenn, 1978) and the Colour Atlas of Pathogenic Fungi (Dorothea *et al.,* 1979). The yeast isolates were also sent to the yeast collection centre in Neitherlands, Centraal Bureau Voors Schimmel culture (CBS) for identification by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

3.9.1 Dalmau Plate Culture (Growth on Cornmeal Agar)

This test was carried out using the method described by Conant *et al.* (1955). A single Petri dish was used for three or four different isolates. The Corn Meal agar (TM MEDIA, TITAN BIOTECH LTD, Rajasthan, India) was prepared according to the manufacturer's instructions. Using a sterile inoculating straight wire, a light inoculum of the pure yeast isolate was streaked by making a deep cut (that is a horizontal furrow) in the cornmeal agar. A flamed coverslip was placed along the line of inoculum. The Petri dishes were then incubated for 24 to 48 hours at 25^{o}C. After incubation, the streaks were examined microscopically (for chlamydospores, pseudohyphae and blastospores), *in situ* through the coverslip, using a low power objective lens (x10) and then x40.

3.9.2 Germ Tube Test (Reynold-Braude Test)

This test provides a rapid presumptive test for the identification of *Candida albicans*. The method of germ tube test as described by Elmer *et al.* (1992) was used for this study. Using a sterile inoculation loop, a pure yeast colony was lightly touched and inoculated into a sterile test tube containing 0.5ml of human serum. The resulting mixture was incubated aerobically at 37^{o}C for 3hrs. After this period; a drop of the yeast-serum mixture was placed on a clean microscope slide, covered with a cover slip and examined using the x10 and x40 objective lens. A short hyphal (filamentous) extension arising laterally from a yeast cell, with no constriction at the point of origin confirmed formation of germ tubes.

3.9.3 Growth on Chromogenic Candida Agar

This medium is highly selective for yeasts and allows presumptive identification of *Candida albicans*, *C. tropicalis*, *C. krusei* and *C. guilliermondii*, and can also be used to identify specimens containing mixtures of yeast species (Baixench *et al*., 2006; Ghelardi *et al*., 2008). Each isolate was inoculated on Chromogenic Candida Agar (TITAN BIOTECH LTD, India) and incubated in an inverted position at 37°C for 48 hours. This ensured the detection of mixed cultures since this agar presumptively identifies the *Candida* species based on the colours produced by the colonies (Ghelardi *et al*., 2008).

3.9.4 Identification by Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Preparation of the Yeasts Isolates: The yeast isolates were subcultured on Sabouraud Dextrose Agar (SDA) plates and incubated at 30°C for 24 hours.

Sample Preparation: The yeast proteins were extracted according to the Bruker Daltonics GmbH protocol using the formic acid/ethanol extraction protocol as described by Marklein *et al*. (2009). Using a pipette tip, cells from five single colonies were transferred from the SDA plate into a 1.5ml extraction tube containing 0.3ml of distilled water and mixed very well. After, 0.9ml of absolute ethanol was added and the contents of the tube mixed well and then centrifuged at 20, 000rpm for 2 minutes. The supernatant was discarded and the pellet was air-dried at room temperature and then resuspended in 0.05ml of 70% formic acid. The pellet was then extracted by the addition of an equal volume of acetonitrile with thorough mixing. The mixture was centrifuged at 20, 000rpm for 2 minutes. After centrifugation, 0.001ml of the clear supernatant was spotted (placed) in duplicate on a 96-spot polished steel target plate (Bruker Daltonics, Bremen, Germany) and allowed to air dry. Subsequently, each tested spot (sample) was overlaid with 0.002ml of matrix (which consisted of a saturated solution of alpha-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile– 2.5% trifluoroacetic acid) and air dried at room temperature.

Sample Analysis: After drying at room temperature they were analyzed in automatic runs operated by flexControl, version 3.3.108.0 (Bruker Daltonics). The yeast identification was achieved by means of MALDI-TOF MS Biotyper RTC software, version 3.0 (Bruker Daltonics), based on the comparison of mass spectra generated by the Microflex LT software with databases. The recognition results were scored according to the manufacturer's criteria. In this regard, the log score values of > 2.0 indicated correct species identification; 1.7 – 2 indicated correct genus level while < 1.7 indicated no reliable identification (Alizadeh *et al*., 2017).

3.10 Extraction of Plant Materials

After weighing the pulverized plant leaves, 300g of each was extracted using analytical grade of ethanol (Anala® BDH Chemicals Ltd, Pool, England) in a Soxhlet extractor at 70^0C for 6hrs. The plant weight to solvent volume was in the ratio of 1:5. That is, for every 100g of pulverized plant leaves, 500ml of ethanol was used for the extraction. The extract recovered was then concentrated by surface evaporation to dryness under room temperature. This helped to remove the solvent leaving a solvent-free extract. The concentrated extract was transferred to a sterile container and kept in the refrigerator at 20^oC until required for analysis.

3.11 Percentage yield

The yield of crude plant extract was obtained by measuring its dry weight before and after extraction and the % yield of extract was given as

$$\%\ yield\ of\ extract = \frac{crude\ \ extract\ \ weig\,ht}{initial\ \ dry\ \ weig\,ht} \times 100$$

3.12 Preparation of Stock Solution of the Extracts

Stock solutions of the ethanolic extracts of the leaves of *Moringa oleifera*, *Vernonia amygdalina*, and *Ocimum gratissimum* were prepared by weighing out 1.6g of each of the extract using electronic weighing machine. This was then dissolved completely in 4ml of Dimethyl sulfoxide (DMSO)(JHD Guangbong Guanghue Sci-TechCo, Ltd, Shamtou Guangdong, China) in sterile bottles to give a stock concentration of 400mg/ml of the individual extracts. A double fold serial dilution was performed on the stock solution by transferring 1ml of the stock solution into an equal volume (1ml) of DMSO in another bottle. This resulted in a concentration of 200mg/ml. The double fold serial dilution was continued to obtain different concentrations of 100mg/ml, 50mg/ml, 25mg/ml and 12.25mg/ml.

3.13 Standardization of Inoculum for Susceptibility Testing

The inoculum was standardized as described by Cheesbrough, (2010) with slight modification. A 1% v/v solution of sulphuric acid (H_2SO_4) was prepared by adding 1ml of concentrated H_2SO_4 to 99ml of sterile distilled water and mixed well. Also, a 1% w/v solution of Barium Chloride ($BaCl_2$) was prepared by dissolving 0.5g of divalent barium chloride ($BaCl_2.2H_2O$) in 50ml of distilled water. A 0.5 McFarland standard was prepared by adding 0.6ml of the barium chloride solution to 99.4 ml of the solution of sulphuric acid in a conical flask.

A Small volume (5ml) of the turbid solution was transferred to a small screw-cap bottle of the same type as used to prepare the inoculum. Using a sterile wire loop, discrete colonies each of 24hours pure culture of the *Candida* isolates was picked and inoculated into 5ml of sterile 0.85% saline. The turbidity of the suspension was adjusted and then matched visually with 0.5 McFarland standard which is equivalent to 1 x 10^6 colony forming units per ml (CFU/ml).

3.14 *In Vitro* Antifungal Susceptibility Testing using Commercial Antifungal Discs

This was carried out using one of four standard methods for antifungal susceptibility testing, that is M44-A for yeast disk diffusion testing (NCCLS, 2004) released by the Clinical and Laboratory Standards Institute (CLSI), formerly the NCCLS (National Committee on Clinical Laboratory Standards). Eight antifungal drugs were used: Fluconazole (25µg), Ketoconazole (10µg), Voriconazole (1µg), Nystatin (100Units), Amphotericin B (20µg), Flucytosine (1µg), Clotrimazole (10µg) and Itraconazole (50µg) (Oxoid, UK and Abtek, Liverpool). Mueller Hinton Agar (TM MEDIA, TITAN BIOTECH LTD, Rajasthan, India) was prepared according to the manufacturer's instructions and poured into Petri dishes (plates). Each plate was seeded with 0.2ml of the standardized inoculum and spread plated evenly on the surface of the agar. The above antifungal discs were then aseptically placed on the surface of the agar plates by pressing each disc down firmly to ensure complete, level contact with the agar. The plates were left for 30 minutes at room temperature on the laboratory bench for pre-diffusion and then incubated in an inverted position at 30°C for 24 hours. After the incubation period, the inhibition zone diameter was measured and recorded in millimeter (mm) using a transparent ruler (CLSI, 2009).

The antifungal susceptibility of the isolates was interpreted as susceptible (S), Susceptible Dose-Dependent (SDD) or Intermediate (I) and Resistant (R). The results were interpreted in line with the Clinical and Laboratory Standards Institute guidelines (CLSI, 2009) and Rosco (2011).

For Voriconazole (1µg); ≥17mm is susceptible, 14-16mm is susceptible-dose dependent and ≤13mm is resistant. Fluconazole (25µg); ≥19mm is susceptible, 15-18mm is susceptible-dose dependent while ≤14mm is resistant. For Itraconazole (10µg); ≥23mm is susceptible, 22-14mm is susceptible-dose dependent and ≤13mm is resistant. For Ketoconazole (10µg); ≥28mm is susceptible, 21-27mm is susceptible-dose dependent and ≤20mm is resistant. For Clotrimazole (10µg) and Flucytosine (1µg); ≥20mm is susceptible, 12-19mm is susceptible-dose dependent and ≤11mm is resistant. For Amphotericin B (20µg); ≥15mm is susceptible,

10-14mm is susceptible-dose dependent and <10mm is resistant. For Nystatin (100Units); ≥15mm is susceptible, 10-14mm is susceptible-dose dependent while no zone of inhibition is resistant.

3.15 *In Vitro* Antifungal Susceptibility Testing using the Crude Plant Extracts

The antifungal potency of the plant extracts was evaluated against selected *Candida* species that were resistant to four or more of the commercial antifungal drugs. These included 2 isolates of *Candida albicans*, 2 isolates of *C. tropicalis*, and 1 each of *C. parapsilosis* and *C. krusei*. This was carried out using agar diffusion method as described by Balouiri *et al.* (2016). A pure culture of some selected resistant strains of the *Candida* species was exposed to different dilutions of the individual crude plant extract for antimicrobial evaluation. Mueller Hinton agar (20 ml) in Bijou bottles previously prepared and allowed to cool to warm touch were introduced into sterile Petri dishes. After, 0.1ml of standardized inoculum (containing approximately 1 x 10^6cfu/ml) was introduced into each of the Mueller Hinton agar medium and shaken for even distribution in the Petri dish. This was allowed to cool and gel. Wells of 9 mm in diameter each were dug into the Mueller Hinton agar using a sterile cork borer. Using a sterile syringe for each concentration, 0.2ml of the different concentrations (200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml and 12.25mg/ml) of the individual plant extracts were introduced into the corresponding labeled wells. In one of the wells in each Mueller Hinton agar plate, 0.2ml of DMSO was introduced to serve as negative control. This was carried out in duplicates and the plates were then incubated at 25^0C±2^0C for 24hrs. After the incubation period, the inhibition zone diameter (in mm) produced in each plate was measured and the mean recorded.

3.16 Determination of Minimum Inhibitory Concentration (MIC)

The agar diffusion method was used to determine the minimum inhibitory concentration (MIC) of the plant extracts and Fluconazole. This was done using linear regression analysis as described by Bonev *et al.* (2008). The free diffusion model was adopted which assumes that the antimicrobial agents (Fluconazole and the plant extracts) diffuse freely through the medium. The X^2 values (squared values of the inhibition zone radii) were plotted against the logarithm of the different concentrations (obtained by double-fold serial dilution as described above) of each of Fluconazole and the plant extracts together with linear fits. From the equation of the graph, the MIC was calculated as the antilog of the value of x when the value of y = 0, (that is the x-intercept). The closer to 1 the regression coefficient (R^2) is, the better the linear fits and the more accurate is the MIC value.

$$X^2 = \left[\frac{IZD - well\ diameter}{2}\right]^2$$

3.17 Combined Activity of Fluconazole and the Plant Extracts

The concentrations and varying proportions of Fluconazole and the extracts were prepared and mixed using the continuous variation Checkerboard method as has been previously described (Ofokansi *et al*., 2013; Afunwa *et al*., 2014). Stock solutions of 26mg/ml, for both *Moringa oleifera* and *Ocimum gratissimum* were prepared by dissolving 1.56g of the individual leaf extract each in 60ml of Dimethyl sulfoxide (DMSO) while stock solutions of 18mg/ml for *Vernonia amygdalina*, was prepared by dissolving 1.08g of the leaf extract of the plant in 60ml DMSO. Stock solutions of 50µg/ml of Fluconazole were prepared by dissolving 3mg of Fluconazole powder (FLUCAMED®, DRUGFIELD PHARMACEUTICALS LIMITED, Nigeria) in 60ml of slightly warm sterilized distilled water.

Following the continuous variation checkerboard method, varying proportions of the stock solution of Fluconazole and the stock solution of each of the individual plant extract were combined in different ratios starting from A10:B0 (10 part of "A" to 0 part of "B") to A0:B10 (0 part of "A" to 10 part of "B") where "A" represents Fluconazole and "B" each of the plant extract combining separately with Fluconazole. A two-fold serial dilution (as described above) was performed on each of these combinations using DMSO as the diluents up to four dilutions in sterile Bijou bottles. The MICs of each combination ratio was determined as describe above. Interaction between the plant extracts and Fluconazole for each of the resistant isolates, across all the ratios of the combined extract and pure drug was determined by calculating their Fractional inhibitory concentration (FIC) index using the equation (Ofokansi *et al*,. 2013) below:

FIC index= FIC A + FIC B

Where,

A and B are the two antimicrobial agents being combined (that is Fluconazole and the ethanolic extract of the leaves of any of the plants).

$$\text{FIC A} = \frac{MIC\ of\ Fluconazole\ \ in\ combination\ \ with\ the\ plant\ \ extract}{MIC\ of\ \ Fluconazole\ \ \ alone}$$

$$\text{FIC B} = \frac{MIC\ of\ \ the\ plant\ \ extract\ \ in\ combination\ \ \ with\ Fluconazole}{MIC\ of\ \ the\ plant\ \ extract\ \ alone}$$

The effect of the interactions of each combination was interpreted as;

•Synergistic if FIC index < 1.0

•Additive if FIC index = 1.0

•Indifference if 1.0 < FIC index < 2.0

•Antagonistic if FIC index > 2.0

•No activity if FIC index = 0

3.18 Determination of Resistant Genes (Against Azoles) from the Selected Resistant *Candida* species

3.18.1 Extraction of the Yeast DNA: The Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research co, USA) was used for the DNA extraction.

After subculturing for 48 hours at 25°C, 50mg (wet weight) yeast cells were resuspended in up to 200µl of isotonic buffer (PSB) and then added to a 0.5mm ZR Bashing Bead Lysis Tube. After, 750µl Bashing Bead™ Buffer was added to the tube. This was then secured in a bead beater fitted with a 2ml tube holder assembly and processed at maximum speed for ≥ 5minutes.

The contents of the 0.5mm ZR Bashing Bead™ Lysis Tube were centrifuged in a micro centrifuge at 10,000 x *g* for 1 minute and 400µl of the supernatant was transferred to a Zymo-Spin™ III-F Filter in a Collection Tube and centrifuge at 28°C at 8000 x *g* for 1 minute.

After, 1,200µl of Genomic Lysis Buffer was added to the filtrate in the Collection Tube from step 4. After addition of the Buffer, 800µl of the mixture from Step 5 was transferred to a Zymo-Spin TM IIC Column in a Collection Tube and Centrifuge at 28°C at 10,000 x *g* for 1 minute. The flow through from the Collection Tube was discarded and Step 6 was repeated.

A 200µl DNA Pre-Wash Buffer was then added to the Zymo-Spin™ IIC Column in a new Collection Tube and afterwards, 500µl g-DNA Wash Buffer was also added to the Zymo-Spin™ IIC Column and centrifuge at 10,000 x *g* for 1 minute.

Finally, the Zymo-Spin™ IIC Column was transferred to a clean 1.5ml micro centrifuge tube and 100 (35 minimum) DNA Elution Buffer was added directly to the column matrix and centrifuge at 28°C at 10,000 x *g* for 30 seconds to elute the DNA.

3.18.2 PCR Amplification of Coding Region of the ERG11 Gene: Two pairs of primers (synthesized by Inqaba Biotec West Africa Ltd.) were used for the amplification;
ERG1A-F (5′-ATGGCTATTGTTGAAACTGTCATT-3′) with
ERG1B-R (5′-GGATCAATATCACCACGTTCTC-3′), and
ERG2A-F (5′-ATTGGAGACGTGATGCTGCTCAA-3′) with
ERG2B-R (5-CCAAATGATTTCTGCTGGTTCAGT-3′) (Ming-Jie *et al*., 2013).

The reaction mixture for the PCR contained 12.5µl of One *Taq* Quick-Load 2X Master Mix with Standard Buffer (New England Biolabs Inc.); 0.5µl each of ERG1 forward and reverse primers; 9.5µl of Nuclease free water and 2µl of DNA template giving a total reaction volume of 25µl. Same was done using the second primer set (ERG2). The reaction was gently mixed and transferred to a preheated thermalcycler (Eppendorf nexus gradient Mastercycler, Germany). Amplification conditions for the PCR were as follows: initial denaturation of the DNA at 95^{o}C for 5 minutes, followed by 35 cycles of denaturation at 95^{o}C for 40secs, primer annealing at 55^{o}C for 40secs and strand extension at 72^{o}C for 5 minutes (Ming-Jie *et al*., 2013). The PCR products were separated on a 2% agarose gel electrophoresis and the bands were visualized by UV transilluminator (syber gold).

3.18.3 Sequencing of the ERG11 Gene: The PCR products of each of the selected resistant isolates that showed visible bands were sent to Inqaba Biotec South Africa Ltd for sequencing. The protocol for the sequencing is found in appendix F.

3.18.4 Analysis of the Sequencing Results: Sequence analysis, alignment and data presentation was done using BioEdit Sequence Allignment Editor (version 7.05.3) (Hall, 1999). A local pair wise alignment between the reference gene (accession number AY856352) and the query gene was done using CLUSTALW 2.0 (Larkin *et al*., 2007). Blossom 62 scoring matrix, a gap opening of 10 and a gap extension penalty of 0.5 were employed as the default setting of CLUSTALW program in the BioEdit software. Point mutations in the ERG11 gene and their resultant amino acid changes were noted.

3.19 Statistical analysis

Demographic characteristics of the respondents were extracted from the questionnaire and statistical analysis and data presentation were performed using Statistical Package for Social Sciences (SPSS) Version 22.0. Chi- square Test was used to compare the differences between the effects of various parameters on the prevalence of vaginal *Candida* colonization. Values of $p < 0.05$ were considered statistically significant. All values of inhibition zone diameters for the plant extracts were expressed as mean ± Standard deviation and difference between the inhibition zone diameters across the plant extracts were also considered significant at $p < 0.05$ using one-way analysis of variance (ANOVA).

CHAPTER FOUR

4.0 RESULTS

4.1 Occurrence of the *Candida* species in the High Vaginal Swab Specimens

Out of the 340 HVS specimens collected, 4 were regarded invalid (the questionnaires were not completed) while 83 yielded growth of yeasts giving a prevalence of vaginal *Candida* colonization of 24.7%. Among the yeasts isolated, *Candida tropicalis* was the most predominant with a distribution frequency of 34 (38.63%) followed by *Candida parapsilosis* 21 (23.86%), *Candida albicans* 20 (22.73), *Candida krusei* (*Issatchenkia orientalis*) 7 (7.95%) and *Candida glabrata* 6 (6.82%). Out of the 83 specimens that were positive for *Candida*, 2 species each were isolated from 5 specimens and single species from 78 specimens giving a total of 88 species. A combination of *Candida albicans* and *Candida tropicalis* was isolated from 2 specimens, a combination of *Candida albicans* and *Candida krusei* was isolated from 1 specimen, a combination of *Candida albicans* and *C. parapsilosis* was isolated from 1 specimen and finally, a combination of *Candida tropicalis and Candida parapsilosis* was isolated from 1 specimen. The overall occurrence of non-albicans species was 61.37% (Table 1).

4.2 Prevalence of Vaginal *Candida* Colonization According to Age Group

The prevalence of vaginal *Candida* colonization according to age group is presented in Table 2. It can be observed from the table that women of ages 50 years and above recorded the highest prevalence (6, 50%), followed by women in the age groups 18 – 25 years (40, 29.6%), 42 - 49 years (6, 23.1%) and 34 – 41 years (12, 19.4%). Women in the age bracket 26 – 33 years had the least prevalence (19, 18.8%). The difference in the prevalence of vaginal *Candida* colonization among the age groups was not statistically significant ($p>0.05$).

4.3 Prevalence of Vaginal *Candida* Colonization amongst the Women in Relation to Pregnancy

The prevalence of vaginal *Candida* colonization was higher amongst the non-pregnant women (28.6%) than the pregnant women (16.0%) though the difference was statistically not significant ($p> 0.05$). The prevalence amongst the women who were not sure of their pregnancy status was 0% while those who never responded vis-à-vis their pregnancy status had a prevalence of 30.8% (Table 3).

Table 1: Occurrence of the *Candida* species in the High Vaginal Swab Specimens

Species	Frequency	Percentage (%)
Candida tropicalis	34	38.63
Candida parapsilosis	21	23.86
Candida albicans	20	22.73
Candida krusei	7	7.95
Candida glabrata	6	6.82
Total	**88**	**100**

Table 2: Prevalence of Vaginal *Candida* Colonization According to Age Group

Age group (in years)	Number sampled	Number of *Candida* positive specimens	Percentage of *Candida* positive Women (%)
18 - 25	135	40	29.6
26 - 33	101	19	18.8
34 – 41	62	12	19.4
42 – 49	26	6	23.1
>50	12	6	50
Total	**336**	**83**	**24.7**

p – value = 0.067 (p> 0.05); df = 4

Table 3: Prevalence of Vaginal *Candida* Colonization among the Women in Relation to Pregnancy

Status	Number sampled	Number of *Candida* positive specimens	Percentage of *Candida* positive Women (%)
Pregnant	100	16	16.0
Non-pregnant	220	63	28.6
Not sure	3	0	0.0
No response	13	4	30.8
Total	**336**	**83**	**24.7**

p - value = 0.067 ($p > 0.05$); df = 3

4.4 Prevalence of Vaginal *Candida* Colonization among the Women According to Trimester

Table 4 shows the prevalence of vaginal *Candida* colonization amongst the women according to trimester. Women in their first trimester had the highest prevalence of 25.0% followed by those in the second trimester and third trimester with prevalence of 11.1% and 5.0% respectively. Statistically, there was no significant difference in the effect of trimester on the prevalence of vaginal *Candida* colonization.

4.5 Prevalence of Vaginal *Candida* Colonization among the Women in Relation to Marital Status

As can be seen in Table 5, the single women had the highest prevalence (26.0%) of vaginal *Candida* colonization when compared to that of the married women (23.8%) and the divorcees (0.0%). However, of the 4 women who never responded vis-à-vis their marital status, 2 were positive for vaginal *Candida* colonization giving a prevalence of 50%.

4.6 Prevalence of Vaginal *Candida* Colonization in Symptomatic and Asymptomatic Women

In Table 6 is presented the prevalence of vaginal *Candida* colonization in symptomatic and asymptomatic women. It can be observed that out of the 254 symptomatic women who took part in the study, 59 were positive for vaginal *Candida* colonization giving a prevalence of 23.3% while out of the 82 asymptomatic women, 24 were positive for vaginal *Candida* colonization giving a prevalence of 29.2%. Thus, the prevalence was higher in asymptomatic women than in the symptomatic women. However, the difference was not significant statistically.

4.7 Prevalence of Vaginal *Candida* Colonization among the Women in Relation to the Different Symptoms

The highest prevalence of vaginal *Candida* colonization among the symptomatic women was observed in the women who presented with both vaginal discharge and dispareuria (42.7%), followed by those with vaginal discharge only (36.7%) and dysuria only (20.0%). There was 0.0% prevalence respectively in the women who presented with burning only; dispareuria only; vaginal discharge, itching and dysuria; vaginal discharge, burning and itching; itching and dysuria; burning and itching; burning and dysuria; vaginal discharge, burning, itching and dysuria (Table 7)

Table 4: Prevalence of Vaginal *Candida* Colonization among the Women According to Trimester

Trimester	**Number Sampled (%)**	***Candida* positive**	***Candida* negative**	**Percentage of *Candida* positive women (%)**
First	44	11	33	**25.0**
Second	36	4	32	**11.1**
Third	20	1	19	**5.0**
Non- pregnant	236	67	169	**28.4**
Total	**336**	**83**	**253**	

p – value = 0.024 ($p < 0.05$); df = 3

Table 5: Prevalence of Vaginal *Candida* Colonization among the Women in Relation to Marital Status

Marital Status	Number sampled	Number of *Candida* positive specimens	Percentage of *Candida* positive Women (%)
Married	181	43	23.8
Single	146	38	26.0
Divorced	5	0	0.0
No response	4	2	50.0
Total	**336**	**83**	**24.7**

p – value = 0.356 (p > 0.050); df = 3

Table 6: Prevalence of Vaginal *Candida* Colonization in Symptomatic and Asymptomatic Women

Clinical Manifestation	Number Sampled	*Candida* positive	*Candida* negative	Percentage of *Candida* positive women (%)
Symptomatic	254	59	195	**23.2**
Asymptomatic	82	24	59	**29.3**
Total	**336(100)**	**83(24.7%)**	**253(75.3%)**	

p – value = 0.305 ($p > 0.05$); df = 1

Table 7: Prevalence of Vaginal *Candida* Colonization among the Women in Relation to the Different Symptoms

Symptoms	Number sampled	Candida Positive	Candida Negative	Percentage of *Candida* positive women (%)
Vaginal discharge	109	40	69	36.7
Burning	4	0	4	0.0
Itching	36	4	32	1.1
Dysuria	5	1	4	20.0
Dispareuria	1	0	1	0.0
V and B	11	2	9	18.2
V and I	57	7	50	12.3
V and D	16	2	14	12.5
V and DP	7	3	4	42.7
V, I and D	2	0	2	0.0
V, B and I	1	0	1	0.0
I and D	2	0	2	0.0
B and I	1	0	1	0.0
B and D	1	0	1	0.0
V, B, I and D	1	0	1	0.0
TOTAL	**254**	**59**	**195**	**23.2**

p – value = 0.039 ($p < 0.05$); df = 14

KEY:
V = Vaginal discharge, B = Burning, I = Itching, D = Dysuria, DP = Dispareuria

4.8 Characteristic features of the Isolates after Growth on Chromogenic *Candida* Agar, Corn Meal Agar and Germ Tube Test

The result of growth on Chromogenic *Candida* Agar, Corn Meal Agar and germ tube test for the identification of the isolates is presented in Table 8 and Plates 8a - 8b (Appendix A).

4.9 Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) Identification of the Isolates

The performance of MALDI-TOF MS for the identification of the isolates is presented in Table 9. This table shows that 70 (79.55%) of the isolates had correct species identification with log score value of >2.0 while 18 (20.45%) had correct genus level identification with log score value of 1.7 – 2.0. There was no isolate (0.00%) with a log score value of <1.7 which indicates an unreliable identification. The scores for the individual species are presented in Appendix A.

4.10: *In Vitro* Susceptibility Profile of the *Candida* Species to Fluconazole

The *in vitro* susceptibility profile of the isolated *Candida* species to Fluconazole is presented in Table 10. From the table, it can be observed that 27 (30.7%) of the *Candida* isolates were susceptible to Fluconazole, 15 (17%) were susceptible dose-dependent and 46 (52.3%) were resistant. *Candida glabrata* was the most susceptible (50%) followed by *Candida parapsilosis* (42.9%). Meanwhile, the highest resistance (85.7%) was shown by *Candida krusei*. In fact, none of the *Candida krusei* isolate was susceptible to Fluconazole.

4.11 *In Vitro* Susceptibility Profile of the *Candida* Species to Ketoconazole

Twelve (13.6%) out of the 88 *Candida* isolates were susceptible, 22 (25%) were susceptible dose-dependent and 54 (61.4%) were resistant (Table 11). *Candida krusei* was the most resistant (71.4%) followed by *Candida albicans* (70%) while *Candida parapsilosis* was the most susceptible dose-dependent species.

Table 8: Characteristic features of the Isolates after Growth on Chromogenic *Candida* Agar, Corn Meal Agar and Germ Tube Test

Candida species	Germ Tube	Colony Characteristics on Chromogenic *Candida* Agar	Growth on Corn Meal Agar		
			Chlamydospores	Pseudohyphae	Blastospores
Candida albicans	+	Light green	+	+	+
Candida tropicalis	-	Blue	-	+	+
Candida parapsilosis	-	Off-white	-	+	+
Candida krusei	-	Large, flat, dry, Spreading, Rose-pink	-	+	+
Candida glabrata	-	Large, glossy, pink to violet	-	-	+

KEY:
+ = Positive, - = Negative

Table 9: MALDI-TOF MS Identification of the Isolates Analyzed with Bruker Daltonics

***Candida* Species**	**No of Isolates Analyzed (%)**	**MALDI-TOF Score**		
		> 2	**1.7 - 2.0**	**< 1.7**
Candida tropicalis	34(38.63)	22	12	0
Candida parapsilosis	21(23.86)	20	1	
Candida albicans	20(22.73)	19	1	
Candida krusei	7(7.95)	5	2	
Candida glabrata	6(6.82)	4	2	
TOTAL	**88**	**70**	**18**	**0**

Key:
MALDI-TOF MS = Matrix assisted laser desorption ionization time of flight mass spectrometry

Table 10: *In Vitro* Susceptibility Profile of the *Candida* species to Fluconazole (25µg)

Species	Total number	S (%)	SDD (%)	R (%)
Candida albicans	20	5(25)	3(15)	12(60)
Candida tropicalis	34	10(29.4)	8(23.5)	16(47.1)
Candida parapsilosis	21	9(42.9)	2(9.5)	10(47.6)
Candida krusei	7	0(0)	1(14.3)	6(85.7)
Candida glabrata	6	3(50)	1(16.7)	2(33.3)
Total	**88**	**27(30.7)**	**15(17.0)**	**46(52.3)**

Key:
S = Susceptible; SDD = Susceptible Dose Dependent; R = Resistant

Table 11: *In Vitro* Susceptibility Profile of the *Candida* species to Ketoconazole (10µg)

Species	Total number	S (%)	SDD (%)	R (%)
Candida albicans	20	3(15)	3(15)	14(70)
Candida tropicalis	34	5(14.7)	8(23.5)	21(61.8)
Candida parapsilosis	21	3(14.3)	8(38.1)	10(47.6)
Candida krusei	7	0(0)	2(28.6)	5(71.4)
Candida glabrata	6	1(16.7)	1(16.7)	4(66.7)
Total	**88**	**12(13.6)**	**22(25.0)**	**54(61.9)**

Key:
S = Susceptible; SDD = Susceptible Dose Dependent; R = Resistant

4.12 *In Vitro* Susceptibility Profile of the *Candida* Species to Clotrimazole

As can be seen from Table 12, 37(42.0%) of the *Candida* isolates were susceptible, 21 (23.9%) were susceptible dose-dependent while 30 (34.1%) were resistant. The highest resistance (71.4%) was observed with *Candida krusei* while *Candida parapsilosis* was the most susceptible (57.1%). *Candida tropicalis* was the most susceptible dose-dependent species (32.4%).

4.13 *In Vitro* Susceptibility Profile of the *Candida* Species to Amphotericin B

Susceptible isolates were 61(69.3%) while 10 (11.4%) and 17 (19.3%) were susceptible dose-dependent and resistant respectively. The species that was most resistant to Amphotericin B was *Candida krusei* (42.9%) followed by *Candida albicans* (25.0%). *Candida tropicalis* was the most susceptible (79.4%) followed by *Candida parapsilosis* (76.2%). There were no susceptible dose-dependent and resistant isolates of *Candida parapsilosis* and *Candida glabrata* respectively (Table 13).

4.14 *In Vitro* Susceptibility Profile of the *Candida* Species to Flucytosine

The in vitro susceptibility profile of the *Candida* isolates to Flucytosine is shown in Table 14. Seventy-six (86.4%) of all the isolates were resistant, 6 (6.8%) were susceptible dose-dependent and also 6 (6.8%) were susceptible. All the isolates of *Candida albicans*, *Candida krusei* and *Candida glabrata* were resistant to Flucytosine. A very low susceptibility of 4% and 2% was observed with *Candida tropicalis* and *Candida parapsilosis* respectively. Only isolates of *Candida tropicalis* (17.6%) were susceptible dose-dependent.

4.15 *In Vitro* Susceptibility Profile of the *Candida* Species to Voriconazole

For Voriconazole, 46 (52.3%) of the *Candida* isolates were susceptible, 11 (12.5%) were susceptible dose-dependent and 31 (35.2%) were resistant. *Candida parapsilosis* isolates were the most susceptible (66.7%) followed by *Candida krusei* (57.1%) and *Candida tropicalis* (52.9%). There were no susceptible dose-dependent isolates of *Candida krusei*.

Table 12: *In Vitro* Susceptibility Profile of the *Candida* species to Clotrimazole (10µg)

Species	Total number	S (%)	SDD (%)	R (%)
Candida albicans	20	6(30.0)	6(30.0)	8(40.0)
Candida tropicalis	34	14(41.2)	11(32.4)	9(26.5)
Candida parapsilosis	21	12(57.1)	3(14.3)	6(28.6)
Candida krusei	7	2(28.6)	0(0.0)	5(71.4)
Candida glabrata	6	3(50.0)	1(16.7)	2(33.3)
Total	**88**	**37(42.0)**	**21(23.9)**	**30(34.1)**

Key:
S = Susceptible; SDD = Susceptible Dose Dependent; R = Resistant

Table 13: *In Vitro* Susceptibility Profile of the *Candida* species to Amphotericin B (20µg)

Species	Total number	S (%)	SDD (%)	R (%)
Candida albicans	20	12(60.0)	(15.0)	5(25.0)
Candida tropicalis	34	27(79.4)	3(8.8)	4(11.8)
Candida parapsilosis	21	16(76.2)	0(0.0)	5(23.8)
Candida krusei	7	3(42.9)	1(14.3)	3(42.9)
Candida glabrata	6	3(50.0)	3(50.0)	0(0.0)
Total	**88**	**61(69.3)**	**10(11.4)**	**17(19.3)**

Key:
S = Susceptible; SDD = Susceptible Dose Dependent; R = Resistant

Table 14: *In Vitro* Susceptibility Profile of the *Candida* species to Flucytosine (1µg)

Species	Total number	S (%)	I (%)	R (%)
Candida albicans	20	0(0.0)	0(0.0)	20(100.0)
Candida tropicalis	34	4(11.8)	6(17.6)	24(70.6)
Candida parapsilosis	21	2(9.5)	0(0.0)	19(90.5)
Candida krusei	7	0(0.0)	0(0.0)	7(100.0)
Candida glabrata	6	0(0.0)	0(0.0)	6(100.0)
Total	**88**	**6(6.8)**	**6(6.8)**	**76(86.4)**

Key:
S = Susceptible; I = Intermediate; R = Resistant

Table 15: *In Vitro* Susceptibility Profile of the *Candida* species to Voriconazole (1µg)

Species	Total number	S (%)	SDD (%)	R (%)
Candida albicans	20	7(35.0)	5(25.0)	8(40.0)
Candida tropicalis	34	18(52.9)	2(5.9)	14(41.2)
Candida parapsilosis	21	14(66.7)	3(14.3)	4(19.0)
Candida krusei	7	4(57.1)	0(0.0)	3(42.9)
Candida glabrata	6	3(50.0)	1(16.7)	2(33.3)
Total	**88**	**46(52.3)**	**11(12.5)**	**31(35.2)**

Key:
S = Susceptible; SDD = Susceptible Dose Dependent; R = Resistant

4.16 *In Vitro* Susceptibility Profile of the *Candida* Species to Itraconazole

Twenty-seven (30.7%) of the *Candida* isolates were susceptible while 21 (23.9%) and 40 (45.5%) were susceptible dose-dependent and resistant respectively. *Candida parapsilosis* was the most susceptible (42.9%) followed by *Candida tropicalis* (35.3%) while *Candida glabrata* was the most resistant (83.3%) followed by *Candida albicans* (65.0%). There were no susceptible isolates of *Candida glabrata* (Table 16).

4.17 *In Vitro* Susceptibility Profile of the *Candida* Species to Nystatin

Table 17 shows the *in vitro* susceptibility profile of the *Candida* isolates to Nystatin. It can be seen from the table that 33 (37.5%), 16 (18.2%) and 39 (44.4%) of the isolates were susceptible, susceptible dose-dependent and resistant respectively. *Candida glabrata* was the most susceptible (66.4%) followed by *Candida parapsilosis* (52.4%) while *Candida albicans* was the most resistant (55.0%) followed by *Candida tropicalis* (50.0%) and *Candida krusei* (42.9%).

4.18 *In vitro* Susceptibility and Resistance Pattern of the *Candida* Species to the Antifungal Drugs

The *in vitro* susceptibility and resistance pattern of the *Candida* species to the antifungal drugs is presented in Table 18. The highest susceptibility was recorded for Amphotericin B to which 61 (69.3%) of all the *Candida* isolates were susceptible followed by Voriconazole (52.3%) and Clotrimazole (43.0%). The highest resistance was observed with Flucytosine to which 76 (86.4%) out of the 88 *Candida* isolates were resistant followed by Ketoconazole (61.4%) and Fluconazole (52.3%). Meanwhile, the highest number of susceptible-dose dependent *Candida* isolates was observed with Ketoconazole (25%), followed by Clotrimazole and Itraconazole, each recording 23.9% and then, Nystatin (18.2%).

Candida tropicalis was the species with the highest susceptibility (79.4%) to Amphotericin B followed by *Candida parapsilosis* (76.2%) and 66.7% respectively to Amphotericin B and Voriconazole. *Candida albicans*, *Candida krusei* and *Candida glabrata* showed 100% resistance to Flucytosine. *Candida krusei* was the species with the least susceptibility showing 0% susceptibility to each of Fluconazole, Ketoconazole and Flucytosine. Also, *Candida glabrata* showed a 0% susceptibility to each of Flucytosine and

Table 16: *In Vitro* Susceptibility Profile of the *Candida* species to Itraconazole (50µg)

Species	Total number	S (%)	SDD (%)	R (%)
Candida albicans	20	4(20.0)	3(15.0)	13(65.0)
Candida tropicalis	34	12(35.3)	11(32.4)	11(32.4)
Candida parapsilosis	21	9(42.9)	4(19.0)	8(38.1)
Candida krusei	7	2(28.6)	2(28.6)	3(42.9)
Candida glabrata	6	0(0.0)	1(16.7)	5(83.3)
Total	**88**	**27(30.7)**	**21(23.9)**	**40(45.5)**

Key:
S = Susceptible; SDD = Susceptible Dose Dependent; R = Resistant

Table 17: *In Vitro* Susceptibility Profile of the *Candida* species to Nystatin (100units)

Species	Total number	S (%)	SDD (%)	R (%)
Candida albicans	20	6(30.0)	3(15.0)	11(55.0)
Candida tropicalis	34	9(26.5)	8(23.5)	17(50.0)
Candida parapsilosis	21	11(52.4)	3(14.3)	7(33.3)
Candida krusei	7	3(42.9)	1(14.3)	3(42.9)
Candida glabrata	6	4(66.4)	1(16.7)	1(16.7)
Total	**88**	**33(37.5)**	**16(18.2)**	**39(44.4)**

Key:
S = Susceptible; SDD = Susceptible Dose Dependent; R = Resistant

Itraconazole while *Candida albicans* showed 0% susceptibility to Flucytosine only. *Candida albicans*, *Candida parapsilosis*, *Candida krusei* and *Candida glabrata* each showed 0% susceptible-dose dependence to Flucytosine. There was 50% susceptibility of *Candida glabrata* to each of Fluconazole, Clotrimazole, Amphotericin B and Voriconazole. Also *Candida glabrata* was the only *Candida* species with 0% resistance to Amphotericin B. Most of the *Candida* isolates were susceptible to Amphotericin B, namely *Candida tropicalis* (79.4%), *Candida parapsilosis* (76.2%), *Candida albicans* (60%), *Candida glabrata* (50%) and *Candida krusei* (42.9%) (Table 18). The inhibition zones produced by some of the antifungal drugs against some of the *Candida* species are shown in Plates 1 to 4 while the IZDs of each antifungal drug against each of the isolated *Candida* species are shown in Appendix A

4.19 Prevalence of Vaginal *Candida* Colonization among the Women in Relation to Antimicrobial Therapy

The prevalence of Vaginal *Candida* Colonization amongst the women in relation to antimicrobial therapy is presented in Table 19. It can be observed that women not on any antimicrobial therapy as at the time of this study had a higher prevalence (27.93%) than those on antimicrobial therapy (4.35%). Statistically, the difference was significant ($p = 0.00$).

4.20 Percentage Yield of the Plant Extracts

The percentage yield of the individual plant extract is shown in Table 20. *Moringa oleifera* had the highest percentage yield of 15.0% followed by *Vernonia amygdalina* (12.0%) and *Ocimum gratissimum* (11.67%).

Table 18: *In vitro* Susceptibility and Resistance Pattern of the *Candida* species to the Antifungal Drugs

DRUGS	*Candida albicans*	*Candida tropicalis*	*Candida parapsilosis*	*Candida Krusei*	*Candida glabrata*	TOTAL (%)
Fluconazole						
S	5	10	9	0	3	27 (30.7)
SDD	3	8	2	1	1	15 (17,0)
R	12	16	10	6	2	46 (52.3)
ketoconazole						
S	3	5	3	0	1	12 (13.6)
SDD	3	8	8	2	1	22 (25.0)
R	14	21	10	5	4	54 (61.4)
Clotrimazole						
S	6	14	12	2	3	37 (42.0)
SDD	6	11	3	0	1	21 (23.9)
R	8	9	6	5	2	30 (34.1)
Amphotericin B						
S	12	27	16	3	3	61 (69.3)
SDD	3	3	0	1	3	10 (11.4)
R	5	4	5	3	0	17 (19.3)
Flucytosine						
S	0	4	2	0	0	6 (6.8)
I	0	6	0	0	0	6 (6.8)
R	20	24	19	7	6	76 (86.4)
Voriconazole						
S	7	18	14	4	3	46 (52.3)
SDD	5	2	3	0	1	11 (12.5)
R	8	14	4	3	2	31 (35.2)
Itraconazole						
S	4	12	9	2	0	27 (30.7)
SDD	3	11	4	2	1	21 (23.9)
R	13	11	8	3	5	40 (45.5)
Nystatin						
S	6	9	11	3	4	33 (37.5)
SDD	3	8	3	1	1	16 (18.2)
R	11	17	7	3	1	39 (44.3)

Key:
S = Susceptible, SDD = Susceptible Dose-Dependent, I = Intermediate, R = Resistant

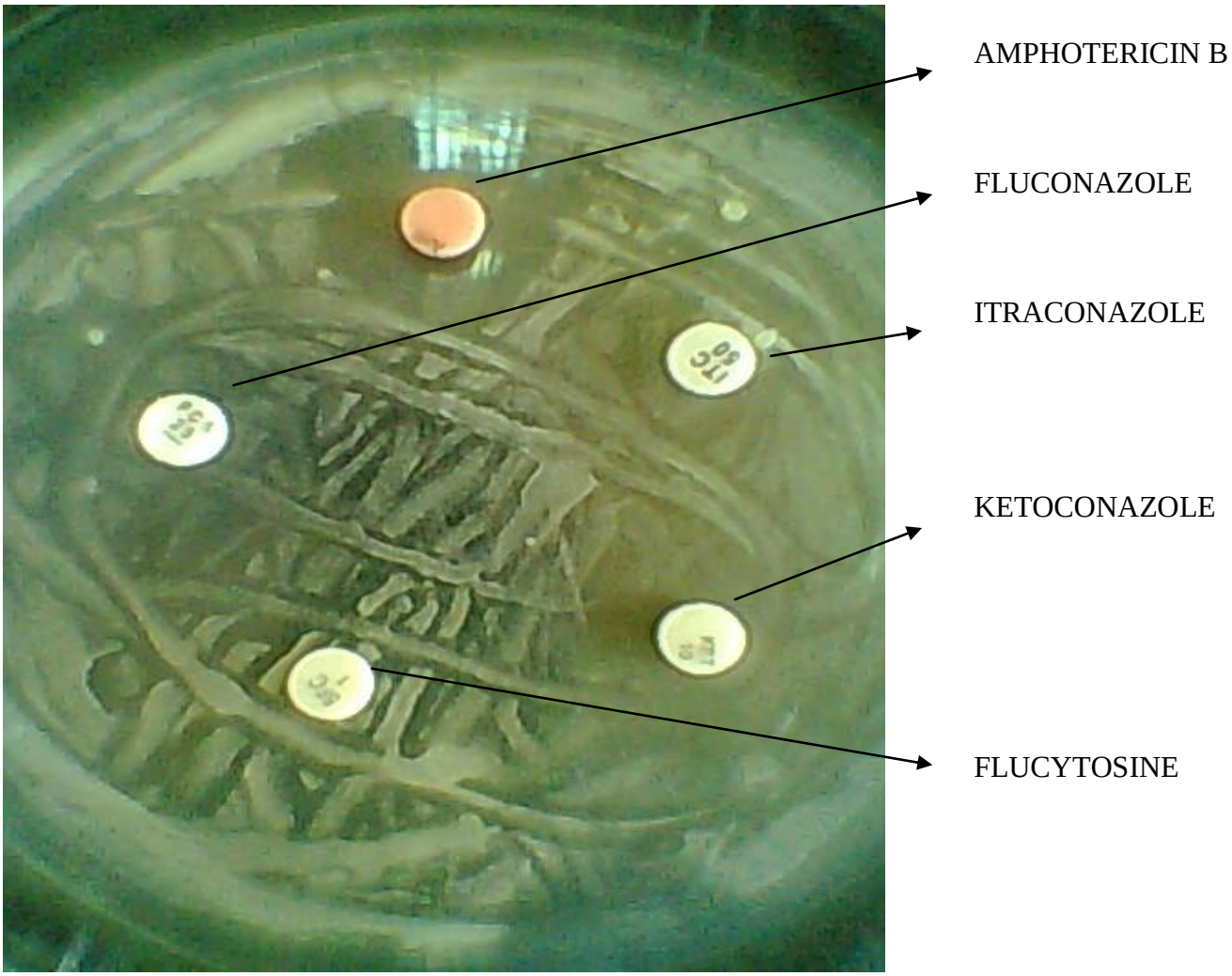

Plate 1: Resistance (No Inhibition Zones) by *Candida albicans*[1] against the Antifungal Drugs, Except Amphotericin B

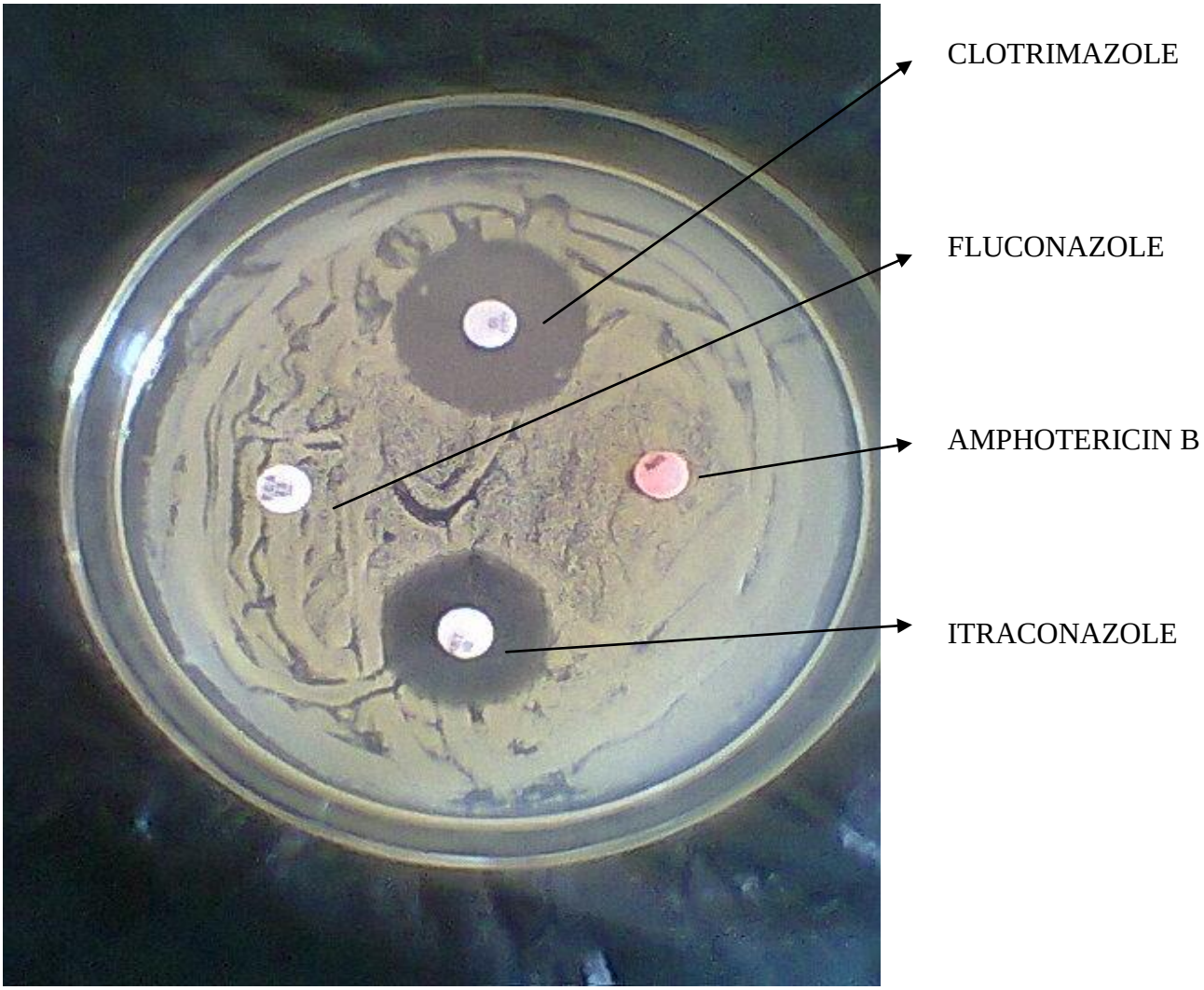

Plate 2: Resistance (No Inhibition Zones) by *Candida albicans*[2] against Fluconazole and Amphotericin B.

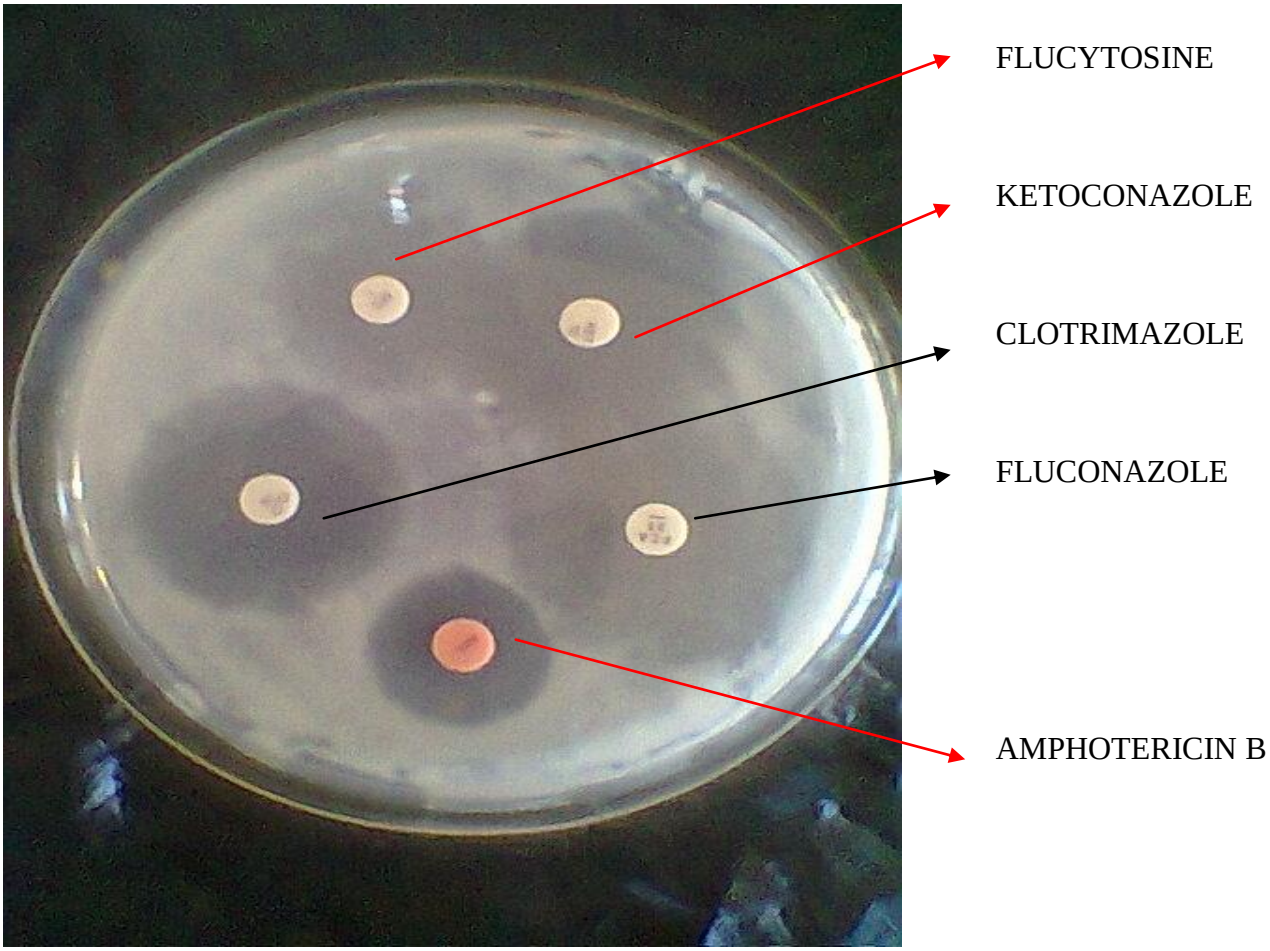

Plate 3: Zones of Inhibition Shown by some of the Antifungal Drugs against Sensitive *Candida tropicalis*.

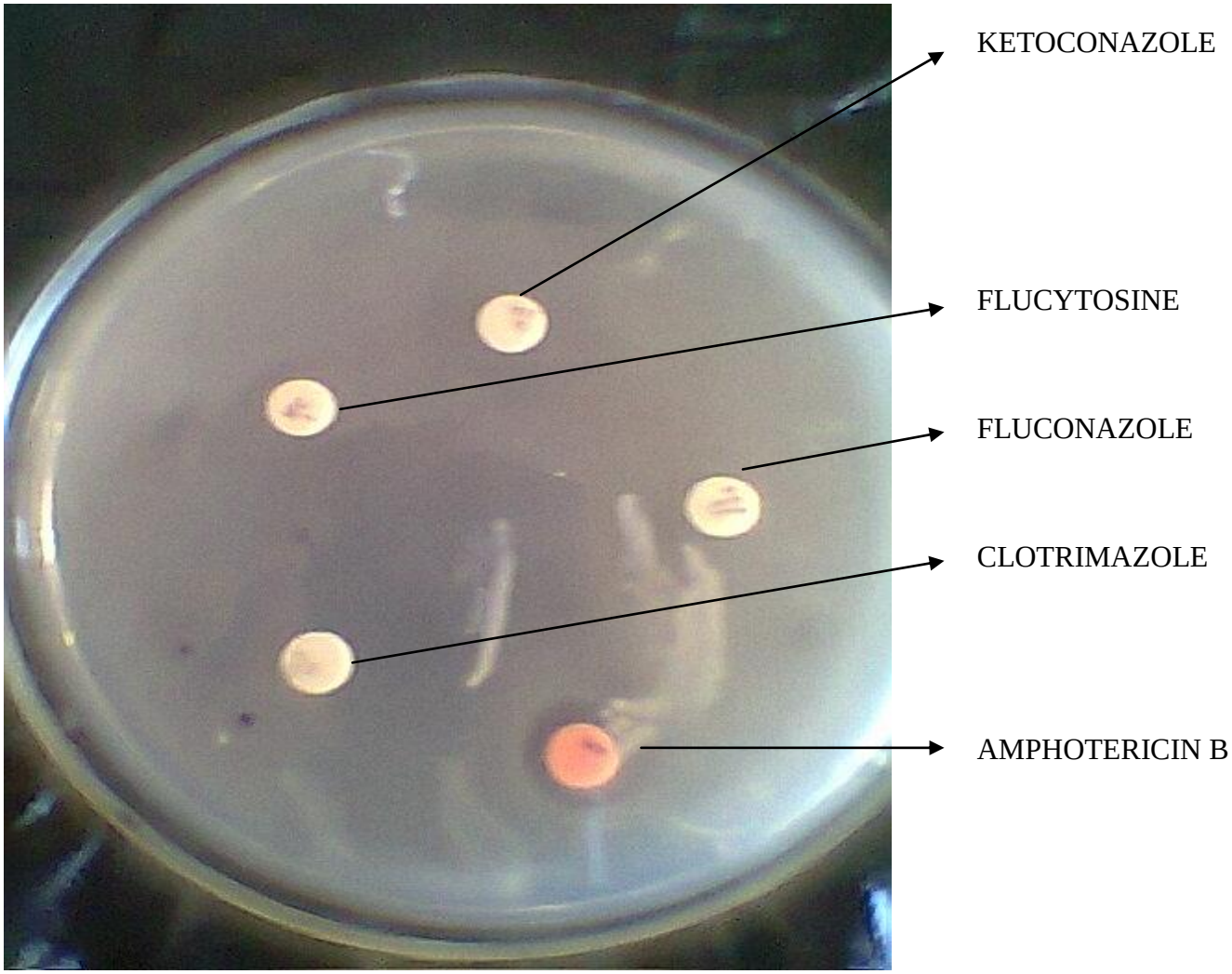

Plate 4: Total Resistance against some of the Antifungal Drugs by *Candida krusei*

Table 19: Prevalence of Vaginal *Candida* Colonization among the Women in Relation to Antimicrobial Therapy

Antimicrobial Therapy	Number sampled	Number of *Candida* positive specimens	Percentage of *Candida* positive Women (%)
Yes	46	2	4.35
No	290	81	27.93
Total	**336**	**83**	**24.7**

$p < 0.05$ ($p = 0.00$); df = 1

Table 20: Percentage Yield of the Plant Extracts

Plants	Initial Dry Weight (g)	Crude Extract Weight (g)	Percentage Yield (%)
Moringa oleifera	300	45	15.0
Vernonia amygdalina	300	36	12.0
Ocimum gratissimum	300	25	11.67

4.21 Inhibition Zone Diameter (IZD) of the Ethanolic Plant Extracts against Some Resistant Strains of the Isolated *Candida albicans*

The inhibition zone diameter (IZD) of the plant extracts against some resistant strains of the isolated *Candida albicans* is presented in Tables 21a and 21b and Plate 6 Extract of *Moringa oleifera* had the highest inhibition zone diameter (19mm) at 200mg/ml against resistant *Candida albicans*[1], followed by extract of *Vernonia amygdalina* and *Ocimum gratissimum* with inhibition zone diameters of 18.67mm and 18mm at 100mg/ml and 200mg/ml respectively. *Candida albicans*[1] was totally resistant to *Ocimum gratissimum* at 25mg/ml and 12.25mg/ml (Table 21a). There was also total resistance by *Candida albicans*[2] to extracts of *Vernonia amygdalina* (at 200mg/ml and 100mg/ml) and *Ocimum gratissimum* (at 25mg/ml and 12.25mg/ml). The highest IZD (18.33mm) against *Candida albicans*[2] was exhibited by extracts of *Vernonia amygdalina* at 50mg/ml followed by *Ocimum gratissimum* (18mm) at 200mg/ml. The extract of *Moringa oleifera* showed the least IZD (11.67mm) at 12.25mg/ml (Table 21b). The IZDs produced by extracts of *Moringa oleifera* and *Ocimum gratissimum* against *Candida albicans*[1] were concentration dependent, ranging from 14.33 (at 12.25 mg/ml) to 19.0 mm (at 200 mg/ml) and zero inhibition (at 12.25 and 25.50mg/ml) to 18.0 mm (at 200 mg/ml) respectively (Table 21a). Similarly, the IZD produced by *Ocimum gratissimum* against *Candida albicans*[2] ranged from zero inhibition (at 12.25 and 25.50 mg/ml) to18.0 mm (at 200 mg/ml).

4.22 Inhibition Zone Diameter (IZD) of the Ethanolic Plant Extracts against Some Resistant Strains of the Isolated *Candida tropicalis*

Tables 22a and 22b show the IZD of the plant extracts against resistant strains of *Candida tropicalis*[1] and *Candida tropicalis*[2] respectively. *Candida tropicalis*[1] was sensitive to all the plant extracts at all concentrations. The IZDs produced by extracts of *Moringa oleifera* and *Ocimum gratissimum* against *Candida tropicalis*[1] were concentration dependent, ranging from the least IZD of 11.33mm (at 12.25 mg/ml) for *Ocimum gratissimum* to the highest IZD of 17.00mm (at 200 mg/ml) for extracts of *Moringa oleifera* and *Vernonia amygdalina* (Table 22a). For *Candida tropicalis*[2], the IZD ranged from 11.67mm for *Moringa oleifera* at 12.25mg/ml to 17.67mm for *Vernonia amygdalina* at 200mg/ml. *Candida tropicalis*[2] was totally resistant to extract of *Moringa oleifera* at 200mg/ml (Table 22b).

Table 21a: Inhibition Zone Diameter of the Ethanolic Plant Extracts against Resistant *Candida albicans*[1]

	Inhibition Zone Diameter (mm)				
	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.25mg/ml
Plant Extracts					
Moringa oleifera	19 ± 0.0	17.33±0.577	15.67± 0.0	15 ± 0.0	14.33±0.577
Vernonia amygdalina	15.33±0.577	18.67±0.577	17 ± 1.0	15 ± 0.0	13 ± 0.0
Ocimum gratissimum	18 ± 0.0	16 ± 0.0	14 ± 0.0	0 ± 0.0	0 ± 0.0

p-value = 0.00 ($p < 0.05$); df =2

Table 21b: Inhibition Zone Diameter of the Ethanolic Plant Extracts against Resistant *Candida albicans*[2]

Plant Extract	Inhibition Zone Diameter (mm)				
	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.25mg/ml
Moringa oleifera	15.00 ± 0.000	16.33 ± 0.577	14.67± 0.577	14.00 ± 0.000	11.67± 0.577
Vernonia amygdalina	0.00 ± 0.000	0.00 ± 0..000	18.33 ±0.577	17.00 ± 0.000	15.33 ±0.577
Ocimum gratissimum	18.00 ± 0.000	15.67 ± 0.577	14.00 ±0.000	0.00 ± 0.000	0.00 ± 0.000

p-value = 0.130 ($p > 0.05$); df = 2

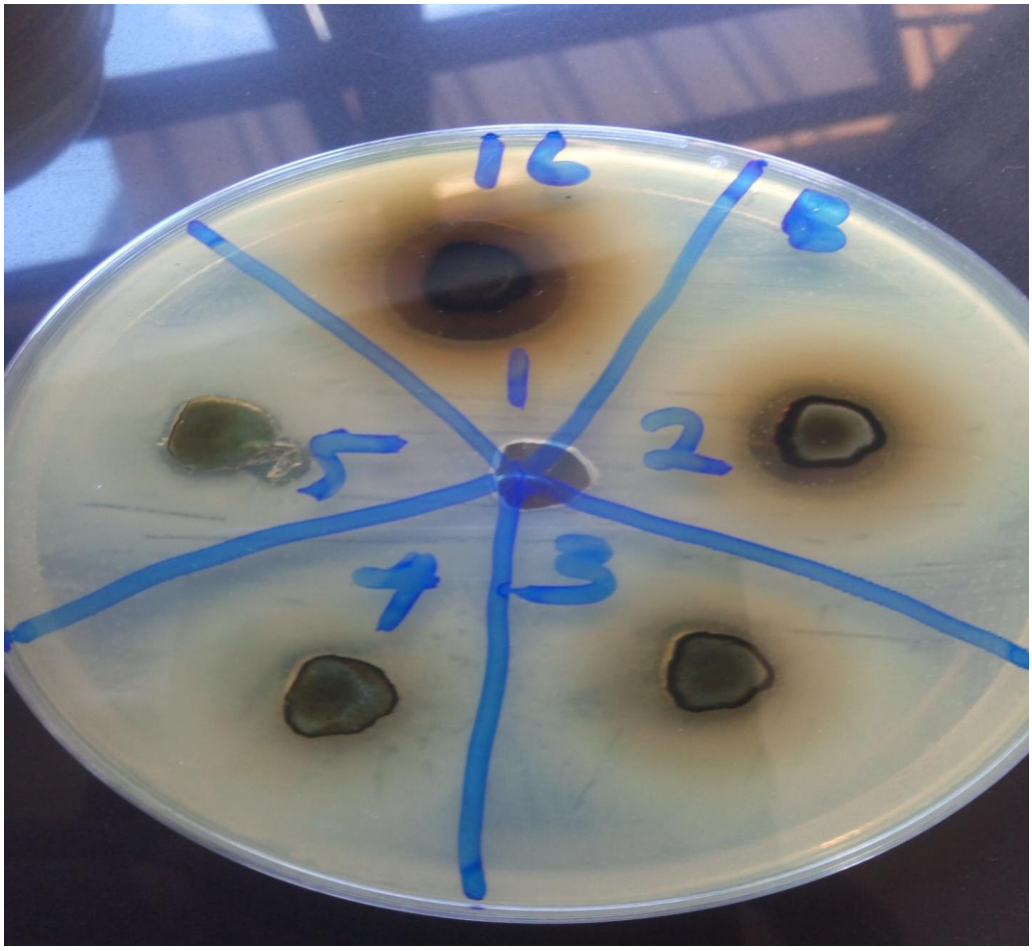

Plate 5: Inhibition Zone Diameters of Ethanolic Extracts of *Vernonia amygdalina* against *Candida albicans*[1]. Numbers 1, 2, 3, 4 and 5 contain 200mg/ml, 100, 50, 25 and 12.5mg/ml of the extract respectively. Centre well contains DMSO (Control).

Table 22a: Inhibition Zone Diameter of the Ethanolic Plant Extracts against Resistant *Candida tropicalis*[1]

Plant Extracts	Inhibition Zone Diameter (mm)				
	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.25mg/ml
Moringa oleifera	17.00 ±0.00	15.67±0.577	15.00±0.00	14.67±1.53	14.00 ±0.00
Vernonia amygdalina	17.00 ±0.00	15.67 ±0.577	15.00 ±0.00	14.67±1.53	14.00±0.00
Ocimum gratissimum	15.00±0.00	13.67±0.577	13.00±0.00	11.67±0.577	11.33±0.00
p-value = 0.00	($p < 0.05$);	df = 2			

Table 22b: Inhibition Zone Diameter of the Ethanolic Plant Extracts against Resistant *Candida tropicalis*[2]

Plant Extract	Inhibition Zone Diameter (mm)				
	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.25mg/ml
Moringa oleifera	0.00 ± 000	17.00 ± 0.000	15.33 ±0.577	14.00 ±1.732	11.67 ±1.155
Vernonia amygdalina	17.67 ± 0.577	16.33 ±0.577	16.00 ±0.000	15.00 ±0.000	13.00 ±0.000
Ocimum gratissimum	17.00 ±.000	16.00 ±.000	14.00 ±1.000	13.67 ±0.577	12.33 ±.577

P-value = 0.021 ($p<0.05$); df =2

4.23 Inhibition Zone Diameter (IZD) of the Ethanolic Plant Extracts against *Candida parapsilosis*

The IZD of the plant extracts against resistant *Candida parapsilosis* is shown in Table 23. The microorganism was resistant to the extract of *Ocimum gratissimum* at all concentrations (Plate 7) while the IZD produced against *Candida parapsilosis* by extracts of *Moringa oleifera* and *Vernonia amygdalina* were concentration dependent, ranging from 14.33mm (at 12.25 mg/ml) to 19.00mm (at 200 mg/ml) and from 12.33mm (at 12.25mg/ml) to 19.00mm (at 200mg/ml) respectively. Some few colonies were observed growing within the inhibition zone at 25mg/ml for *Moringa oleifera* (Plate 6).

4.24 Inhibition Zone Diameter (IZD) of the Ethanolic Plant Extracts against *Candida krusei*

Candida krusei was totally resistant to all the extracts of the plants at 200mg/ml concentration and also resistant to *Ocimum gratissimum* at 12.25mg/ml. The highest IZD (18mm) was shown by extract of *Vernonia amygdalina* at 100mg/ml followed by *Ocimum gratissimum* (16mm) at the same concentration. Extract of *Moringa oleifera* at 12.25mg/ml showed the least IZD (11.67mm) against *Candida krusei* (Table 24).

4.25 MIC Values of the Extracts and Fluconazole against the *Candida* species Using the Continuous Variation Checkerboard Techniques

The MIC values of the plant extracts and that of Fluconazole are presented in Table 25a. These values ranged from 2.21mg/ml (for *M. oleifera*) against *Candida krusei* to 12.88mg/ml (for *O. gratissimum*) against *Candida tropicalis*[1] while the MIC of Fluconazole ranged from 0.01271mg/ml against *Candida albicans*[1] to 0.02512mg/ml against *Candida tropicalis*[1]. The MIC values produced by Fluconazole against the microorganisms were much lower than the MIC values produced by the plant extracts. Tables 25b – 25f and Figures 1 – 6 show how some of the MICs were determined.

Table 23: Inhibition Zone Diameter of the Ethanolic Plant Extracts against Resistant *Candida parapsilosis*

	Inhibition Zone Diameter (mm)				
Plant Extract	200mg/m	100mg/ml	50mg/ml	25mg/ml	12.25mg/ml
Moringa oleifera	19.00± 0.000	17.33± 0.577	15.67± 0.577	15.00± 0.000	14.33± 0.577
Vernonia amygdalina	19.00±1.000	17.67± 0.577	15.33±1.528	13.33±1.528	12.33± 0.577
Ocimum gratissimum	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0

$p = 0.00$ $(p < 0.05)$ $df = 2$

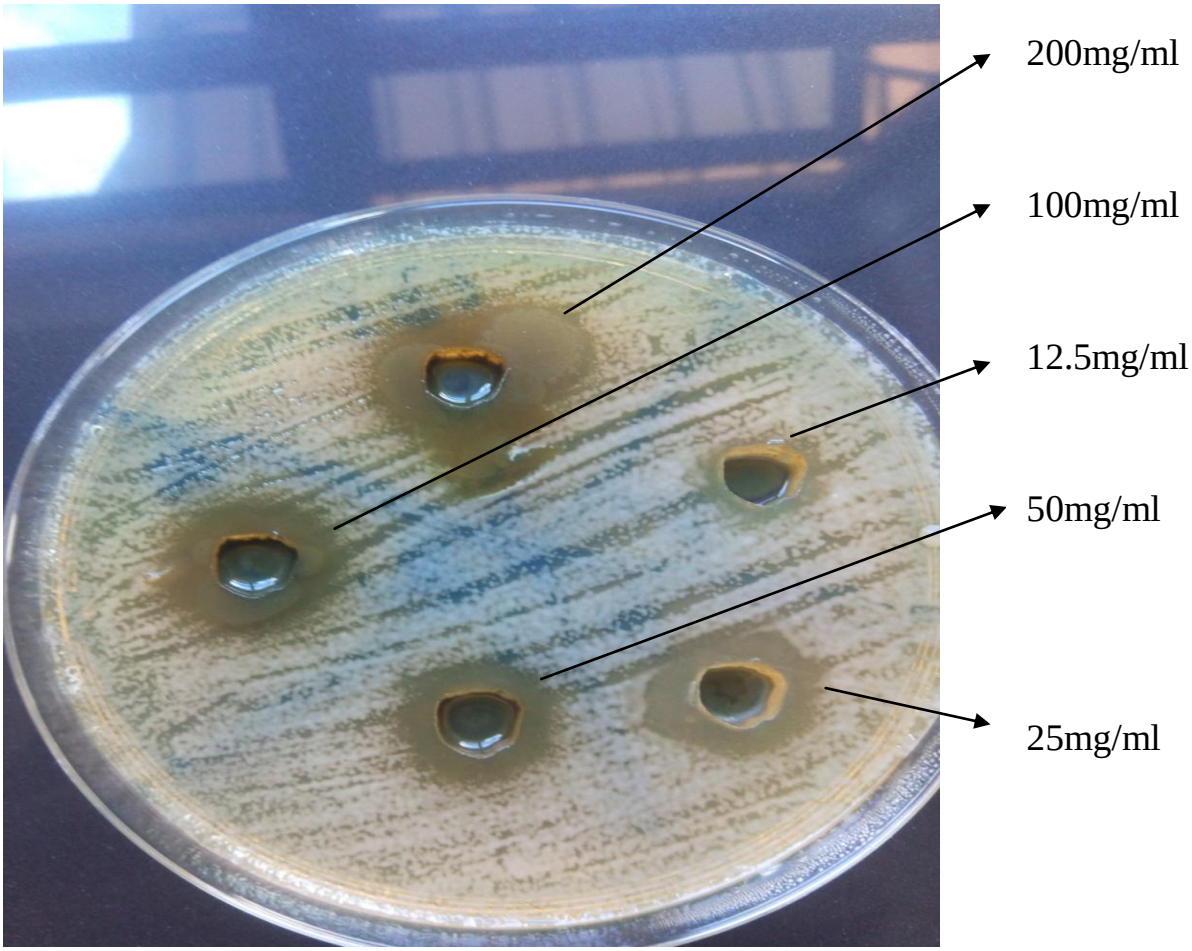

Plate 6: Inhibition Zone Diameters of Ethanolic Extracts of *Moringa oleifera* (at Different Concentrations) against *Candida parapsilosis.*

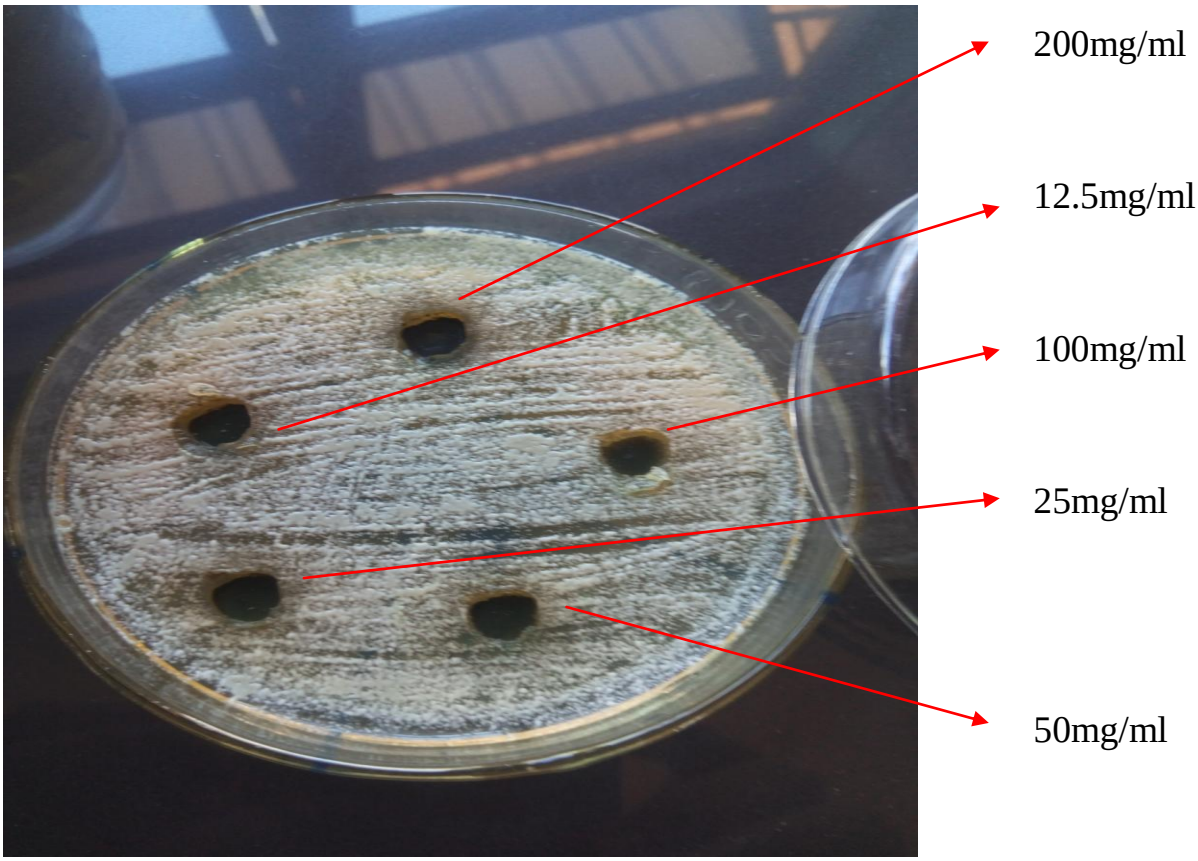

Plate 7: Total Resistance (No Inhibition Zone Diameters) to the Ethanolic Extracts of *Ocimum gratissimum* (at all Concentrations) by *Candida parapsilosis*

Table 24: Inhibition Zone Diameter of the Ethanolic Plant Extracts against Resistant *Candida krusei*

Plant Extract	Inhibition Zone Diameter (mm)				
	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.25mg/ml
Moringa oleifera	0.00 ± 0.000	14.33 ± 0.577	13.00 ±0.000	12.00± 0.000	11.67 ±0.577
Vernonia amygdalina	0.00 ± 0.000	18.00 ± 0.000	15.00 ±0.000	14.00± 0.000	13.00± 0.000
Ocimum gratissimum	0.00 ± 0.000	16.00 ± 0.000	14.00± 0.000	13.00± 0.000	0.00± 0.000

p-value = 0.364 ($p>0.05$); df = 2

Table 25a: MIC Values of the Extracts and Fluconazole against the Resistant Strains of *Candida* species Using the Continuous Variation Checkerboard Techniques

	Minimum Inhibitory Concentration (mg/ml)					
Plant Extracts	*Candida albicans*[1]	*Candida albicans*[2]	*Candida tropicalis*[1]	*Candida tropicalis*[2]	*Candida krusei*	*Candida parapsilosis*
M. oleifera	4.51	3.95	6.48	3.73	2.21	2.64
V. amygdalina	4.03	4.10	8.91	2.59	8.91	2.39
O. gratissimum	5.97	-	12.88	5.80	12.74	-
Fluconazole	0.01271	-	0.02512	-	-	-

Key:
M. oleifera = Moringa oleifera, V. amygdalina = Vernonia amygdalina, O. gratissimum = Ocimum gratissimum, - = No activity

Table 25b: MIC Determination of Fluconazole in A10:B0 Combination against *Candida albicans*[1]

Concentration (µg/ml)	Log Concentration	Well Diameter (mm)	Inhibition Zone Diameter (mm)	X^2	x-intercept	MIC
50	1.69897	9	22	42.25		
25	1.39794	9	18	20.25		
12.5	1.09691	9	9	0		
6.25	0.79588	9	9	0		
3.13	0.495544	9	9	0	1.104014	12.70616

Key:
A = Fluconazole, B = Extract of *Moringa oleifera*

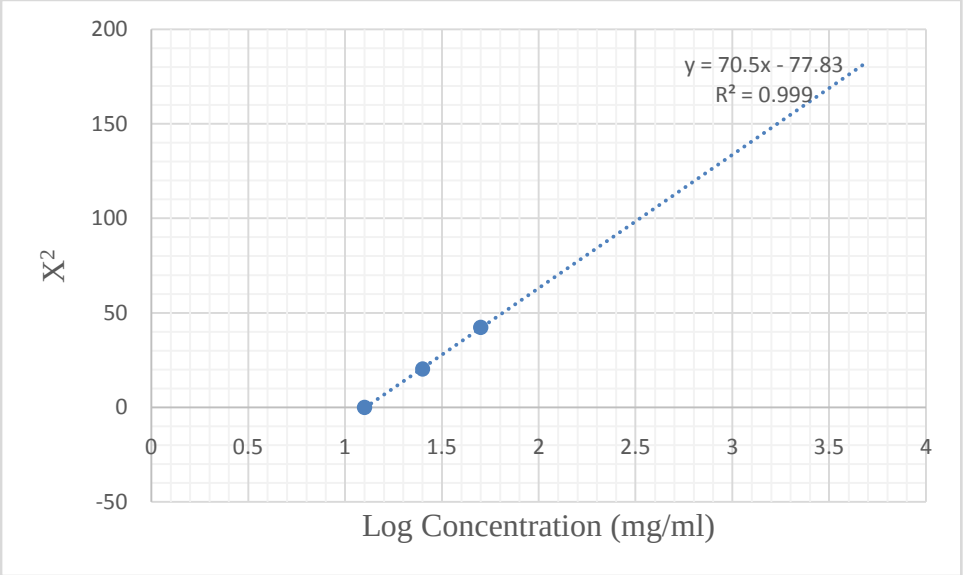

Fig 1: MIC Determination of Fluconazole in A10:B0 Combination against *Candida albicans*[1]

Table 25c: MIC Determination of Extract of *Moringa oleifera* in A0:B10 Combination against *Candida albicans*[1]

Concentration (mg/ml)	Log Concentration	Well Diameter (mm)	Inhibition Zone Diameter (mm)	X^2	x-intercept	MIC
26	1.414973	9	20	30.25		
13	1.113943	9	15	9		
6.5	0.812913	9	11	1		
3.25	0.511883	9	9	0		
1.63	0.212188	9	9	0	0.654382	4.512131

Key:
A = Fluconazole, B = Extract of *Moringa oleifera*

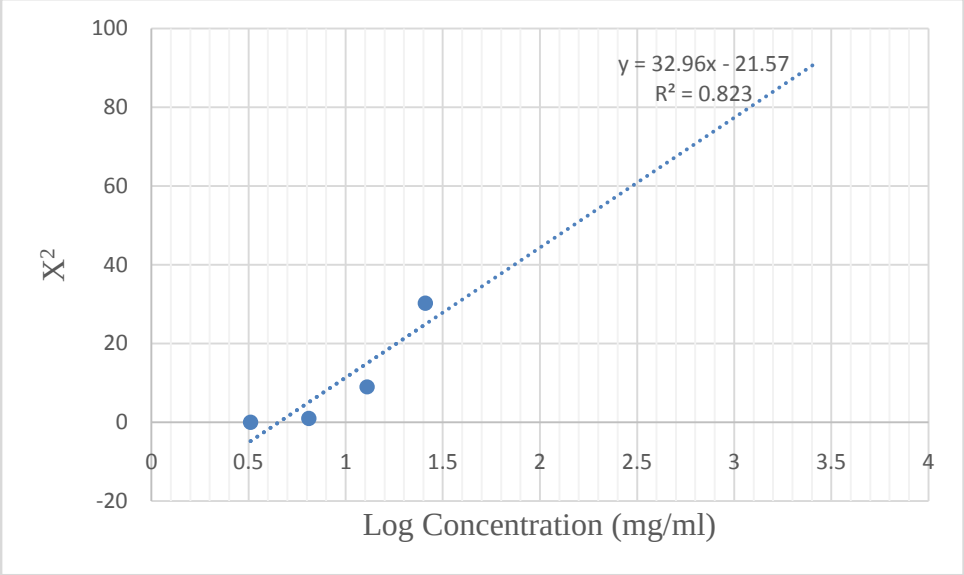

Fig 2: MIC Determination of Extract of *Moringa oleifera* in A0:B10 Combination against *Candida albicans*[1]

Table 25d: MIC Determination of Fluconazole in A10:B0 Combination against *Candida tropicalis*[1]

Concentration (mg/ml)	Log Concentration	Well Diameter (mm)	Inhibition Zone Diameter (mm)	X^2	x-intercept	MIC
50	1.69897	9	16	12.25		
25	1.39794	9	9	0		
12.5	1.09691	9	9	0		
6.25	0.79588	9	9	0		
1.13	0.49554	9	9	0	1.4	25.11886

Key:

A = Fluconazole, B = Extract of *Moringa oleifera*

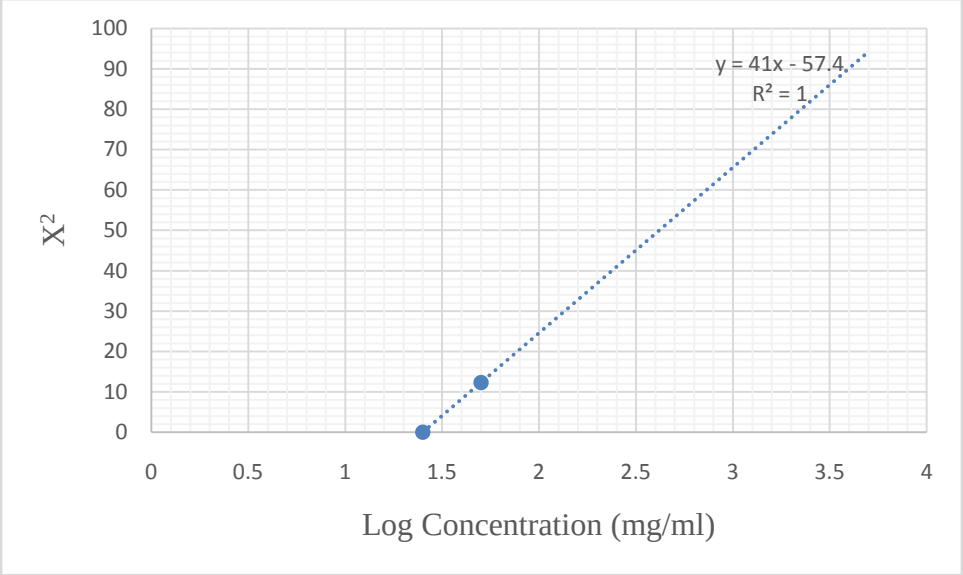

Fig 3: MIC Determination of Fluconazole in A10:B0 Combination against *Candida tropicalis*[1]

Table 25e: MIC Determination of Extract of *Ocimum gratissimum* in A0:B10 Combination against *Candida tropicalis*[1]

Concentration (mg/ml)	Log Concentration	Well Diameter (mm)	Inhibition Zone Diameter (mm)	X^2	x-intercept	MIC
26	1.414973	9	15	9		
13	1.113943	9	9	0		
6.5	0.812913	9	9	0		
3.25	0.511883	9	9	0		
1.63	0.212188	9	9	0	1.11	12.8825

Key:

A = Fluconazole, B = Extract of *Ocimum gratissimum*

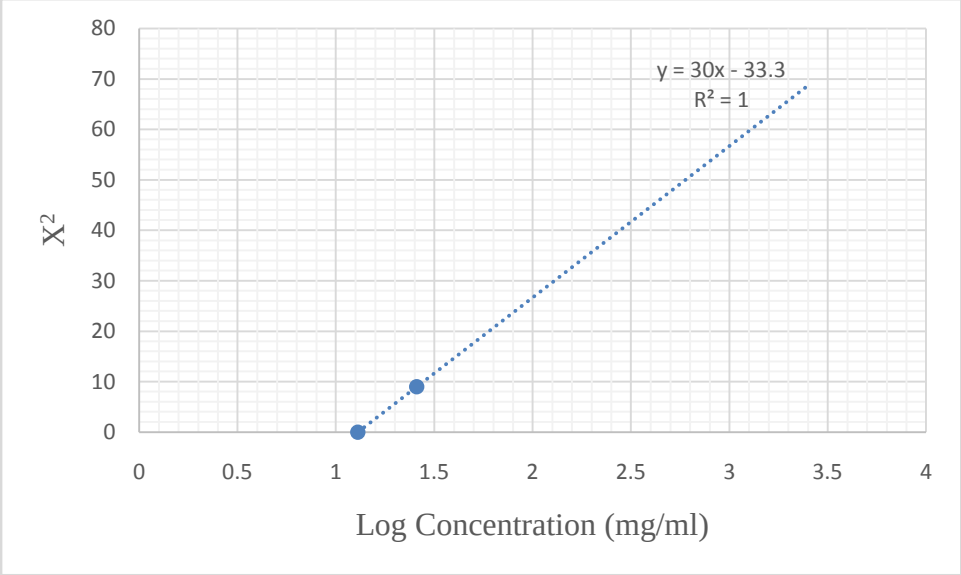

Fig 4: MIC Determination of Extract of *Ocimum gratissimum* in A0:B10 Combination against *Candida tropicalis*[1]

Table 25f: MIC Determination of *Vernonia amygdalina* in A0:B10 Combination against *Candida parapsilosis*

Concentration (mg/ml)	Log Concentration	Well Diameter (mm)	Inhibition Zone Diameter (mm)	X^2	x-intercept	MIC
18	1.255273	9	18	20.25		
9	0.954243	9	15	9		
4.5	0.653213	9	14	6.25		
2.25	0.352183	9	9	0		
1.13	0.053078	9	9	0	0.379286	2.394891

Key:

A = Fluconazole, B = Extract of *Vernonia amygdalina*

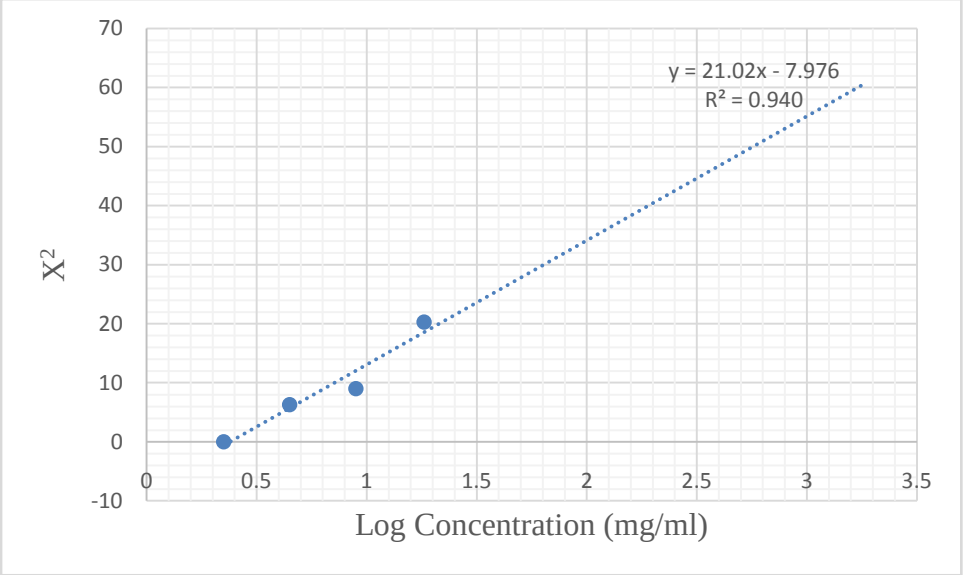

Fig 5: MIC Determination of *Vernonia amygdalina* in A0:B10 Combination against *Candida parapsilosis*

Table 25g: MIC Determination of Fluconazole in A10:B0 Combination against *Candida krusei*

Concentration (mg/ml)	Log Concentration	Well Diameter (mm)	Inhibition Zone Diameter (mm)	X^2	x-intercept	MIC
18	1.69897	9	9	0		
9	1.39794	9	9	0		
4.5	1.09691	9	9	0		
2.25	0.79588	9	9	0		
1.13	0.49554	9	9	0	0.00	0.00

Key:

A = Fluconazole, B = Extract of *Moringa oleifera*

Table 25h: MIC Determination of Fluconazole in A6:B4 Combination against *Candida albicans*[1]

Concentration (mg/ml)	Log Concentration	Well Diameter (mm)	Inhibition Zone Diameter (mm)	X^2	x-intercept	MIC
30	1.477121	9	17	16		
15	1.176091	9	9	0		
7.5	0.875061	9	9	0		
3.75	0.574031	9	9	0		
1.88	0.274158	9	9	0	1.20008	15.84921

Key:

A = Fluconazole, B = Extract of *Moringa oleifera*

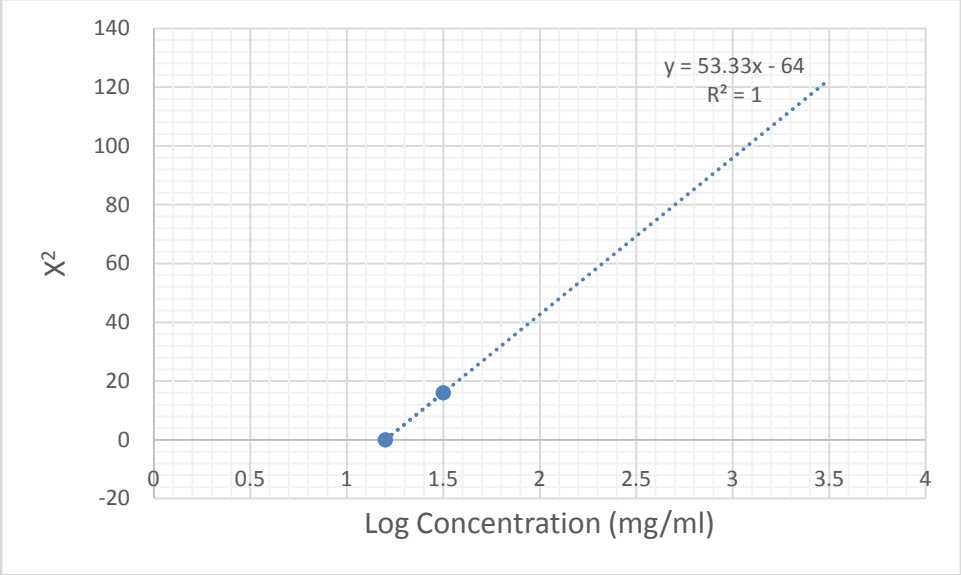

Fig 6: MIC Determination of Fluconazole in A6:B4 Combination against *Candida albicans*[1]

4.26 Combined Antifungal Activity of Fluconazole and Extract of *Moringa oleifera* against Some Selected Resistant *Candida* species

The results of the combined antifungal activity of Fluconazole and extract of *Moringa oleifera* against some selected resistant *Candida* species are shown in Table 26a. Most of the combination ratios produced indifference and antagonism against *Candida albicans*[1] and *Candida krusei*. However, there was synergy at the ratio of 2:8 against *Candida albicans*[1] and the combination ratios of 9:1 against *Candida krusei*. Synergy was produced in most of the combination ratios against *Candida albicans*[2], *Candida tropicalis*[2] and *Candida parapsilosis*. While there was no activity at the ratio of 9:1 against *Candida tropicalis*[2], synergy was produced in all the combination ratios against *Candida tropicalis*[1]. Table 26b shows how the FIC Index (combined activity) of each combination ratio of Fluconazole and extract of *Moringa oleifera* against *Candida albicans*[1] was determined.

4.27 Combined Antifungal Activity of Fluconazole and Extract of *Vernonia amygdalina* against Some Selected Resistant *Candida* species

Synergism was produced by most of the combination ratios against *Candida albicans*[1] and *Candida albicans*[2] while indifference was produced by the combination ratios of 3:7 and 1:9 against *Candida albicans*[1] and 5:5 and 3:7 against *Candida albicans*[2]. Also, synergism was produced against *Candida tropicalis*[1] at combination ratios 9:1 to 6:4 while there was no activity at combination ratios of 5:5 to 1:9. The combination ratios of 9:1 to 5:5 produced no activity against *Candida tropicalis*[2] while 4:6 to 1:9 produced antagonism. There was no antifungal activity in all the combination ratios against *Candida krusei* meanwhile synergy was observed in the combination ratios of 9:1, 8:2 and 6:4 against *Candida parapsilosis* (Table 27a). The FIC Index (combined activity) of each combination ratio of Fluconazole and extract of *Vernonia amygdalina* against Candida *tropicalis*[2] is shown in Table 27b.

Table 26a: Combined Antifungal Activity of Fluconazole and Extract of *Moringa oleifera* against Some Selected Resistant *Candida* species

Combination Ratio A:B	*Candida albicans*[1] FIC Index	*Candida albicans*[2] FIC Index	*Candida tropicalis*[1] FIC Index	*Candida tropicalis*[2] FIC Index	*Candida krusei* FIC Index	*Candida parapsilosis* FIC Index
10:0	-	-	-	-	-	-
9:1	1.22[c]	0.38[a]	0.13[a]	0.00[e]	0.59[a]	0.42[a]
8:2	1.23[c]	0.60[a]	0.16[a]	0.60[a]	1.37[c]	0.41[a]
7:3	2.13[d]	0.73[a]	0.17[a]	0.28[a]	2.12[d]	0.54[a]
6:4	2.36[d]	0.82[a]	0.22[a]	0.43[a]	1.48[c]	0.67[a]
5:5	1.43[c]	0.95[a]	0.24[a]	0.16[a]	1.44[c]	0.85[a]
4:6	1.31[c]	2.21[d]	0.50[a]	2.33[d]	2.20[d]	1.09[c]
3:7	1.55[c]	1.66[c]	0.61[a]	1.56[c]	1.32[c]	1.35[c]
2:8	0.85[a]	1.24[c]	0.53[a]	1.03[c]	1.52[c]	1.31[c]
1:9	1.65[c]	0.68[a]	0.57[a]	1.22[c]	2.05[d]	1.38[c]
0:10	-	-	-	-	-	-

Key:

a = Synergism, b = Additivity, c = Indifference, d = Antagonism, e = No activity, A = Fluconazole; B = Extract of *Moringa oleifera*, FIC = Fractional inhibitory Concentration

Table 26b: Combined Activity of Fluconazole and Extract of *Moringa oleifera* against *Candida albicans*[1]

Combination ratio A:B	MIC of A (mg/ml)	MIC of B (mg/ml)	FIC A	FIC B	FIC index	Inferences
10:0	0.01271					
9:1	0.01365	0.68	1.073958	0.150776	1.224734	indifference
8:2	0.01155	1.45	0.908733	0.321508	1.230241	indifference
7:3	0.01585	3.98	1.24705	0.882483	2.129533	Antagonism
6:4	0.01585	5.01	1.24705	1.110865	2.357914	Antagonism
5:5	0.00755	3.78	0.59402	0.838137	1.432158	indifference
4:6	0.00514	4.09	0.404406	0.906874	1.31128	indifference
3:7	0.00434	5.47	0.341463	1.21286	1.554324	indifference
2:8	0.00164	3.27	0.129032	0.725055	0.854088	synergism
1:9	0.00138	6.93	0.108576	1.536585	1.645161	indifference
0:10		4.51				

Key:
A = Fluconazole; B = Extract of *Moringa oleifera*
FIC = Fractional inhibitory concentration
MIC = Minimum Inhibitory Concentration

Table 27a: Combined Antifungal Activity of Fluconazole and Extract of *Vernonia amygdalina* against Some Selected Resistant *Candida* species

Combination Ratio A:B	*Candida albicans*[1] FIC Index	*Candida albicans*[2] FIC Index	*Candida tropicalis*[1] FIC Index	*Candida tropicalis*[2] FIC Index	*Candida krusei* FIC Index	*Candida parapsilosis* FIC Index
10:0	-	-	-	-	-	-
9:1	0.20[a]	0.00[e]	0.56[a]	0.00[e]	0.00[e]	0.47[a]
8:2	0.26[a]	0.89[a]	0.53[a]	0.00[e]	0.00[e]	0.61[a]
7:3	0.21[a]	0.41[a]	0.42[a]	0.00[e]	0.00[e]	1.37[c]
6:4	0.19[a]	0.39[a]	0.67[a]	0.00[e]	0.00[e]	0.83[a]
5:5	0.30[a]	1.47[c]	0.00[e]	0.00[e]	0.00[e]	1.09[c]
4:6	0.60[a]	0.32[a]	0.00[e]	2.13[d]	0.00[e]	1.16[c]
3:7	1.13[c]	1.70[c]	0.00[e]	2.60[d]	0.00[e]	1.44[c]
2:8	0.64[a]	0.34[a]	0.00[e]	3.09[d]	0.00[e]	1.86[c]
1:9	1.17[c]	0.36[a]	0.00[e]	3.12[d]	0.00[e]	3.37[d]
0:10	-	-	-	-	-	-

Key:
a = Synergism, b = Additivity, c = Indifference, d = Antagonism, e = No activity,
A = Fluconazole; B = Extract of *Vernonia amygdalina*, FIC = Fractional inhibitory concentration

Table 27b: Combined Activity of Fluconazole and Extract of *Vernonia amygdalina* against *Candida tropicalis*[2]

Combination ratio A:B	MIC of A (mg/ml)	MIC of B (mg/ml)	FIC A	FIC B	FIC index	Inferences
10:0	0.05					
9:1	0	0	0	0	0	No activity
8:2	0	0	0	0	0	No activity
7:3	0	0	0	0	0	No activity
6:4	0	0	0	0	0	No activity
5:5	0	0	0	0	0	No activity
4:6	0.01	5.01	0.2	1.934363	2.134363	Antagonism
3:7	0.00794	6.31	0.1588	2.436293	2.595093	Antagonism
2:8	0.00137	7.94	0.0274	3.065637	3.093037	Antagonism
1:9	0.00251	7.94	0.0502	3.065637	3.115837	Antagonism
0:10		2.59				

Key:
A = Fluconazole; B = Extract of *Vernonia amygdalina*
FIC = Fractional inhibitory concentration
FIC = Minimum inhibitory concentration

4.28 Combined Antifungal Activity of Fluconazole and Extract of *Ocimum gratissimum* against Some Selected Resistant *Candida* species

There was no activity for all the combination ratios of Fluconazole and extract of *Ocimum gratissimum* against *Candida albicans*[1], *Candida krusei* and *Candida parapsilosis* except for the combination ratios 2:8 and 1:9 which was synergistic and produced indifference respectively against *Candida krusei*. Synergy was observed in all the combination ratios against *Candida albicans*[2] except combination ratio 3:7 and also most of the combination ratios against *Candida tropicalis*[1] produced synergy. The combination ratios of 9:1 and 7:3 produced synergy against *Candida tropicalis*[2], 4:6 and 3:7 showed no activity while the remaining combination ratios produced indifference (Table 28a). The FIC Index (combined activity) of each combination ratio of Fluconazole and extract of *Ocimum gratissimum* against Candida *krusei* is presented in Table 28b.

4.29: Interactions of the Combination Ratios of the Ethanolic Plant Extracts and Fluconazole against the *Candida* species

Table 29 summarizes the interactions of the combination ratios of the ethanolic plant extracts and Fluconazole. It can be seen that none (0%) of the combination ratios of any of the plant extracts with Fluconazole had any additive effect against the *Candida* species. Also, there was no antagonism in all the combination ratios of *O. gratissimum* with Fluconazole. Meanwhile, the highest number of synergistic combination ratios (48.15%) was observed in the combination of *M. oleifera* with Fluconazole. Only 1 (1.85%) out of the 54 combination ratios of *M. oleifera* with Fluconazole showed no activity.

4.30: PCR Amplification of the Coding Region of ERG11 Gene

The result of the PCR amplification of the coding region of ERG11 gene shows that two fragments were produced for *Candida albicans*[1] (A1), that is E1 and E3 using the two different pairs of primers ERG1A and ERG 1B respectively while only one fragment was produced for *Candida tropicalis*[1] (T1), that is E2 (Fig 7b). The ERG11 genes of the other selected resistant isolates; *Candida tropicalis*[2] (T2), *Candida krusei* (K) and *Candida parapsilosis* were not amplified using both primers (Fig 7a).

Table 28a: Combined Antifungal Activity of Fluconazole and Extract of *Ocimum gratissimum* against Some Selected Resistant *Candida* species

Combination Ratio A:B	*Candida albicans*[1] FIC Index	*Candida albicans*[2] FIC Index	*Candida tropicalis*[1] FIC Index	*Candida tropicalis*[2] FIC Index	*Candida krusei* FIC Index	*Candida parapsilosis* FIC Index
10:0	-	-	-	-	-	-
9:1	0.00[e]	0.30[a]	0.00[e]	0.43[a]	0.00[e]	0.00[e]
8:2	0.00[e]	0.22[a]	0.47[a]	1.11[c]	0.00[e]	0.00[e]
7:3	0.00[e]	0.47[a]	0.94[a]	0.40[a]	0.00[e]	0.00[e]
6:4	0.00[e]	0.51[a]	1.02[c]	1.18[c]	0.00[e]	0.00[e]
5:5	0.00[e]	0.25[a]	0.00[e]	1.34[c]	0.00[e]	0.00[e]
4:6	0.00[e]	0.17[a]	0.74[a]	0.00[e]	0.00[e]	0.00[e]
3:7	0.00[e]	1.18[c]	1.09[c]	0.00[e]	0.00[e]	0.00[e]
2:8	0.00[e]	0.15[a]	0.61[a]	1.82[c]	0.89[a]	0.00[e]
1:9	0.00[e]	0.53[a]	0.59[a]	1.22[c]	1.04[c]	0.00[e]
0:10	-	-	-	-	-	-

Key:
a = Synergism, b = Additivity, c = Indifference, d = Antagonism, e = No activity,
A = Fluconazole; B = Extract of *Ocimum gratissimum,* FIC = Fractional inhibitory concentration

Table 28b: Combined Activity of Fluconazole and Extract of *Ocimum gratissimum* against *Candida krusei*

Combination ratio A:B	MIC of A (mg/ml)	MIC of B (mg/ml)	FIC A	FIC B	FIC index	Inferences
10:00	0.05					
9:01	0	0	0	0	0	No activity
8:02	0	0	0	0	0	No activity
7:03	0	0	0	0	0	No activity
6:04	0	0	0	0	0	No activity
5:05	0	0	0	0	0	No activity
4:06	0	0	0	0	0	No activity
3:07	0	0	0	0	0	No activity
2:08	0.00501	10	0.1002	0.784929	0.885129	synergism
1:09	0.00251	12.59	0.0502	0.988226	1.038426	Indifference
0:10		12.74				

Key:
A = Fluconazole; B = Extract of *Ocimum gratissimum*
FIC = Fractional inhibitory concentration
MIC = Minimum inhibitory concentration

Table 29: Interactions of the Combination Ratios of the Ethanolic Plant Extracts and Fluconazole against the *Candida* species

Plant Extract Combined with FLUCZ	Number of Combination ratios (%)				
	Synergism	*Additivity*	*Indifference*	*Antagonism*	*No Activity*
Moringa oleifera	26(48.15)	0(0.00)	20(37.04)	7(12.96)	1(1.85)
Vernonia amygdalina	20(37.04)	0(0.00)	9(16.67)	5(9.26)	20(37.04)
Ocimum gratissimum	16(29.63)	0(0.00)	9(16.67)	0(0.00)	29(53.70)
TOTAL	**62(38.27)**	**0(0.00)**	**38(23.46)**	**12(7.41)**	**50(30.86)**

Key:
FLUCZ = Fluconazole

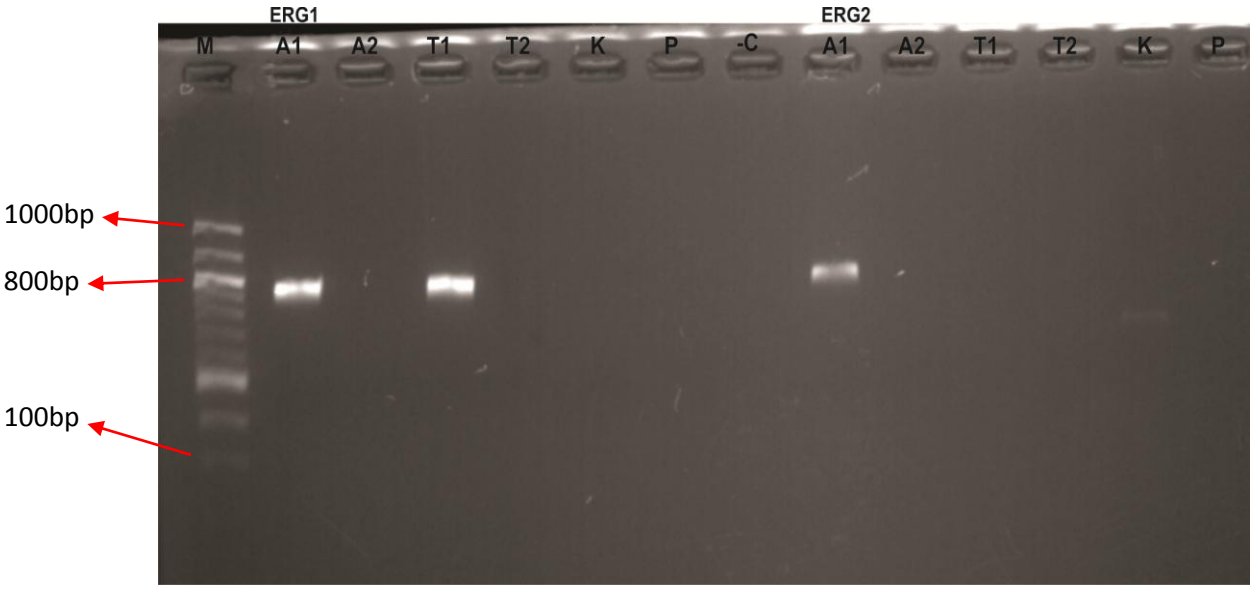

Fig 7a: Agarose Gel Electrophoresis of the Amplicon. Lane M: DNA marker; lane C: Negative Control and Lanes A1 and T1 Showing Visible Amplification of ERG11 Gene for Resistant *Candida albicans*[1] (A1) and *Candida tropicalis*[1] (T1) respectively.

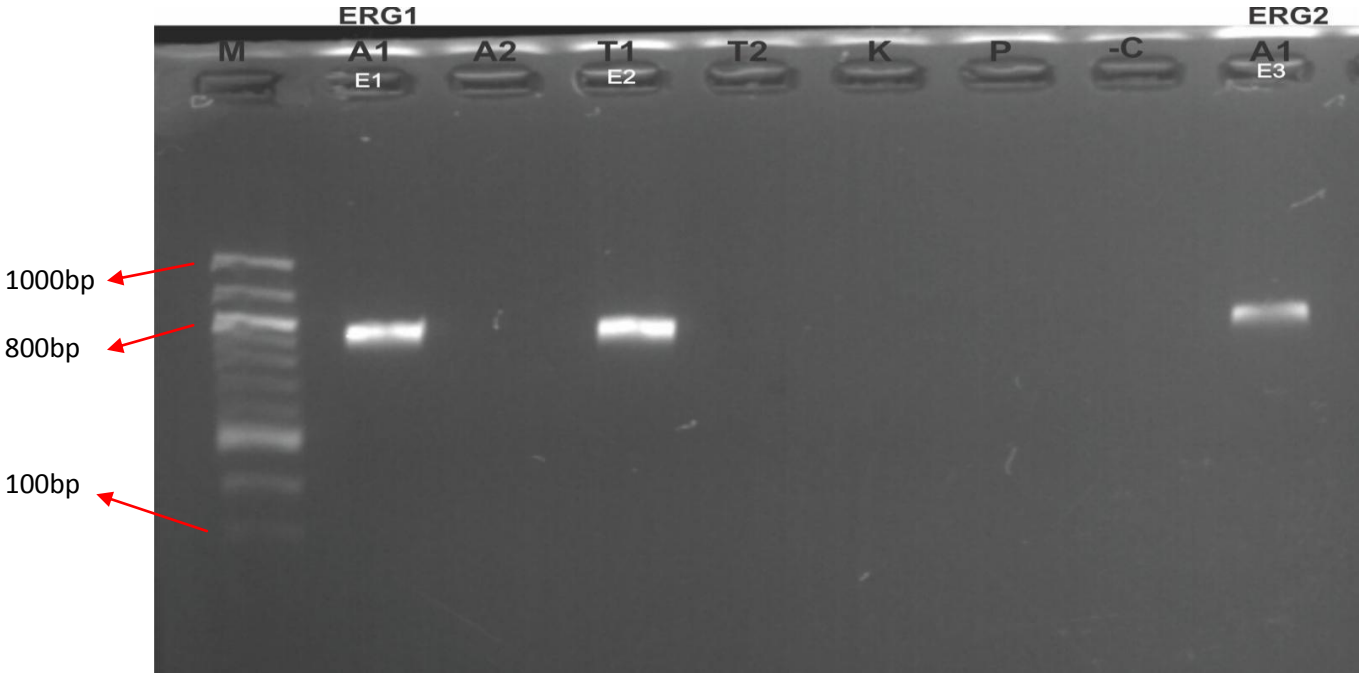

Fig 7b: Agarose Gel Electrophoresis of the Amplicon. Lane M: DNA marker; lane C: Negative Control and Lanes A1 and T1 Showing Visible Amplification of ERG11 Gene for Resistant *Candida albicans*[1] (A1) and *Candida tropicalis*[1] (T1) respectively.

4.31: Sequencing of the ERG11 Gene

The sequencing result of the two fragments of the ERG11 gene of *Candida albicans*[1] (A1) showed that one (E1) was 774 bp (base pairs) in length extending from 55 to 828 bp and the other (E3) 794 bp in length, extending from 769 to 1563 bp (Figures 8a and 8b). Also, the ERG11 gene (E2) of *Candida tropicalis*[1] (T1) was 320 bp extending from 46 to 365 bp (Fig 9). After translation, the length of the amino acid sequences was 258 and 264 for E1 and E3 respectively (figures 11b and 12b) while that for E2 was 106 (fig 13b). The chromatograms of these fragments are shown in figures 14a to 14c (Appendix A). Fig 4.10 shows the nucleotide sequence of the reference *Candida albicans* ERG11 gene (AY856352) as obtained through BLAST. This reference gene is 1587 bp in length.

4.32: Analysis of the Sequencing Results of the ERG11 Gene in *Candida albicans*[1]

The results of the alignment (comparison) of the nucleotide sequence and amino acid sequence (protein) of the reference ERG11 gene and that of the first fragment of *Candida albicans*[1] (E+ERG1A) are shown in Fig 11a and Fig 11b respectively. Also figures 12a and 12b respectively show the alignment of the nucleotide sequence and amino acid sequence (protein) of the reference ERG11 gene with that of the second fragment of *Candida albicans*[1] (E3+ERG2A). On comparing the nucleotide sequence of the two fragments of the ERG11 coding region of *Candida albicans*[1] with that of the reference *Candida albicans* ERG11 gene (AY856352), 11 synonymous mutations and 60 non synonymous mutations which encoded distinct amino acid substitutions were identified. Six (6) of these amino acid substitutions (I162T, R163H, E164G, V272D, F273I and K276Q) occurred within the three hot spot regions in ERG11 gene as shown in Table 30.

CTTKMMMCCCTTAGWGTTACACAACAGATCAGTATATTATTAGGGGTTCCATTTGTTTACAACTTAGTATGG
CAATATTTATATTCATTAAGAAAAGATAGAGCTCCATTAGTGTTTTATTGGATTCCTTGGTTTGGTTCTGCAGCT
TCATATGGTCAACAACCTTATGAATTTTTCGAATCATGTCGTCAAAAGTATGGTGATGTATTTTCATTTATGTTA
TTAGGGAAAATTATGACGGTTTATTTAGGTCCAAAAGGTCATGAATTTGTTTTTAATGCTAAATTATCTGATGT
TTCTGCTGAAGATGCTTATAAACATTTAACTACTCCAGTTTTCGGTAAAGGGGTTATTTATGATTGTCCAAATTC
CAGATTAATGGAACAAAAAAAATTTGCTAAATTTGCTTTGACTACTGATTCATTTAAAAGATATGTTCCTAAGA
TTAGAGAAGAAATTTTGAATTATTTTGTTACTGATGAAAGTTTCAAATTGAAAGAAAAAACTCATGGGGTTGC
CAATGTTATGAAAACTCAACCAGAAATTACTATTTTCACTGCTTCAAGATCTTTATTTGGTGATGAAATGAGAA
GAATTTTTGACCGTTCATTTGCTCAACTATATTCTGATTTAGATAAAGGTTTTACCCCTATTAATTTTGTTTTCCC
TAATTTACCTTTACCTCATTATTGGAGACGTGATGCTGCTCAAAAGAAAATCTCTGCTACTTATATGAAAGAAA
TTAAACTGAGAAGAGAACGTGGTGTWWWTTTGATCAAA

Fig 8a: Nucleotide Sequence of the ERG 11 Gene in *Candida albicans*[1] (E+ERG1A)

GCMWCTTWTATGAAGAATTAAACTGAGAAGAGAACGTGGTGATATTGATCCAAATCGTGATTTAATTGATTC
CTTATTGATTCATTCAACTTATAAAGATGGTGTGAAAATGACTGATCAAGAAATTGCTAATCTTTTAATTGGTAT
TCTTATGGGTGGTCAACATACTTCTGCTTCTACTTCTGCTTGGTTCTTGTTACATTTAGGTGAAAAACCTCATTT
ACAAGATGTTATTTATCAAGAAGTTGTTGAATTATTGAAAGAAAAAGGTGGTGATTTGAATGATTTGACTTAT
GAAGATTTACAAAAATTACCATCAGTCAATAACACTATTAAGGAAACTCTCAGAATGCATATGCCATTACATTC
TATTTTTAGAAAAGTTACTAACCCATTAAGAATCCCTGAAACCAATTATATTGTTCCAAAAGGTCATTATGTTTT
AGTTTCTCCAGGTTATGCTCATACTAGTGAAAGATATTTTGATAACCCTGAAGATTTTGATCCAACTAGATGGG
ATACTGCTGCTGCCAAAGCTAATTCTGTTTCATTTAACTCTTCTGATGAAGTTGATTATGGGTTTGGGAAAGTTT
CTAAAGGGGTTTCTTCACCTTATTTACCATTTGGTGGTGGTAGACATAGATGTATTGGGGAACAATTTGCTTAT
GTTCAATTAGGAACCATTTTAACTACTTTTGTTTATAATTTAAGATGGACTATTGATGGTTATAAAGTGCCTGAC
CCTGATTATAGTTCAATGGTGGTTTTACCTACTGAACCAGCAGAAATCATTTGGAAA

Fig 8b: Nucleotide Sequence of the ERG 11 Gene in *Candida albicans*[1] (E3+ERG2A)

GTTCCGGTTCTTATCTTAACTCAACAAATCACCATCTTGGTTGTTTTCCCATTCATCTACAACATAGCATGGCAA
TTACTTTACTCCTTAAGAAAAGATAGAGTTCCAATGGTTTTCTACTGGATCCCATGGTTTGGTTCTGCTGCTAGT
TATGGTATGCAACCATACGAATTCTTTGAAAAGTGTAGATTGAAATATGGTGATGTTTTTTCMTTTATGTTATT
GGGWAAAGTYATGAYTGKWTATTTGKGTCCWMWMSGWYACGAATTCATTTACAATGCTAAATTATCCGAT
CTTTCTTTGAGCAGCATCACGTCTCCAAT

Fig 9: Nucleotide Sequence of the ERG 11 Gene in *Candida tropicalis*[1] (E2+ERG1A)

ATGGCTATTGTTGAAACTGTCATTGATGGCATTAATTATTTTTTGTCCCTTAGTGTTACACAACAGATCA
GTATATTATTAGGGGTTCCATTTGTTTACAACTTAGTATGGCAATATTTATATTCATTAAGAAAAGATAG
AGCTCCATTAGTGTTTTATTGGATTCCTTGGTTTGGTTCTGCAGCTTCATATGGTCAACAACCTTATGAA
TTTTTCGAATCATGTCGTCAAAAGTATGGTGATGTATTTTCATTTATGTTATTAGGGAAAATTATGACGG
TTTATTTAGGTCCAAAAGGTCATGAATTTGTTTTCAATGCTAAATTATCTGATGTTTCTGCTGAAGATGC
TTATAAACATTTAACTACTCCAGTTTTCGGTAAAGGGGTTATTTATGATTGTCCAAATTCTAGATTAATG
GAACAAAAAAAATTTGCTAAATTTGCTTTGACTACTGATTCATTTAAAAGATATGTTCCTAAAACTCATG
GGGTTGCCAATGTTATGAAAACTCAACCAGAAATTACTATTTTCACTGCTTCAAGATCTTTATTTGGTGA
TGAAATGAGAAGAATTTTTGACCGTTCATTTGCTCAATTAAAGATTAGAGAATACATTTTGAATTATTTT
GTTACTGATGAAAGTTTCAAATTGAAAGAATATTCTGATTTAGATAAAGGTTTTACCCCTATTAATTTTG
TTTTCCCTAATTTACCTTTACCTCATTATTGGAGACGTGATGCTGCTCAAAAGAAAATCTCTGCTACTTA
TATGAAAGAAATTAAACTGAGAAGAGAACGTGGTGATATTGATCCAAATCGTGATTTAATTGATTCCTTA
TTGATTCATTCAACTTATAAAGATGGTGTGAAAATGACTGATCAAGAAATTGCTAATCTTTTAATTGGTA
TTCTTATGGGTGGTCAACATACTTCTGCTTCTACTTCTGCTTGGTTCTTGTTACATTTAGGTGAAAAACC
TCATTTACAAGATGTCATTTATCAAGAAGTTGTTGAATTATTGAAAGAAAAAGGTGGTGATTTGAATGAT
TTGACTTATGAAGATTTACAAAAATTACCATCAGTCAATAACACTATTAAGGAAACTCTTAGAATGCATA
TGCCATTACATTCTATTTTTAGAAAAGTTACTAACCCATTAAGAATCCCTGAAACCAATTATATTGTTCC
AAAAGGTCATTACGTTTTAGTTTCTCCAGGTTATGCTCATACTAGTGAAAGATATTTTGATAACCCTGAA
GATTTTGATCCAACTAGATGGGATACTGCTGCTGCCAAAGCTAATTCTATTTCATTTAACTCTTCTGATG
AAGTTGATTATGGGTTTGGGAAAGTTTCTAAAGGGGTTTCTTCACCTTATTTACCATTTGGTGGTGGTAG
ACATAGATGTATTGGGGAACAATTTGCTTATGTTCAATTGGGAACCATTTTAACTACTTTTGTTTATAAC
TTAAGATGGACTATTGATGGTTATAAAGTGCCTGACCCTGATTATAGTTCAATGGTGGTTTTACCTACTG
AACCAGCAGAAATCATTTGGGAAAAAAGAGAAACTTGTATGTTTTAA

Fig 10: Nucleotide Sequence of the Reference *Candida albicans* ERG11 Gene (AY856352)

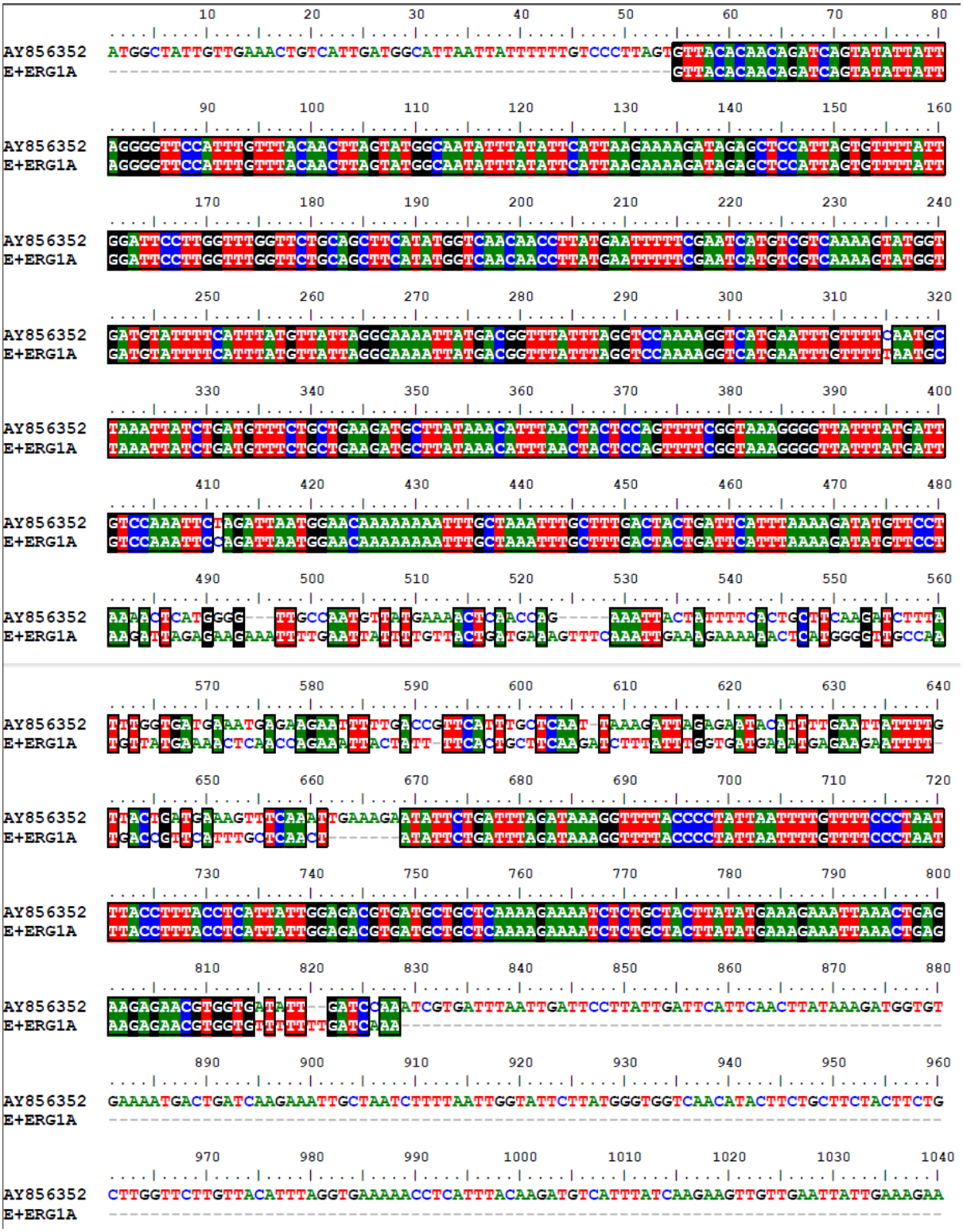

Fig 11a: Alignment (comparison) of the Nucleotide Sequence of the Reference ERG11 Gene (AY856352) and that of the First fragment of *Candida albicans*[1] (E+ERG1A)

Fig 11b: Alignment (comparison) of the Amino Acid Sequence (Protein) of the Reference ERG11 Gene (AY856352) and that of the First fragment of *Candida albicans*[1] (E+ERG1A).

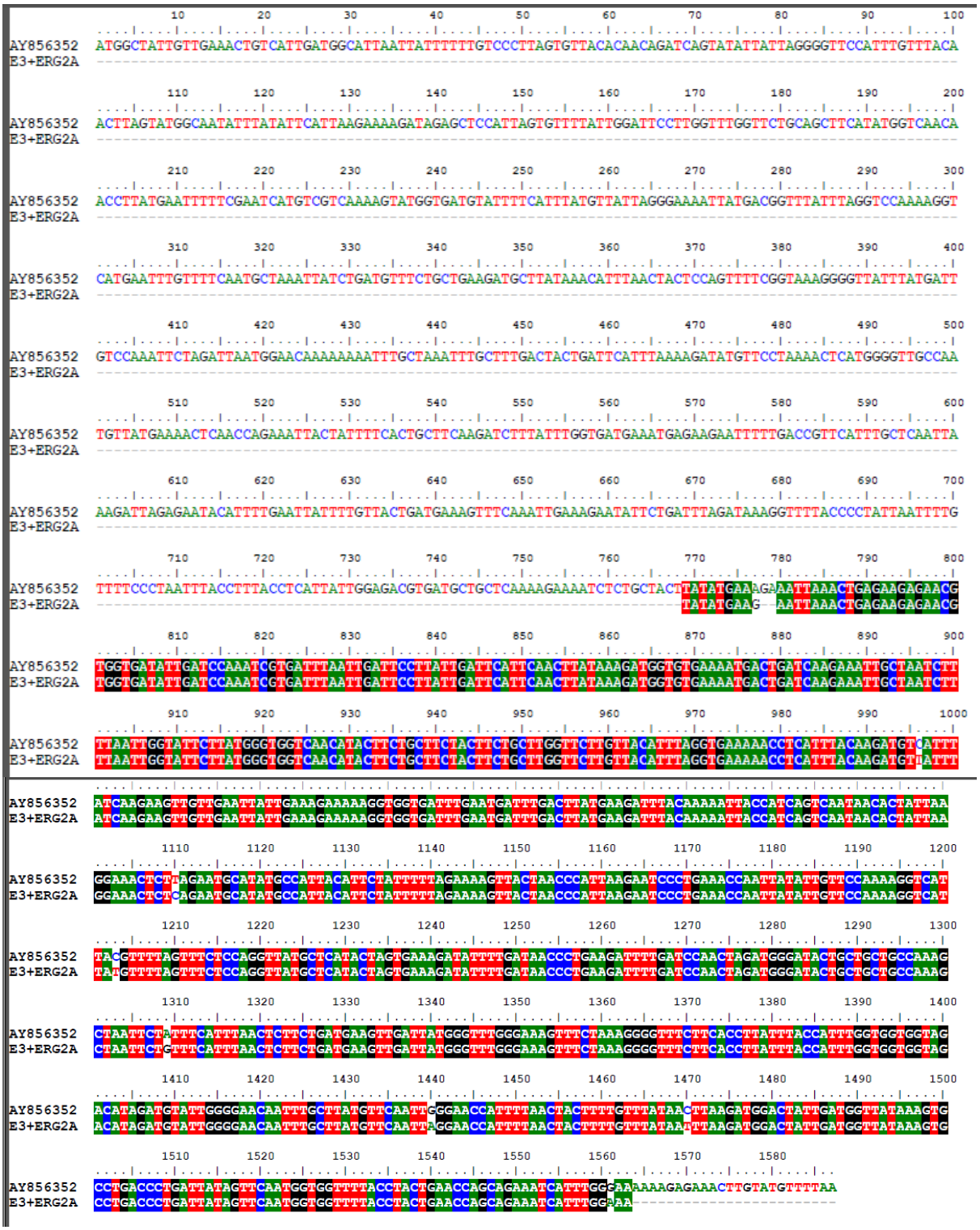

Fig 12a: Alignment (comparison) of the Nucleotide Sequence of the Reference ERG11 Gene (AY856352) and that of the Second fragment of *Candida albicans*[1] (E3+ERG2A).

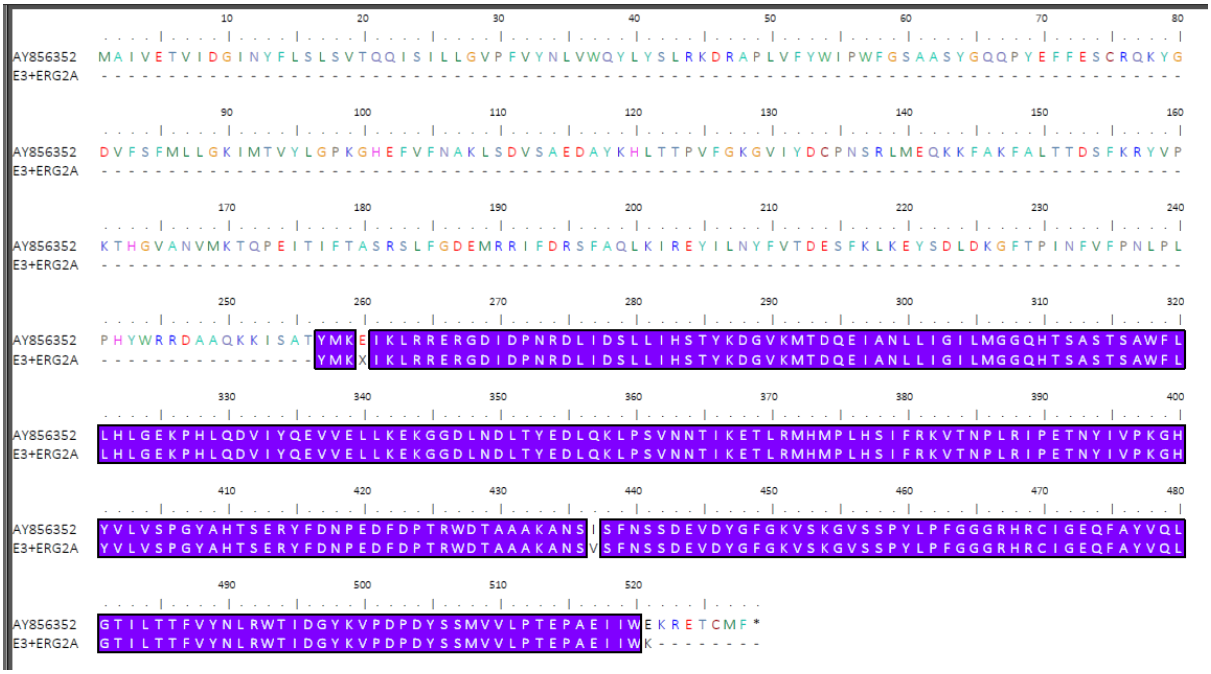

Fig 12b: Alignment (comparison) of the Amino Acid Sequence (Protein) of the Reference ERG11 Gene (AY856352) and that of the Second fragment of *Candida albicans*[1] (E3+ERG2A).

Table 30: Mutations Leading to Amino Acid Substitutions in the Hot Spot Regions of ERG11 Gene in *Candida albicans*[1]

ERG11 Gene Mutations	Amino Acid Substitutions
T486C	I162*T
A487C, G488A, A489T	R163H
A491G	E164G
T815A	V272D
T817A	F273I
A826C	K276Q

Key:
A= Adenine, C = Cytosine, G = Guanine, T = Thymine, I = Isoleucine,,
R = Arginine, H = Histidine, E = Glutamic Acid, G = Glycine, V = Valine,
D = Aspartic Acid, F = Phenylalanine, K = Lysine, Q = Glutamine, *T = Threonine

4.33: Analysis of the Sequencing Results of the ERG11 Gene in *Candida tropicalis*[1]

Figures 13a and 13b respectively show the results of the alignment (comparison) of the nucleotide sequence and amino acid sequence (protein) of the reference ERG11 gene and that of *Candida tropicalis*[1] (E2+ERG1A). There were 27 synonymous and also 27 non synonymous mutations which encoded distinct amino acid substitutions. Six (6) of these mutations (Y105F, L112V, A116D, T119K, S120H and P121L) were found within the 3 hot spot regions in ERG11 gene (Table 31).

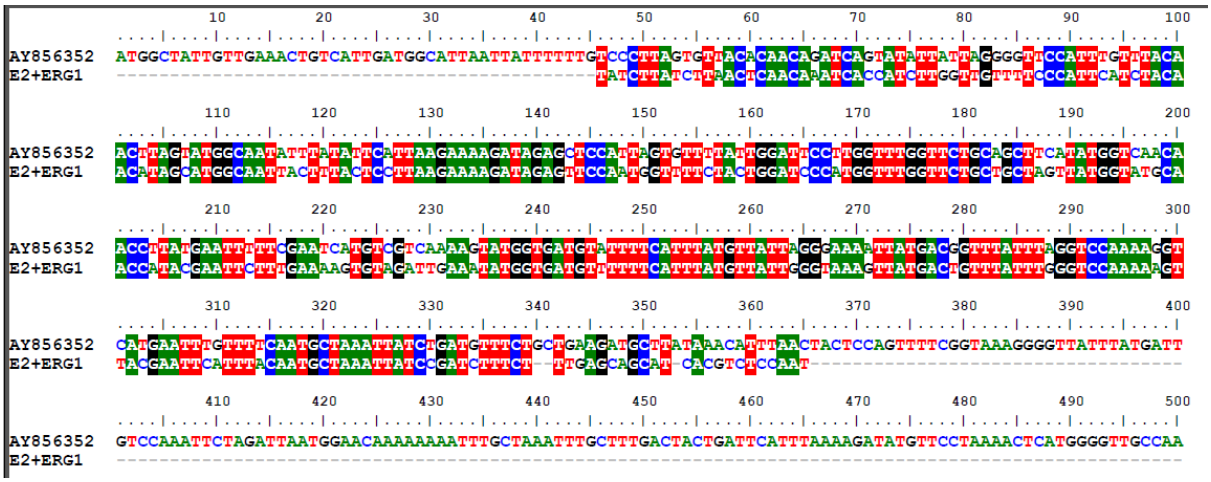

Fig 13a: Alignment (comparison) of the Nucleotide Sequence of the Reference ERG11 Gene (AY856352) and that of *Candida tropicalis*[1] (E2+ERG1A).

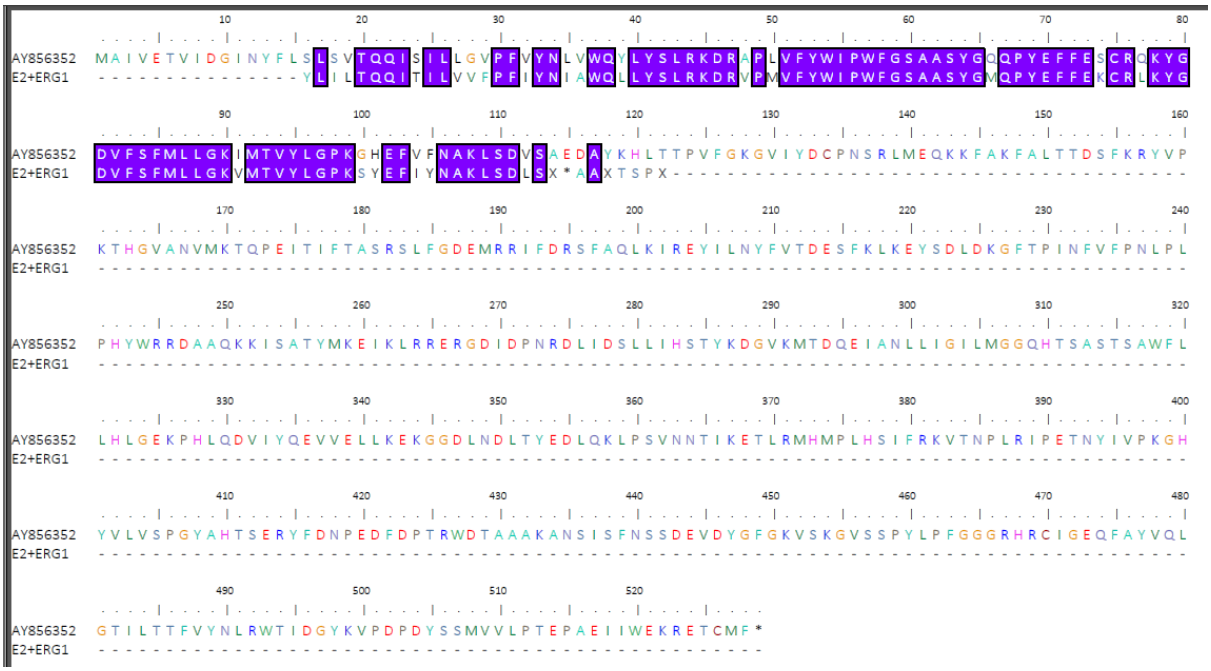

Fig 13b: Alignment (comparison) of the Amino Acid Sequence (Protein) of the Reference ERG11 Gene (AY856352) and that of the Second fragment of *Candida tropicalis*[1] (E2+ERG1A).

Table 31: Mutations Leading to Amino Acid Substitutions in the Hot Spot Regions of ERG11 Gene in *Candida tropicalis*[1]

ERG11 Gene Mutations	Amino Acid Substitutions
A314T	Y105F
C334G	L112V
C347A, A348T	*A116D
C356A, G357A	*T119K
T358C, C359A	S120H
C361T, C362T	P121L

Key:
A= Adenine, C = Cytosine, G = Guanine, T = Thymine, Y = Tyrosine, *T = Threonine, H = Histidine, V = Valine, S = Serine, D = Aspartic Acid, F = Phenylalanine, L = Leucine, *A = Alanine, K = Lysine, P = Proline

CHAPTER FIVE

5.0 DISCUSSION

Out of the 340 high vaginal swab (HVS) specimens collected from each of the 340 women who took part in this study, 4 were considered invalid because the questionnaires were not completed.

In this study, eighty-three (83) out of the 336 women who took part in the study were positive for *Candida* species giving a prevalence of vaginal *Candida* colonization of 24.7%. A similar result was reported by Amutaigwe *et al*. (2017) in which a 33.3% prevalence of vaginal *Candida* colonization was observed among the women studied. Okolo *et al*. (2017) reported a prevalence of 22.5% in their study. There are other similar reports by other researchers (Abruquah, 2012; Maikenti *et al.*, 2016; Damen *et al.*, 2017). The findings of Amar *et al*. (2014) and Udayalaxmi *et al*. (2014) showed a much lower prevalence of 11.7% and 14% respectively. The result of this study contradicts that of Elfeky *et al*. (2015) who observed a vaginal *Candida* colonization prevalence of 50.4%. Also, the result of a similar research by Akortha *et al*. (2009) doesn't agree with that of this study. In their work, out of a total of 239 high vaginal swab (HVS) specimens collected, 179 yielded growth of *Candida* species giving a vaginal *Candida* colonization of 74.9%. Uzoh, *et al*. (2016) also recorded a high prevalence of 41% in their study. Differences in the periods during which the specimens where collected as well as the population types may likely explain the variations in the reports, vis-à-vis the prevalence rates of vaginal *Candida* colonization (Eweani *et al*., 2001).

This study revealed *Candida tropicalis* as the most predominant species isolated from the HVS specimens. *Candida tropicalis* constituted 38.63% followed by *Candida parapsilosis* (23.86%), *Candida albicans* (22.73%), *Candida krusei* (7.95%) and *Candida glabrata* (6.82%). This result agrees with that of Amutaigwe *et al*. (2017) in which *Candida tropicalis* was the most predominant species constituting 38.2%. The results of Almeida *et al*. (2013) also indicated *Candida tropicalis* as the most commonly isolated yeast species from patients treated at the University Hospital of the Federal University at Grande Dourados, Central-western Brazil. Also, in a research carried out by Shrivastav *et al*. (2015), in Madhya Pradesh, India, *Candida albicans* was the third (after *Candida krusei* and *C. glabrata*) most predominant species isolated constituting 21.1% which is very close to the 22.73% recorded in the present study. This result, however doesn't agree with those of many other researchers who reported *Candida albicans* as the most predominant species isolated (Akortha *et al*., 2009; Abruquah, 2012; Amar *et al*., 2014; Madhumati and Rajendriani, 2015; Efunshile *et*

al., 2016; Alizadeh *et al*., 2017). In a similar study by Okungbowa *et al*. (2003), in seven cities in Nigeria, *Candida parapsilosis* had the highest frequency of occurrence (33.7%) while *Candida glabrata* had the least (5%). This also disagrees with this study. A possible explanation for these differences in the results of the various studies is the changes that occur in the study populations vis-à-vis menopausal and postmenopausal women and those that might have some underlying disorders like diabetes. Thus, there can be changes in patient physiology, hormone balance, and decrease in immune function (Vermitsky *et al*., 2008). One other reason why *Candida albicans* often predominates non-albicans *Candida* (NAC) species especially from vaginal specimens may be due to the fact that *C. albicans* adheres to vaginal epithelial cells in higher numbers than do other *Candida* species (Payne *et al*., 2016). Also, the rising trend in the isolation of NAC from vaginal specimens can be attributed to the indiscriminate use of anti-fungal agents which eliminates the more sensitive *C. albicans* and selects resistant NAC species (Pirotta *et al*., 2003).

It was observed in this study that women of ages 50 years and above recorded the highest prevalence (6, 50%) of vaginal *Candida* colonization, followed by women in the age groups 18 – 25 years (40, 29.6%), 42 - 49 years (6, 23.1%) and 34 – 41 years (12, 19.4%). Women in the age bracket 26 – 33 years had the least prevalence (19, 18.8%). The difference in the prevalence of vaginal *Candida* colonization among the age groups was not statistically significant ($p > 0.05$). This agrees with the findings of Okungbowa *et al*. (2003) who reported that the age bracket 21 – 25 ranked second (28%) in the frequency of *Candida* positive samples, in their research carried out in seven cities in the Southern part of Nigeria. Also, Maikenti *et al*. (2016) reported a prevalence rate of 30.2% within the age group 21 – 25years among female students in Bingham University, Nasarawa State. The results of this study, however, contradicts that of Akortha *et al*. (2009). In their work, the highest frequency of *Candida* species was seen among the age bracket 26 - 35 years (57.4%), followed by the age group 16 - 25 years (29.6%) while ages 46 and above recorded the lowest (1.0%). Results of similar work by some other researches also disagree with that of this study; the highest prevalence of vaginal *Candida* species was recorded in age groups 26 – 30 (35%), 15 – 20 (25.6%), 16 – 20 (15.6%) and 21 – 30 (46.8%) by Okungbowa *et al*. (2003), Abruquah (2012), Maikenti *et al*. (2016) and Okolo *et al*. (2017) respectively. This can be attributed to the fact that women aged 21 - 30 years are the most sexually active age group with highest risk of pregnancies, indulgence in family planning pills (Nwadioha *et al*., 2010) and more often engage in sexual promiscuity making them more vulnerable.

Contrary to the result of this study, Okungbowa *et al*. (2003), Abruquah (2012), Uzoh *et al*. (2016) and Okolo *et al*. (2017) reported the least prevalence of vaginal *Candida* isolates within the age groups 41years and above (1.2%), 51 years and above (2.6%), 40 – 49 (2%) and above 41 years (4.3%) respectively. The high prevalence (50%) of vaginal *Candida* colonization seen in the women aged 50 years and above as observed in this study, may be because the women had reached menopause. The usual age range is 45 – 55 years. At menopause, the vaginal walls become thinner, dryer, less elastic, and possibly irritated. This increases the risk of vaginal yeast infections (Grady and Barrett-Connor, 2016; Lamberts and van den Beld, 2016; Lobo, 2017). Also, it has been reported that postmenopausal women taking hormone replacement therapy (HRT) are significantly more prone to develop vulvovaginal candidiasis (VVC) than women who are not and those with VVC are likely to have been susceptible to it before menopause (Fischer and Bradford, 2011).

The prevalence of vaginal *Candida* colonization was higher among the non-pregnant women (28.6%) than the pregnant women (16.0%) though the difference was statistically not significant ($p > 0.05$). This agrees with the findings of Ugochukwu *et al*. (2013) who reported a higher prevalence of *Candida albicans* in non-pregnant women (76.8%) than in the pregnant women (23.2%) but because the difference was statistically significant. They suggested the higher prevalence in non-pregnant women than their pregnant counterparts could be as a result of the number of pregnant women whose data were reviewed when compared to the number of non-pregnant women. The result of this study disagrees with that of Uzoh *et al*. (2016) and Amutaigwe *et al*. (2017) who demonstrated a higher prevalence of vaginal *Candida* colonization in the pregnant women (40% and 36.8%) than in their non-pregnant counterparts (17% and 21.7%) respectively. Vroumsia *et al*. (2013) also reported a higher prevalence rate of vulvovaginal candidiasis among pregnant women (55.4%) than among non-pregnant women (35.4%) in Maroua, Far-North, Cameroon. Vroumsia *et al*. (2013) attributed the higher prevalence in the pregnant women to changes in the levels of female sex hormones, such as estrogen and progesterone (Tarry *et al*., 2005) during pregnancy. However, pregnancy is not the only predisposing factor of vaginal candidiasis in women. The higher prevalence in the non-pregnant women as observed in the present study can possibly be explained by differences in the population types; the non-pregnant women would have had other more predisposing factors. In their study, Amar *et al*. (2014) reported that diabetes mellitus was the major predisposing factor causing candidiasis constituting 33% followed by pregnancy (22.3%). Oviasogie and Okungbowa (2009) showed there was a significant difference in the rate of colonization by *Candida* between women who used

contraceptives (58.3%) and those who never used contraceptives (35.3%). Also, Eweani *et al*. (2001) showed that women who used contraceptive had a prevalence of 51.5% of vaginal colonization compared to 40.6% for non-contraceptive users in Edo State, Nigeria.

Among the pregnant women, the highest prevalence (25%) of vaginal *Candida* colonization was observed in the first trimester followed by those in the second trimester (11.1%) and third trimester (5.0%). Statistically, there was no significant difference in the effect of trimester on the prevalence of vaginal *Candida* colonization. This finding is in agreement with that of Ehan (2017) who reported that the highest frequency of vaginal candidiasis was among those in first trimester of pregnancy (37%) followed by those in second trimester (34%) and the lowest among those in 3rd trimester (29%). Oviasogie and Okungbowa (2009) reported that the least prevalence of *Candida* species was also recorded in the third trimester, although the prevalence (30.6%) was much higher than the 5.0% recorded in this study. Masri *et al*. (2015) showed that first and second trimester pregnant women were at a higher risk of getting vaginal candidiasis when compared to those in the third trimester. However, the findings of Oviasogie and Okungbowa (2009) contradicts that of the present study in that they recorded the highest occurrence (68.8%) of *Candida* species among women in their second trimester, followed by those in their first trimester (33.3%). Amutaigwe *et al*. (2007), in a similar study observed the highest prevalence (43.2%) of vaginal *Candida* colonization amongst women in their second trimester of pregnancy followed by those in their third trimester (35.3%) while the women in their first trimester of pregnancy had zero (0%) prevalence. Uzoh *et al*. (2016), working on prevalence of *Candida albicans* among women attending Federal Medical Centre Asaba, South-South, Nigeria, recorded the highest prevalence of 47% among women in their third year of pregnancy. During pregnancy, there is an increase in the level of estrogens. As the pregnancy ages, the level of this hormone increases providing an increased amount of glycogen in the vagina. Glycogen provides a ready source of utilizable sugar that favours the growth of yeasts like *Candida* species (Akingbade *et al*., 2013) and thus a higher prevalence in the third and second trimester. However, the least prevalence and highest prevalence respectively observed in the third and first trimester can be explained in relation to the number sampled. Women in their first and third trimester were least and most in number respectively.

The single women had the highest prevalence (26.0%) of vaginal *Candida* colonization when compared to that of the married women (23.8%) and the divorcees (0.0%). There was statistically, no significant difference in the prevalence of vaginal *Candida* colonization between the married and single women ($p > 0.05$) However, of the 4 women who never

responded vis-à-vis their marital status, 2 were positive for vaginal *Candida* colonization giving a prevalence of 50%. In a retrospective study to determine the prevalence and distribution of *Candida* vaginitis in women of reproductive age in Onitsha metropolis and its environs, Ugochukwu *et al*. (2013) also recorded that single women had a higher prevalence (57.2%) than the married women (42,8%). The result of Enweani *et al*. (2001) contradicts that of the present study in that they reported a higher prevalence (50.9%) of vaginal candidiasis among married women than the single women (48.4%).

Of the 254 symptomatic women who took part in the present study, 59 were positive for vaginal *Candida* colonization giving a prevalence of 23.3% while out of the 82 asymptomatic women, 24 were positive for vaginal *Candida* colonization giving a prevalence of 29.2%. Thus, the prevalence was higher in asymptomatic women than in the symptomatic women. However, the difference was not significant statistically. These results disagree with those of similar researches in which there was a higher prevalence of vaginal *Candida* colonization in the symptomatic women than their asymptomatic counterparts (Amutaigwe *et al*., 2017; Yadav and Prokash, 2016). Also, Akingbade *et al*. (2013) recorded a higher prevalence of *Candida albicans* in symptomatic women (26.7%) compared to the asymptomatic women (21.7%) though; the difference was statistically not significant. The higher prevalence among the asymptomatic women in the present study suggests that vaginal candidiasis should not be ruled out if there are no symptoms. This is because infections by *Candida* species that do not present with symptoms could likely lead to other severe complications (Akingbade *et al*., 2013). Thus, it is of utmost importance that diagnosis be carried out especially in pregnant women notwithstanding the absence of symptoms in order to avoid complications. Though there was a higher prevalence in symptomatic women than their asymptomatic counterparts in the research carried out by some other researchers (Akingbade *et al*., 2013; Yadav and Prokash, 2016; Amutaigwe *et al*., 2017), it is worthy to note that vaginal candidiasis should not be diagnosed based on clinical features only. This is because an appreciable number of women may present with symptoms as a result of some other conditions like allergies (Akah *et al*., 2010).

Among the symptomatic women, those who presented with both vaginal discharge and dispareuria had the highest prevalence (42.7%) of vaginal *Candida* colonization followed by those with vaginal discharge only (36.7%) and dysuria only (20.0%). Out of the 254 symptomatic women, 109 (42%) presented with vaginal discharge only, 57 (22.44%) presented with vaginal discharge and itching followed by 36 (14.17%) who presented with itching only. This agrees with Akingbade *et al*. (2013) who reported vaginal discharge is one

of most frequent gynecological problems encountered in females especially during their reproductive stage. Ibrahim *et al*. (2016), in their work in a Gynecological Clinic at South Libya, also reported that vaginal discharge was the most common symptom seen in pregnant women followed by itching.

The performance of MALDI-TOF MS for the identification of the isolates is presented in Table 4.9. A total of 70 (79.55%) of the isolates had correct species identification with log score value >2.0 while 18 (20.45%) had correct genus level identification with log score value of 1.7 – 2.0. There was no isolate (0.00%) with a log score value of <1.7 which indicates an unreliable identification. Though one of the isolates had a score of 1.545 (<1.7), the higher score of 1.747 (>1.7) was used for the matching (Marklein *et al*., 2009). The identification was considered précised if at least one spot from the duplicates gave a score of > 1.7 (Alizadeh *et al*., 2017). In the overall, 100% of the isolates were recognized by MALDI-TOF MS. This agrees with the results of Yaman *et al*. (2012) and Pulcrano *et al*. (2013) who reported the identification of 100% of *Candida* isolates. Alizadeh *et al*. (2017) and Marklein *et al*. (2009) respectively reported the identification of 94% and 92.5% of the isolates using MALDI-TOF MS. Bader *et al*. (2011) reported that both MALDI-TOF MS systems (Biotyper 2.0 and Saramis) demonstrated an overall species identification rate of 99.0% and 99.5%, respectively of 1192 clinical yeasts and yeast-like isolates. The identification of isolates with 99.4% accuracy by MALDI-TOF MS has also been reported (Cameron *et al*., 2017). Thus, MALDI-TOF MS is a highly reliable tool for identification.

In the present study, there were varied degrees of susceptibility by the different *Candida* species to the different antifungal agents. It was observed that 27 (30.7%) of the *Candida* isolates were susceptible to Fluconazole, 15 (17%) were susceptible dose-dependent and 46 (52.3%) were resistant. *Candida glabrata* was the most susceptible (50%) followed by *Candida parapsilosis* (42.9%). Meanwhile, the highest resistance (85.7%) was shown by *Candida krusei*. In fact, none of the *Candida krusei* isolate was susceptible to Fluconazole. This resistance (52.3%) is very much higher than the 2.6% reported by Won *et al*. (2015) in Korea. Also, the 25%, 29.4%, 42.9% and 50% susceptibilities respectively observed for *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis* and *Candida glabrata* does not agree with the > 90% respectively observed for *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis* and 84.3% for *Candida glabrata* by Fairuz *et al*. (2011) in Kuala Lumpur, Malaysia. The 17% susceptible dose-dependent isolates recorded in this study is not too far from the 13.8% susceptible dose-dependent isolates recorded by Won *et al*. (2015). Also, the 0% susceptibility of *Candida krusei* to Fluconazole observed in thi study agrees

with the 0% susceptibility also reported by Won *et al*. (2015). Similarly, Adesiji *et al*. (2011) reported 100% resistance of *Candida krusei* to Fluconazole. Isolates of *Candida krusei* are considered resistant to Fluconazole irrespective of the MIC (Won *et al*., 2015). This calls for identification of the particular etiologic agent (*Candida* species) and sensitivity testing to avoid ineffective and inappropriate therapy.

For ketoconazole, 12 (13.6%) out of the 88 *Candida* isolates were susceptible, 22 (25%) were susceptible dose-dependent and 54 (61.4%) were resistant. *Candida krusei* was the most resistant (71.4%) followed by *Candida albicans* (70%) while *Candida parapsilosis* was the most susceptible dose-dependent species. *Candida albicans* had a 15% susceptibility to ketoconazole which is very much lower than the 73 susceptibility reported by Gandhi *et al*. (2015). Gandhi *et al*. (2015) also reported a 33.55 resistance of *Candida krusei* against ketoconazole which is lower than the 71.4% resistance observed in the present study. However, Jayalakshmi *et al.* (2014) reported a high resistance (83.3%) for *Candida krusei* against ketoconazole which is similar to the high resistance (71.4%) observed in the present study.

From the *in vitro* susceptibility profile of the *Candida* species to Clotrimazole, it was observed that 37(42.0%) of the *Candida* isolates were susceptible, 21 (23.9%) were susceptible dose-dependent while 30 (34.1%) were resistant. The highest resistance (71.4%) was observed with *Candida krusei* while *Candida parapsilosis* was the most susceptible (57.1%). *Candida tropicalis* was the most susceptible dose-dependent species (32.4%) and also the least resistant species (26.5%). *Candida albicans* had 30% susceptibility and 40% resistance to Clotrimazole. Okolo *et al*. (2017) reported 36.2% sensitivity and 63.8% resistance of *Candida albicans* against Clotrimazole. Tsega and Mekonnem (2019) reported a much higher susceptibility (77.2%) of *Candida albicans* in their study in Northwest Ethiopia. The 66.7% resistance of *Candida tropicalis* against Clotrimazole reported by Okolo *et al*. (2017) in Jos, North Central Nigeria is much higher than the 26.5% resistance recorded in the present study. However, Okolo *et al*. (2017) reported 75.4% resistance against Clotrimazole by *Candida krusei* which is similar to the 71.4% observed in the present study.

For Amphotericin B, susceptible isolates were 61(69.3%) while 10 (11.4%) and 17 (19.3%) were susceptible dose-dependent and resistant respectively. The species that was most resistant to Amphotericin B was *Candida krusei* (42.9%) followed by *Candida albicans* (25.0%). Deepa *et al*. (2013) reported a much lower resistance (15.75%) of *Candida krusei* against Amphotericin B. *Candida tropicalis* was the most susceptible (79.4%) followed by *Candida parapsilosis* (76.2%). Dagi *et al*. (2016) reported 100% susceptibility of *Candida*

albicans, *Candida tropicalis*, *Candida parapsilosis* and *Candida glabrata* to Amphotericin B. There were no susceptible dose-dependent isolates of *Candida parapsilosis* while *Candida glabrata* showed no resistance to Amphotericin B. This agrees with the findings of Fiaruz *et al*. (2011) who reported there were no susceptible dose-dependent isolates of *Candida parapsilosis* and also 0% resistance of *Candida glabrata* against Amphotericin B. Other researchers have also documented 0% resistance of *Candida glabrata* against Amphotericin B (Jayalakshmi *et al*., 2014; Gandhi *et al*., 2015).

The *in vitro* susceptibility profile of the *Candida* isolates to Flucytosine shows that seventy-six (86.4%) of all the isolates were resistant, 6 (6.8%) were intermediate and also 6 (6.8%) were susceptible. The interpretive categories for Flucytosine are the same categories used to interpret bacterial testing. These categories include susceptible (S), intermediate (I), and resistant (R), with Intermediate (I) being substituted for the susceptible dose- dependent category. All the isolates (100%) of *Candida albicans*, *Candida krusei* and *Candida glabrata* were resistant to Flucytosine. Hope *et al*. (2004) reported 80% resistance of *Candida albicans* in the United Kingdom. The method used to determine the susceptibility of the isolates can influence the results. It has been suggested that the disk method is a sensitive but not necessarily specific method to determine Flucytosine susceptibility of *Candida albicans* (Hope *et al*., 2004). A very low susceptibility of 4% and 2% was observed with *Candida tropicalis* and *Candida parapsilosis* respectively. Only isolates of *Candida tropicalis* (17.6%) were susceptible dose-dependent.

For Voriconazole, 46 (52.3%) of the *Candida* isolates were susceptible, 11 (12.5%) were susceptible dose-dependent and 31 (35.2%) were resistant. *Candida parapsilosis* isolates were the most susceptible (66.7%) followed by *Candida krusei* (57.1%) and *Candida tropicalis* (52.9%). There were no susceptible dose-dependent isolates of *Candida krusei*. A 100% susceptibility of *Candida tropicalis* and *Candida parapsilosis* to Voriconazole has been observed by some other researchers (Fairuz *et al*., 2011; Won *et al*., 2015) which conflicts with the present study. Also, in a similar research carried out by Panizo *et al*. (2009) in Venezuela, none of the *Candida* species was found to be resistant to Voriconazole. In another study by Dagi *et al*. (2016) in Turkey, all 200 (100%) isolates of *Candida* species were susceptible to Voriconazole. Jasem *et al*. (2014) recorded 89.9% susceptibility of all the *Candida* species isolated from samples from oral candidiasis and diaper dermatitis lesions collected from children referring to private and public clinics in Ilam, Iran. This 89.9% susceptibility is much higher than the 52.3% observed in the present study. These variations in susceptibility profile may be explained by the differences in the hospital, the underlying

disease of the patient, clinical specimen analyzed as well as the geographical location where the studies were carried out (Panizo *et al*., 2009).

In the present study, 27 (30.7%) of the *Candida* isolates were susceptible to Itraconazole while 21 (23.9%) and 40 (45.5%) were susceptible dose-dependent and resistant respectively. Panizo *et al*., (2009) reported 27.6% resistance of *Candida* isolates in Venezuela while Jasem *et al*. (2014) recorded 38.3% susceptibility in Iran. The present study records *Candida parapsilosis* as the most susceptible (42.9%) to Itraconazole followed by *Candida tropicalis* (35.3%) while *Candida glabrata* was the most resistant (83.3%) followed by *Candida albicans* (65.0%). In one study, 85.7% of *Candida parapsilosis* and more than 90% of *Candida tropicalis* isolates were susceptible to Itraconazole (Won *et al*., 2015). In their research, Jasem *et al*. (2014) found out that the resistance of *Candida albicans* to Itraconazole was 43.8% which is lower than that (65.0%) observed in the present study. There were no susceptible isolates of *Candida glabrata*. This does not agree with the 83.4% susceptibility reported by Won *et al*. (2015).

The *in vitro* susceptibility profile of the *Candida* isolates to Nystatin shows that 33 (37.5%), 16 (18.2%) and 39 (44.4%) of the isolates were susceptible, susceptible dose-dependent and resistant respectively. This disagrees with the findings of Emam *et al*. (2012) and Gandhi *et al*. (2015) that showed a 100% susceptibility of all *Candida* isolates to Nystatin. Okolo *et al*. (2017) reported that out of 139 *Candida* isolates, 26 (18.7%) and 113 (81.3%) were sensitive and resistant to Nystatin respectively which does not agree with the results of the present study. Jasem *et al*. (2014) observed 95.3% susceptibility of all different *Candida* species in Ilam, Iran. *Candida glabrata* was the most susceptible (66.4%) followed by *Candida parapsilosis* (52.4%) while *Candida albicans* was the most resistant (55.0%) followed by *Candida tropicalis* (50.0%) and *Candida krusei* (42.9%) (Table 4.17). This resistance is much lower than the 70.7%, 100% and 82.0% respectively reported for *Candida albicans, Candida tropicalis* and *Candida krusei* by Okolo *et al*. (2017).

The highest susceptibility was recorded for Amphotericin B to which 61 (69.3%) of all the *Candida* isolates were susceptible followed by Voriconazole (52.3%) and Clotrimazole (43.0%). Jasem *et al*., (2014) also recorded the highest susceptibility (99.3%) by all different *Candid*a isolates to Amphotericin B. Fahriye *et al*. (2013) detected 100% susceptibility to Amphotericin B by all strains of *Candida* isolated in Turkey. Almeida *et al*., (2013) reported that all of 50 *Candida* species (except 1 strain of *Candida tropicalis*) isolated in Central-Western Brazil were susceptible to Amphotericin B. Other reports of very high susceptibility of *Candida* species to Amphotericin B have been documented (Fairuz *et al*., 2011; Silva *et*

al., 2013; Dagi *et al*., 2016). Amphotericin has a broad spectrum of action and presents a low incidence of fungal resistance even after a half century of clinical use. One major disadvantage of Amphotericin B is its nephrotoxicity (Nicolaou *et al*., 2009; Volmer *et al*., 2010).

The highest resistance was observed with Flucytosine to which 76 (86.4%) out of the 88 *Candida* isolates were resistant followed by Ketoconazole (61.4%) and Fluconazole (52.3%). Fluconazole has been one of the most widely used drugs for treating candidiasis (Nasira *et al*., 2013). In fact, Fluconazole is the most commonly prescribed antifungal used for most *Candida albicans* infections. (Pfaller *et al*., 2010). Thus, wide spread and prolonged use of azoles promotes rapid development of multidrug resistance, which poses a major problem in antifungal therapy (Nasira *et al*., 2013). In the present study, Voriconazole is the second antifungal drug to which most of the *Candida* species were susceptible. Thus, it can be used to treat infections caused by Fluconazole-resistant *Candida* species. Meanwhile, the highest number of susceptible-dose dependent *Candida* isolates was observed with Ketoconazole (25%), followed by Clotrimazole and Itraconazole, each recording 23.9% and then, Nystatin (18.2%). The *Candida* isolates categorized as being susceptible-dose dependent (SDD) is in recognition that yeast susceptibility is dependent on achieving maximum blood levels. Thus, an isolate with an SDD category implies clinical efficacy when higher than normal dosage of a drug can be used and maximal possible blood levels achieved (CLSI, 2008).

Candida tropicalis was the highest susceptible species (79.4%) to Amphotericin B followed by *Candida parapsilosis* (76.2%) and 66.7% respectively to Amphotericin B and Voriconazole. *Candida albicans*, *Candida krusei* and *Candida glabrata* showed 100% resistance to Flucytosine. *Candida krusei* was the species with the least susceptibility showing 0% susceptibility to each of Fluconazole, Ketoconazole and Flucytosine. It is critically noted that Fluconazole is not recommended for *Candida krusei* and it has also been stated that *Candida krusei* should not be tested against Fluconazole to which it is intrinsically resistant (Fothergill *et al*., 2006). In a review by Whaley *et al*. (2017), it was documented that out of 1075 *Candida krusei* isolates tested against Fluconazole, 96.6% were resistant to the drug. Also, *Candida glabrata* showed a 0% susceptibility to each of Flucytosine and Itraconazole while *Candida albicans* showed 0% susceptibility to Flucytosine only. *Candida glabrata* has been documented of being able to develop high-level resistance after exposure to azole antifungals (Lee *et al*., 2009) *Candida albicans*, *Candida parapsilosis*, *Candida krusei* and *Candida glabrata* each showed 0% susceptible-dose dependence to Flucytosine. There was 50% susceptibility of *Candida glabrata* to each of Fluconazole, Clotrimazole,

Amphotericin B and Voriconazole. Also *Candida glabrata* was the only *Candida* species with 0% resistance to Amphotericin B. Most of the *Candida* isolates were susceptible to Amphotericin B, namely *Candida tropicalis* (79.4%), *Candida parapsilosis* (76.2%), *Candida albicans* (60%), *Candida glabrata* (50%) and *Candida krusei* (42.9%).

The prevalence of Vaginal *Candida* Colonization among the women who were not on any antimicrobial therapy as at the time of this study was higher (27.93%) than amongst those on antimicrobial therapy (4.35%). Statistically, the difference was significant ($p = 0.00$). This contradicts the results of similar researches. Jinping *et al*. (2008) found that a short course of oral antibiotics was associated with both increased prevalence of positive vaginal *Candida* colonization and increased incidence of symptomatic VVC during 4 to 6 weeks of using antibiotic therapy. Also, in a study by Emeribe *et al*. (2015), all subjects with *Candida*-positive culture had been on antibacterial therapy prior to participating in the study. In India, a study by Ahmad and Khan (2009), showed an increased incidence of VVC in women that have taken antibiotics than in those who have not taken antibiotics. Prolonged antibacterial use usually affects vaginal bacteria microflora population especially *Lactobacillus* species (mainly *L. acidophilus*) which represent the dominant vaginal defense mechanism against *Candida*. This (together with other factors) encourages the overgrowth of *Candida*. The discrepancy in the results of the present study and others may have been influenced not only by the location where the study was made, but also by the type of antibiotic used and the duration of the treatment (Gonçalves *et al*., 2015)

Extract of *Moringa oleifera* had the highest inhibition zone diameter (19mm) at 200mg/ml against resistant *Candida albicans*[1], followed by extract of *Vernonia amygdalina* and *Ocimum gratissimum* with inhibition zone diameters of 18.67mm and 18mm at 100mg/ml and 200mg/ml respectively. *Candida albicans*[1] was totally resistant to *Ocimum gratissimum* at 25mg/ml and 12.25mg/ml. There was also total resistance by *Candida albicans*[2] to extracts of *Vernonia amygdalina* (at 200mg/ml and 100mg/ml) and *Ocimum gratissimum* (at 25mg/ml and 12.25mg/ml). The highest IZD (18.33mm) against *Candida albicans*[2] was exhibited by extracts of *Vernonia amygdalina* at 50mg/ml followed by *Ocimum gratissimum* (18mm) at 200mg/ml. The extract of *Moringa oleifera* showed the least IZD (11.67mm) at 12.25mg/ml. Similar researches support the antifungal activity of extract of *Moringa oleifera* against *Candida albicans*. In a study carried out by Aisha *et al*, (2016) in Dutse, Jigawa State, the ethanol extract of *M. oleifera* was found to possess antifungal activity against *Candida albicans* with the inhibition zone diameter being up to 22mm at a concentration of 5000µg/ml. Bassey *et al*. (2016), showed the ethanolic leaf extracts of

Moringa oleifera exhibited antifungal activity against *Candida albicans* giving inhibition zone diameter of 11mm at 100mg/ml concentration. However, the findings of the present study does not agree with that of Pinal *et al*. (2014) who reported that *Candida albicans* (MTCC No. 183) was resistant to both aqueous and ethanolic extracts of *Moringa oleifera*. Also, Bassey *et al*. (2016) reported that *Candida albicans* was resistant to ethanolic extracts of *Moringa oleifera* at concentrations of 50, 25 and 12.5mg/ml unlike in this study in which ethanolic extracts of *Moringa oleifera* at concentrations of 50, 25 and 12,5mg/ml showed activity against both *Candida albicans*[1] and *Candida albicans*[2].

This study agrees with some other studies that ethanolic extracts of *V. amygdalina* has some antifungal properties against *Candida albicans* though with lower IZDs. For example, Erute and Egboduka (2013) showed that ethanolic extracts of *V. amygdalina* had antifungal activity against *Candida albicans* with inhibition zone diameters (IZD) of 10.67±1.15mm at 100mg/ml. In another study by Ghamba *et al*. (2014) in Maiduguri, Borno State, ethanolic leaf extracts of *V. amygdalina* displayed zones of inhibition of 12.4mm against *Candida albicans* isolated from urine specimens. Contrary to the result of this study, the findings of Oshim *et al*. (2016) showed that clinical wound isolates of *Candida albicans* from patients in the surgical wards at Nnamdi Azikiwe Teaching Hospital (NAUTH), Nnewi were resistant to both the ethanol and methanol extracts of *V. amygdalina* at all concentrations (6.25mg/ml to 100mg/ml). However, there was also total resistance by *Candida albicans*[2] to extracts of *Vernonia amygdalina* at concentrations of 200mg/ml and 100mg/ml.

The results of the study of Nweze and Eze (2009) are in conformity with that of this study. Their results showed that ethanolic extract of the leaves of *Ocimum gratissimum* was active against both the clinical isolate of *Candida albicans* and the control strain (*Candida albicans* ATCC 90028) giving inhibition zone diameters of 16mm and 13mm at 100mg/ml and 13mm and 10mm at 50mg/ml respectively. Both the clinical isolate and control strains were resistant to concentrations of 25, 12.5 and 6.25mg/ml of the extracts as also recorded in this study.

Candida tropicalis[1] was sensitive to all the plant extracts at all concentrations. The IZDs produced by extracts of *Moringa oleifera* and *Ocimum gratissimum* against *Candida tropicalis*[1] were concentration dependent, ranging from the least IZD of 11.33mm (at 12.25 mg/ml) for *Ocimum gratissimum* to the highest IZD of 17.00mm (at 200 mg/ml) for extracts of *Moringa oleifera* and *Vernonia amygdalina.* For *Candida tropicalis*[2], the IZD ranged from 11.67mm for *Moringa oleifera* at 12,25mg/ml to 17.67mm for *Vernonia amygdalina* at 200mg/ml. *Candida tropicalis*[2] was totally resistant to extract of *Moringa oleifera* at

200mg/ml. This agrees with the work of Rocha *et al*. (2014) who demonstrated the antifungal activity of chloroform and ethanolic extracts (especially from leaves and flowers) of *Moringa oleifera* against some strains of *Candida* species including *Candida tropicalis*. In India, Pinal *et al*. (2014) reported that both aqueous and ethanolic extracts of *Moringa oleifera* had little activity against *Candida tropicalis* (MTCC No.1000). Nakamura *et al*. (2004) showed that the *Ocimum gratissimum* essential oil had fungicidal activity against some *Candida* species including *Candida tropicalis* though *Candida tropicalis* was the least susceptible.

The IZD of the plant extracts against resistant *Candida parapsilosis* showed that the microorganism was resistant to the extract of *Ocimum gratissimum* at all concentrations while the extracts of both *Moringa oleifera* and *Vernonia amygdalina* had activity at all concentrations with various IZDs. This agrees with the study of Rocha *et al*. (2014) who demonstrated the antifungal activity of chloroform and ethanolic extracts of *Moringa oleifera* against some strains of *Candida* species (*Candida ciferrii*, *Candida famata*, *Candida guilliermondii*, *Candida parapsilosis* and *Candida tropicalis*). Nakamura *et al*. (2004) showed that *Candida parapsilosis* was most susceptible to *Ocimum gratissimum* essential oil. This study observed the growth of some few colomies of *Candida parapsilosis* within the zone of inhibition at 25mg/ml for *Moringa oleifera*. These are considered resistant mutants (NCCLS, 2008).

Candida krusei was totally resistant to all the extracts of the plants at 200mg/ml concentration and also resistant to *Ocimum gratissimum* at 12.25mg/ml. It is expected that the higher the concentration, the higher the IZD. However, at the highest concentration of 200mg/ml, *Candida krusei* was totally resistant to all the plant extracts. A possible explanation may be the fact that at that high concentration the diffusion of the extracts through the medium was very slow and the growth of the microorganism was faster than the extract could diffuse (Trease and Evans, 1978). The highest IZD (18mm) was shown by extract of *Vernonia amygdalina* at 100mg/ml followed by *Ocimum gratissimum* (16mm) at the same concentration. Extract of *Moringa oleifera* at 12.25mg/ml showed the least IZD (11.67mm) against *Candida krusei*. Nakamura *et al*. (2004) showed that the *Ocimum gratissimum* essential oil had fungicidal activity against *Candida krusei, Candida parapsilosis*, *Candida tropicalis* and *Candida albicans*. Also, the ethanolic and methanolic extracts of the leaves of *Ocimum gratissimum* showed antifungal effect against *Candida krusei* producing inhibition zone diameters of 13.00±2.00mm and 14.67±1.53mm respectively (Erute and Egboduku, 2013). However, the result of this study doesn't agree with that of Erute and Egboduka (2013) who reported that both the ethanolic and methanolic

extracts of *V. amygdalina* had no activity against *Candida krusei*

The differences observed in the activities of the plant extracts in this study, when compared to the findings of other studies may be as a result of different strains of the *Candida* species used in the different studies. Also, the antimicrobial activity of the plant extracts can be influenced by the method of their preparation as well as the choice of solvents used (Anibjuiwon *et al*., 2010; Foo *et al*., 2014). The the time of harvest of the plant can determine the amount of active constituents (phytochemical substances) and hence, the potency of the plants (Geyid *et al*., 2005). In cases where there was no activity by any of the plant extracts, it may be due to the absence of some secondary metabolites or the presence of some in low concentration; or it may be due to the type of strains used or a slight change in any of the factors that are likely to affect rate of microbial growth or rate of diffusion of the test agent (Nweze and Eze, 2009). The antifungal activities shown by these plant extracts is not unrelated to the presence of secondary metabolites (such as saponins, flavonoids, tannins, carbohydrates, glycosides, reducing sugar and other active ingredients of plants) which have been shown to be responsible for the antimicrobial activities shown by these extracts (Nweze *et al*., 2004). Also, the activities of these plant extracts against these *Candida* species implies these plants have great therapeutic potentials that can be harnessed for the formulation of drugs especially against those diseases caused by microorganisms that have developed resistance to the commonly used antifungal drugs like Fluconazole.

Most of the combination ratios produced indifference and antagonism against *Candida albicans*[1] and *Candida krusei*. However, there was synergy at the ratio of 2:8 against *Candida albicans*[1] and the combination ratios of 9:1 against *Candida krusei*. Synergy was produced in most of the combination ratios against *Candida albicans*[2], *Candida tropicalis*[2] and *Candida parapsilosis*. Where there is synergy, it suggests that therapeutically, it could be more beneficial to use the combined extract and Fluconazole in the ratios indicated against infections caused by these *Candida* species. While there was no activity at the ratio of 9:1 against *Candida tropicalis*[2], synergy was produced in all the combination ratios against *Candida tropicalis*[1].

Synergism was produced by most of the combination ratios against *Candida albicans*[1] and *Candida albicans*[2] while indifference was produced by the combination ratios of 3:7 and 1:9 against *Candida albicans*[1] and 5:5 and 3:7 against *Candida albicans*[2]. Also, synergism was produced against *Candida tropicalis*[1] at combination ratios 9:1 to 6:4 while there was no activity at combination ratios of 5:5 to 1:9. The combination ratios of 9:1 to 5:5 produced no activity against *Candida tropicalis*[2] while 4:6 to 1:9 produced antagonism. There was no

antifungal activity in all the combination ratios against *Candida krusei* meanwhile synergy was observed in the combination ratios of 9:1, 8:2 and 6:4 against *Candida parapsilosis*.

There was no activity for all the combination ratios of Fluconazole and extract of *Ocimum gratissimum* against *Candida albicans*[1], *Candida krusei* and *Candida parapsilosis* except for the combination ratios 2:8 and 1:9 which was synergistic and produced indifference respectively against *Candida krusei*. Synergy was observed in all the combination ratios against *Candida albicans*[2] except combination ratio 3:7 and also most of the combination ratios against *Candida tropicalis*[1] produced synergy. This agrees with the findings of Nweze and Eze (2009) who reported that the effect of interaction between the ethanolic extract of *O. gratissimum* and an azole (Ketoconazole) was synergistic on *C. albicans*. The combination ratios of 9:1 and 7:3 produced synergy against *Candida tropicalis*[2], 4:6 and 3:7 showed no activity while the remaining combination ratios produced indifference.

In cases where the combination ratios produced indifference against the *Candida* species, it implies that the combined effect of these combination ratios is neither greater than the singular action of the more effective agent nor lower than the singular action of the less effective agent in the combination (Okore, 2009). This also suggests that the combination of ethanolic extract of any of the plants (*M. oleifera, V. amygdalina* or *O. gratissimum*) with Fluconazole in the indicated ratios (as shown in Tables 26a to 28a) may not possess any advantage over any of either ethanolic extract of the plant or Fluconazole used alone against the *Candida* species (Ofokansi *et al*., 2013). Antagonistic effect implies the combined action is less than the action of the more effective component in the combination. One possible explanation for this antagonistic effect may be as a result of physicochemical incompatibility between the agents in the combination or a competitive inhibition at the site of action (Okore, 2009). Where there is synergy, it suggests that therapeutically, it could be more beneficial to use the combined extract and Fluconazole in the ratios indicated against infections caused by these *Candida* species. Synergy means the fungistatic or fungicidal effect of the combination exceeds the arithmetic summation of the individual antimicrobial agents (Okore, 2009). This also implies that greater antifungal effect could be obtained when lower doses of each agent is used thus, minimizing some possible adverse effects (Ofokansi *et al*., 2013) which are often associated with higher doses of antimicrobial agents. It can also be observed in Tables 4.26a to 4.28a that different ratios of the combination of Fluconazole with any of the plant extract (*M. oleifera, V. amygdalina* or *O. gratissimum*) showed different types of synergy. This indicates that synergy of the agents in combination is not only a function of the nature of

the agents involved (in this case, Fluconazole and the plant extracts) but also a function of their ratios in combination (Okore, 2009) since not all the combination ratios were synergistic against the *Candida* species.

It can be seen that none (0%) of the combination ratios of any of the plant extracts with Fluconazole had any additive effect against the *Candida* species. Additivity or additive action means the combined action of the individual agents in the combination ratios is equal to the arithmetic summation of the effects of the single agents in the combination (Okore, 2009). Also, there was no antagonism in all the combination ratios of *O. gratissimum* with Fluconazole. Meanwhile, the highest number of synergistic combination ratios (48.15%) was observed in the combination of *M. oleifera* with Fluconazole. Only 1 (1.85%) out of the 54 combination ratios of *M. oleifera* with Fluconazole showed no activity. The present study shows that the combination of the ethanolic extract of *M. oleifera* with Fluconazole was the best as 26 (48.15%) out of the 54 combination ratios were synergistic against the *Candida* species.

Sequencing of the two fragments (E1 and E3) of the ERG11 coding region of *Candida albicans*[1] showed 71 mutations of which 60 were non synonymous (encoded distinct amino acid substitutions)(Figures 11a to 12b). Six (6) of these amino acid substitutions (I162T, R163H, E164G, V272D, F273I and K276Q) occurred within the three hot spot regions in ERG11 gene ranging from amino acid (aa) 105-165, 266-287, and 405-488. These regions have been reported to be associated with resistance of *Candida* species to azoles (Flowers *et al*., 2015; Grossman *et al*., 2015; Tan *et al*., 2015). *Candida albicans*[1] isolated in this study was resistant to Fluconazole, Ketoconazole and Itraconazole which resistance may have been as a result of the above mutations. This is because one of the mechanisms of resistance by *Candida* species (such as *Candida albicans*) to azole drugs is the presence of point mutations in the *ERG11* gene that encodes lanosterol 14α demethylase which is the primary target for the azoles (Katarzyna *et al*., 2013). The other point mutations that encoded distinct amino acid substitutions as observed in this study may not necessarily be involved in azole resistance. There have been reports of mutations in the ERG11 gene encoding more than 160 distinct amino acid substitutions (Manastır *et al*., 2011; Morio *et al*., 2010). However, of these amino acid substitutions, only 10 have been confirmed to cause Fluconazole resistance. Mutations resulting in amino acid substitutions that lead to changes in the tertiary structure of Lanosterol 14α demethylase can alter the affinity of the enzyme for an azole (Ming-Jie *et al*., 2013). It is also worthy to note that other molecular mechanisms, such as efflux pumps and over expression of genes involved in the biosynthesis of ergosterol might be involved in the

development of the resistant phenotype. (Lamping *et al*., 2009). Thus, a combination of mutations may work together resulting in a high level of resistance to azoles like Fluconazole (Sasse *et al*., 2012).

The point mutation, T1203C observed in this study has been reported by Katarzyna *et al*. (2013). However, they reported that this mutation was found in 52% and 47.5% of *Candida albicans* respectively sensitive and resistant to Fluconazole, Voriconazole and Itraconazole. The T1203C mutation observed in this study led to the amino acid Y401Y as shown in fig 12b. Thus, it is a synonymous mutation where the amino acid tyrosine (Y) at position 401 was not substituted. Danielly *et al*. (2016) reported a non synonymous mutation G1470A in *Candida krusei* isolates with dose-dependent susceptibility to Voriconazole. This study also observed a point mutation at position 1470 (T1470C) of the aa sequence of the ERG11 gene of *Candida albicans*[1]. However, this was a synonymous mutation as there was no change in the amino acids (N490N) (Fig 12b). The difference may be as a result of the different species of *Candida*.

For *Candida tropicalis*[1] (E2+ERG1A), there were 27 synonymous and also 27 non synonymous mutations. Six (6) of these mutations (Y105F, L112V, A116D, T119K, S120H and P121L) were found within the 3 hot spot regions in ERG11 gene (Table 31). *Candida tropicalis*[1] was resistant to Fluconazole and ketoconazole but was susceptible dose-dependent to Clotrimazole. Not all the amino acid alterations above may actually be linked to the resistance. Katarzyna *et al*. (2013) in their study also identified amino acid substitution at position 116, though it was D116E and not A116D as reported in the present study. However, according to Goldman *et al*. (2004), this amino acid alteration is located in 14α sterol demethylase in the region between the B' and C helices and this was suggested to be involved in inhibitor or substrate-elicited structural lesions. Therefore, this substitution may play a very important role in the development of resistance. Unlike D116E which has been frequently reported (Morio *et al*., 2010), the other 5 amino acid alterations (Y105F, L112V, T119K, S120H and P121L) observed in this study, to the best of our knowledge have not been reported.

There were other non synonymous mutations that actually resulted in distinct amino acid substitutions (such as M189V, V4371 and T24S in figures 11b, 12b and 13b respectively). However, these substitutions were conservative in that a given amino acid was replaced by a different amino acid with similar biochemical properties *(*Zhang, 2000; Dagan *et al*., 2002). These mutations may not have had any major effect on the enzyme (protein) since

conservative replacements in proteins often have a smaller effect on function than non-conservative replacement.

5.1 CONCLUSION

In this study, a prevalence of vaginal *Candida* colonization of 24.7% was observed. Women of ages 50 years and above recorded the highest prevalence of vaginal *Candida* colonization, followed by women in the age groups 18 – 25 years. The prevalence of vaginal *Candida* colonization was higher amongst the non-pregnant women (28.6%) than the pregnant women (16.0%) though the difference was statistically not significant ($p > 0.05$). Also, among the pregnant women, the highest prevalence (25%) of vaginal *Candida* colonization was observed in the first trimester. The prevalence was higher in asymptomatic women than in the symptomatic women though statistically, the difference was not significant.

Candida tropicalis was the most predominant species isolated from the HVS specimens followed by *Candida parapsilosis*. Also, *Candida tropicalis* was the species with the highest susceptibility (79.4%) to Amphotericin B followed by *Candida parapsilosis*. Meanwhile, *Candida krusei* was the species with the least susceptibility showing 0% susceptibility to each of Fluconazole, Ketoconazole and Flucytosine. The drug with the highest susceptibility was Amphotericin B followed by Voriconazole while Flucytosine was the drug with the highest resistance followed by Ketoconazole and Fluconazole.

Extract of *Moringa oleifera* had the highest inhibition zone diameter (19mm) at 200mg/ml against *Candida albicans*[1]. *Candida tropicalis*[1] was sensitive to all the plant extracts at all concentrations while *Candida parapsilosis* was resistant to the extract of *Ocimum gratissimum* at all concentrations. The present study revealed that none (0%) of the combination ratios of any of the plant extracts with Fluconazole had any additive effect against the *Candida* species while the highest number of synergistic combination ratios (48.15%) was observed in the combination of *M. oleifera* with Fluconazole.

5.2 RECOMMENDATIONS

The high prevalence of Non-albicans *Candida* (NAC) from vaginal specimens as observed in this study, suggests that proper diagnosis and identification of causal agents be carried out to avoid misuse of drugs. Also indiscriminate use of antifungal drugs should be avoided. In the present study, there was a higher prevalence of vaginal *Candida* colonization among the asymptomatic women than the symptomatic women which suggests that vaginal candidiasis should not be ruled out if there are no symptoms. It is thus, recommended that diagnosis be carried out especially in pregnant women notwithstanding the absence of

symptoms in order to avoid complications. This also implies that vaginal candidiasis should not be diagnosed based on clinical features only since an appreciable number of women may present with symptoms as a result of some other conditions like allergies.

Based on the findings of this study, Voriconazole is recommended for vaginal candidiasis especially for infections caused by Fluconazole-resistant *Candida* species. The ethanolic extracts of the three plants used in this study showed varying degrees of antifungal activities, they should be investigated further for possible use in the formulation of antifungal drugs. This study also recommends further purification of the plant extracts in order to isolate and characterize the active principles that can be used for combination studies with other azoles against resistant *Candida* species.

The non synonymous point mutations, especially the ones that occurred within the 3 hot spot regions as observed in this study, together with the knowledge of further specific point mutations associated with azole resistance could be used to identify resistant strains. This study also suggests further research into the functional consequence of the non synonymous mutations observed in this study as these mutations may serve as markers of resistance to azoles. Such knowledge could be used in the prescription and design of new antifungal drugs which are less proned to resistance.

5.3 CONTRIBUTIONS TO KNOWLEDGE:

Combination of the ethanol extract of *Moringa oleifera* with Fluconazole showed synergistic effect against the *Candida* species resistant to Fluconazole and other azoles.

Sequencing of the ERG11 coding region of *Candida tropicalis*[1] showed 6 non synonymous mutations encoding distinct amino acid substitutions (Y105F, L112V, A116D, T119K, S120H and P121L) which occurred within the three hot spot regions in ERG11 gene. Unlike D116E which has been frequently reported, the other 5 amino acid alterations observed in this study, to the best of our knowledge have not been reported.

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APPENDIX A

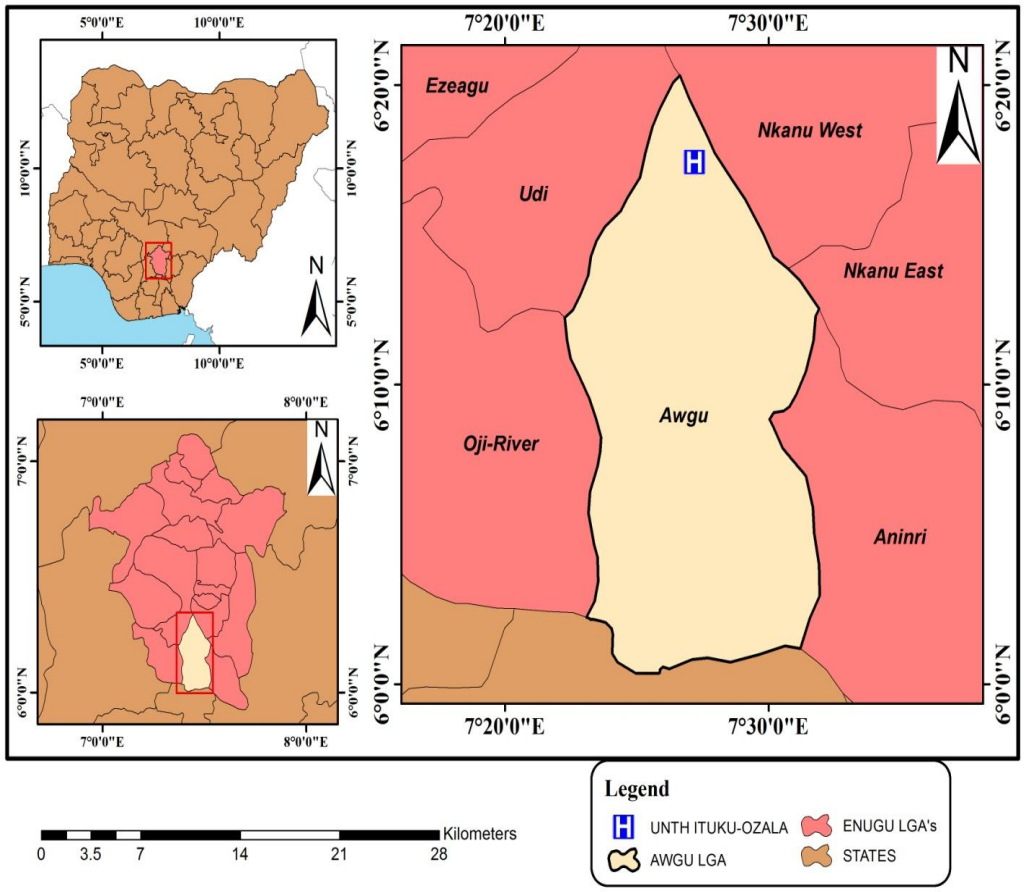

Fig 14: Map Showing Geographical Location of the Study Area

Source: Author's Geographic Information System (GIS) Analysis Using Arcmap 10.4

Determination of Sample Size

To figure out what sample size is needed, the formula $n = Z^2P (1-P) / d^2$ (Daniel, 1999) was used;

$n = Z^2P (1-P) / d^2$

Where:
n = sample size
Z = Z statistic for a level of confidence,
P = expected prevalence or proportion
d = precision

Using the level of confidence of 95%, Z value is 1.96 (Naing *et al*., 2006). The estimate of prevalence (P) is got from previous studies (Akortha *et al*., 2009; Okonkwo and Umeanaeto, 2010; Abruquah, 2012; Faraji *et al*., 2012; Adebiyi *et al*., 2015). With an estimated prevalence of 28%, it is appropriate to have a precision (*d*) of 5% since the estimated prevalence is between 10% and 90% (Naing *et al*., 2006). Thus, if

Z = 1.96, P = 28% = 0.28 and *d* = 5% = 0.05, then

$n = (1.96)^2\ 0.28(1- 0.28) / (0.05)^2$

n = 0.774 / 0.0025

n = 309.79

In order to accomadate for problems with non-response or missing values, 10% of the computed number required is added (Naing *et al*., 2006). Thus, the final sample size

= 309.79 + (10% of 309.79)

n = 340.769 ~ 341.

The sample size used for this study was 340.

Inhibition Zone Diameter (mm) of the Antifungal Agents against the Different Strains of *Candida albicans*

Strains of *Candida albicans*	Inhibition Zone Diameter (mm)							
	FLUCZ	KETOC	CTRIM	AMPH	FLU 1	VOR 1	ITRAC	NYSTA
1	0	0	18	19	0	15	0	0
2	0	15	18	0	0	0	0	0
3	27	26	18	14	0	19	0	0
4	15	0	0	18	0	15	0	20
5	0	0	16	21	0	0	0	0
6	28	27	28	18	0	18	23	0
7	25	28	28	13	0	38	31	9
8	0	0	0	20	0	18	20	23
9	16	20	0	20	0	0	0	16
10	0	0	16	17	0	0	0	15
11	0	0	20	16	0	0	20	13
12	8	0	21	9	0	20	0	17
13	24	0	0	19	0	19	25	0
14	28	0	0	18	0	0	19	10
15	0	29	11	22	0	0	0	0
16	18	17	7	8	0	24	12	0
17	0	0	22	13	0	15	0	17
18	0	22	10	17	0	0	0	13
19	0	30	14	0	0	16	11	0
20	0	10	23	0	0	15	26	0

KEY:
FLUCZ = Fluconazole (25µg), KETOC = Ketoconazole (10µg), CTRIM = Clotrimazole (10µg), AMPH = Amphotericin B (20µg), FLU 1 = Flucytosine (1µg), VOR 1 = Voriconazole (1ug), ITRA C= Itraconazole (50µg), NYSTA = Nystatin (100units).

Inhibition Zone Diameter (mm) of the Antifungal Agents against the Different *Strains of Candida tropicalis*

Strains of *Candida tropicalis*	Inhibition Zone Diameter (mm)							
	FLUCZ	KETOC	CTRIM	AMPH	FLU 1	VOR 1	ITRAC	NYSTA
1	28	25	30	20	10	30	27	8
2	0	0	16	16	0	30	26	9
3	18	25	27	15	0	33	26	12
4	16	19	20	20	18	21	18	0
5	0	16	15	0	0	0	0	0
6	20	20	19	18	22	24	19	0
7	0	0	14	16	25	32	29	0
8	23	25	19	20	0	0	23	0
9	18	21	20	15	18	20	20	18
10	0	20	15	19	0	20	23	16
11	0	16	26	19	0	24	0	0
12	18	0	15	20	0	0	14	0
13	0	0	0	20	18	0	22	15
14	26	28	0	9	20	28	0	10
15	0	14	22	18	16	0	26	19
16	17	0	15	21	0	25	24	0
17	20	30	0	17	0	22	0	0
18	13	10	21	20	0	0	20	8
19	0	29	10	19	0	21	20	13
20	0	18	0	15	0	18	19	16
21	0	13	26	0	0	15	0	10
22	21	0	21	18	0	0	25	0
23	24	0	15	22	21	23	28	0
24	20	25	22	16	12	32	14	0
25	10	31	11	24	0	0	0	13
26	0	0	20	16	0	26	0	0
27	0	13	13	12	0	0	17	19
28	21	23	8	21	0	0	0	16
29	16	28	0	13	0	0	26	11
30	9	0	22	26	11	0	0	11
31	16	0	20	16	0	0	20	14
32	0	23	0	0	0	19	12	16
33	16	18	21	12	0	16	24	0
34	20	27	13	26	14	12	0	17

KEY:

FLUCZ = Fluconazole (25µg), KETOC = Ketoconazole (10µg), CTRIM = Clotrimazole (10µg), AMPH = Amphotericin B (20µg), FLU 1 = Flucytosine (1µg), VOR 1 = Voriconazole (1ug), ITRA C= Itraconazole (50µg), NYSTA = Nystatin (100units).

Inhibition Zone Diameter (mm) of the Antifungal Agents against the Different *Strains of Candida parapsilosis*

Strains of *Candida parapsilosis*	Inhibition Zone Diameter (mm)							
	FLUCZ	KETOC	CTRIM	AMPH	FLU 1	VOR 1	ITRAC	NYSTA
1	18	33	25	18	0	36	30	8
2	0	0	0	16	0	0	0	8
3	0	19	22	0	0	38	31	0
4	35	24	27	16	0	35	27	0
5	27	26	26	17	25	31	28	27
6	0	12	0	0	0	0	0	20
7	20	18	24	18	0	28	21	18
8	20	22	16	16	0	16	0	17
9	0	0	20	18	0	20	0	15
10	0	0	0	0	0	0	0	0
11	25	25	20	20	0	25	26	20
12	0	18	23	21	0	24	20	19
13	19	30	0	19	0	30	25	12
14	20	27	0	0	0	27	20	8
15	13	0	25	17	23	24	12	26
16	23	25	21	22	0	17	26	0
17	16	28	13	19	0	22	30	11
18	0	24	16	18	0	0	24	16
19	8	15	21	0	0	20	13	17
20	25	14	20	20	0	15	0	13
21	12	22	0	16	0	14	22	18

KEY:
FLUCZ = Fluconazole (25µg), KETOC = Ketoconazole (10µg), CTRIM = Clotrimazole (10µg), AMPH = Amphotericin B (20µg), FLU 1 = Flucytosine (1µg), VOR 1 = Voriconazole (1ug), ITRA C= Itraconazole (50µg), NYSTA = Nystatin (100units).

Inhibition Zone Diameter (mm) of the Antifungal Agents against the Different Strains of *Issatchenkia orientalis* (*Candida krusei*)

Strains of *Candida krusei*	Inhibition Zone Diameter (mm)							
	FLUCZ	KETOC	CTRIM	AMPH	FLU 1	VOR 1	ITRAC	NYSTA
1	0	0	0	0	0	30	25	0
2	0	24	0	0	0	21	26	0
3	17	0	0	14	0	0	0	11
4	0	23	0	18	0	0	15	0
5	0	0	25	0	0	28	0	15
6	0	0	21	18	0	0	0	16
7	0	0	0	19	0	22	21	15

KEY:

FLUCZ = Fluconazole (25µg), KETOC = Ketoconazole (10µg), CTRIM = Clotrimazole (10µg), AMPH = Amphotericin B (20µg), FLU 1 = Flucytosine (1µg), VOR 1 = Voriconazole (1ug), ITRA C= Itraconazole (50µg), NYSTA = Nystatin (100units).

Inhibition Zone Diameter (mm) of the Antifungal Agents against the Differen Stains of *Candida glabrata*

Strains of *Candida glabrata*	Inhibition Zone Diameter (mm)							
	FLUCZ	KETOC	CTRIM	AMPH	FLU 1	VOR 1	ITRAC	NYSTA
1	25	20	25	10	0	28	22	15
2	0	23	0	14	0	15	0	16
3	20	15	15	21	0	0	0	14
4	17	29	0	17	0	19	0	0
5	0	0	25	13	0	28	0	15
6	20	0	21	18	0	0	0	16

KEY:
FLUCZ = Fluconazole (25µg), KETOC = Ketoconazole (10µg), CTRIM = Clotrimazole (10µg), AMPH = Amphotericin B (20µg), FLU 1 = Flucytosine (1µg), VOR 1 = Voriconazole (1ug), ITRA C= Itraconazole (50µg), NYSTA = Nystatin (100units).

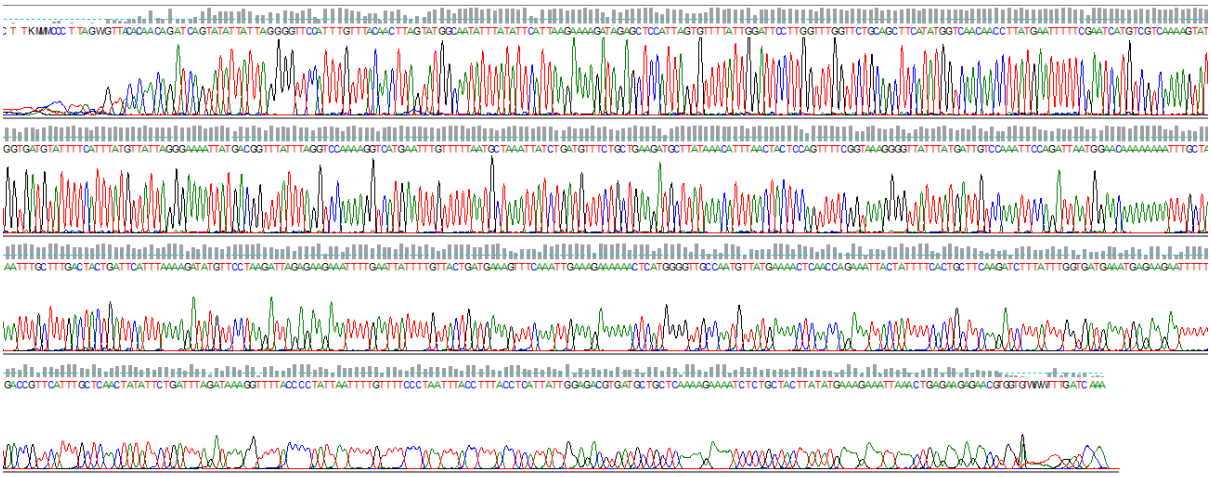

Fig 15a: Chromatogram of the ERG 11 Gene in *Candida albicans*[1] (E+ERG1A)

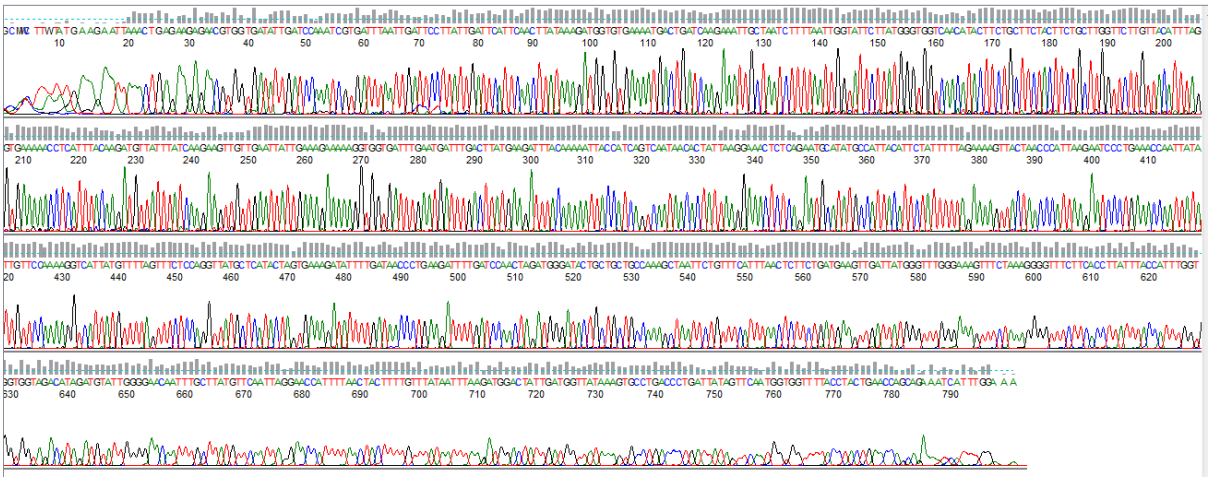

Fig 15b: Chromatogram of the ERG 11 Gene in *Candida albicans*[1] (E3+ERG2A)

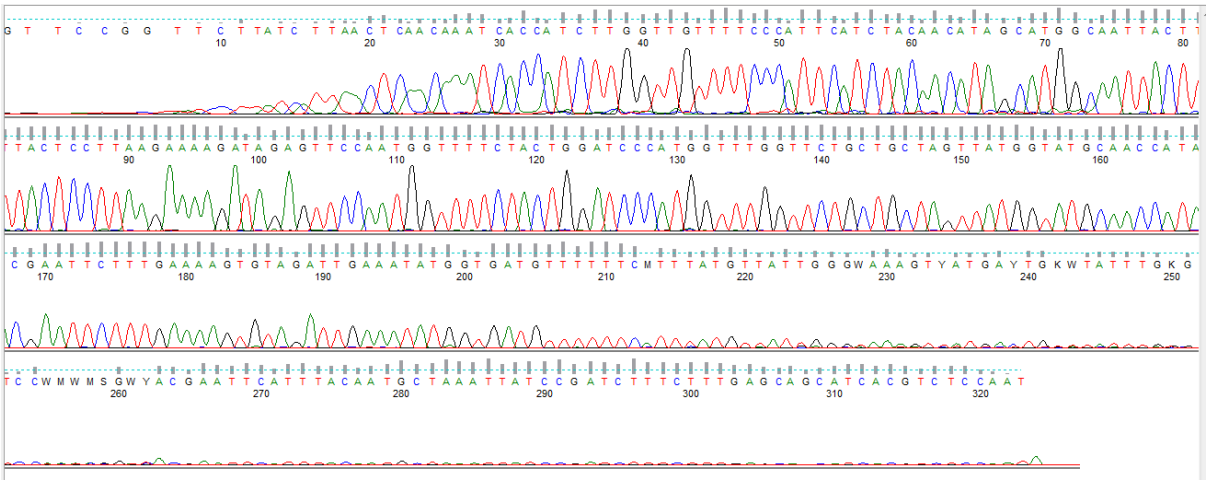

Fig 15c: Chromatogram of the ERG 11 Gene in *Candida tropicalis*[1] (E2+ERG1A)

MALDI-TOF MS Identification of the Isolates

Vaginal Yeasts Samples Africa						
Name Strain	Description	Maldi ID first best match	Score	Maldi ID second best match	Score	Final ID
F-1A	White/Crème Matt Hyphae	Candida tropicalis ATCC 13803 THL	2.133	Candida tropicalis DSM 9419 DSM	2.051	*C. tropicalis*
F-1B	W/C Matt Hyphae	Candida tropicalis ATCC 13803 THL	1.973	Candida tropicalis VML	1.952	*C. tropicalis*
F-2A	W/C Shiny	Candida parapsilosis ATCC 22019 THL	2.056	Candida parapsilosis CBS 2216 CBS	1.800	*C. parapsilosis*
F-2B	W/C Shiny	Candida parapsilosis ATCC 22019 THL	1.970	Candida parapsilosis DSM 70125 DSM	1.800	*C. parapsilosis*
F-3A	W/C Shiny Hyphae some wrinkles	Candida tropicalis ATCC 13803 THL	1.964	Candida tropicalis CBS 2313 CBS	1.883	*C. tropicalis*
F-3B	W/C Shiny Hyphea	Candida tropicalis RV_03 VML	2.059	Candida tropicalis VML	1.927	*C. tropicalis*
F-4A a	Crème Slimy	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	2.363	Klebsiella pneumoniae RV_BA_03_B LBK	2.254	*bacteria (Klebsiella)*
F-4A b	Crème Shiny	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	2.308	Klebsiella pneumoniae RV_BA_03_B LBK	2.165	*bacteria (Klebsiella)*
F-4B a	Crème Slimy	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	2.349	Klebsiella pneumoniae 37585 PFM	2.205	*bacteria (Klebsiella)*
F-4B b	Crème Shiny	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	2.191	Klebsiella pneumoniae 37585 PFM	2.044	*bacteria (Klebsiella)*
F-5A	W/C Shiny	Candida Albicans, CBS 1912, manual entry	2.209	Candida albicans DSM 1665 DSM	2.160	*C. albicans*
F-5B	W/C Shiny	Candida albicans ATCC 10231 THL	2.262	Candida Albicans, CBS 1912, manual entry	2.262	*C. albicans*
F-6A a	W/C Matt	Candida parapsilosis ATCC 22019 THL	2.195	Candida parapsilosis DSM 70125 DSM	1.866	*C. parapsilosis*
F-6A b	Crème Slimey	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	2.163	Klebsiella pneumoniae RV_BA_03_B LBK	2.162	*bacteria (Klebsiella)*
F-6B a	W/C Matt	Candida parapsilosis ATCC 22019 THL	2.181	Candida parapsilosis DSM 70125 DSM	1.867	*C. parapsilosis*
F-6B b	Crème Slimey	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	2.183	Klebsiella pneumoniae 37585 PFM	2.062	*bacteria (Klebsiella)*
F-7A	W/C Shiny Hyphae	Candida tropicalis ATCC 13803 THL	2.172	Candida tropicalis VML	2.116	*C. tropicalis*
F-7B	W/C Shiny	Candida tropicalis DSM 9419 DSM	1.994	Candida tropicalis ATCC 13803 THL	1.956	*C. tropicalis*
F-8A a	W/C Matt Wrinkled/Brainy	Candida parapsilosis ATCC 22019 THL	2.392	Candida parapsilosis CBS 2216 CBS	2.120	*C. parapsilosis*
F-8A b	Crème Slimy	Klebsiella (bact)				*bacteria (Klebsiella)*
F-8B a	W/C Matt Wrinkled	Candida parapsilosis ATCC 22019 THL	2.447	Candida parapsilosis DSM 4237 DSM	2.193	*C. parapsilosis*
F-8b b	Crème Slimy	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	2.183	Klebsiella pneumoniae RV_BA_03_B LBK	2.135	*bacteria (Klebsiella)*
F-9A	W/C Shiny	Candida Albicans, CBS 1912, manual entry	2.229	Candida albicans CBS 1893 CBS	2.103	*C. albicans*
F-9B	W/C Shiny	Candida Albicans, CBS 1912, manual entry	2.224	Candida albicans DSM 1665 DSM	2.196	*C. albicans*
F-10A	W/C Shiny	Candida albicans DSM 1665 DSM	2.222	Candida Albicans, CBS 1912, manual entry	2.199	*C. albicans*
F-10B	W/C Shiny	Candida Albicans, CBS 1912, manual entry	2.274	Candida albicans ATCC 10231 THL	2.224	*C. albicans*
F-11A	W/C Shiny Wrinkled Hyphae	Candida tropicalis ATCC 13803 THL	2.018	Candida tropicalis VML	1.919	*C. tropicalis*
F-11B a	W/C Wrinkled/Vulcano	Candida tropicalis VML	1.974	Candida tropicalis RV_03 VML		*C. tropicalis*
F-11B b	W/C Matt Wrinkled	Candida tropicalis ATCC 13803 THL	2.128	Candida tropicalis VML	1.992	*C. tropicalis*
F-12A	W/C Matt Hyphae	Candida tropicalis ATCC 13803 THL	2.074	Candida tropicalis VML	2.048	*C. tropicalis*
F-12B	W/C Matt Hyphae	Candida tropicalis ATCC 13803 THL	2.007	Candida tropicalis VML	1.842	*C. tropicalis*
F-13A a	W/C Matt	Candida parapsilosis ATCC 22019 THL	2.192	Candida parapsilosis CBS 8836 CBS	1.987	*C. parapsilosis*
F-13A b	W/C Wrinkled/Brainy	Candida parapsilosis CBS 8836 CBS	2.246	Candida parapsilosis MY924_09 ERL	2.202	*C. parapsilosis*
F-13B a	W/C Matt	Candida parapsilosis ATCC 22019 THL	2.149	Candida parapsilosis CBS 8836 CBS	2.045	*C. parapsilosis*
F-13B b	W/C Wrinkled/Brainy	Candida parapsilosis DSM 11224 DSM	2.119	Candida parapsilosis ATCC 22019 THL	2.112	*C. parapsilosis*
F-14A a	Crème Slimey	Klebsiella (bact)				*bacteria (Klebsiella)*
F-14A b	W/C Shiny	Klebsiella (bact)				*bacteria (Klebsiella)*
F-14B a	Crème Slimey	Klebsiella (bact)				*bacteria (Klebsiella)*
F-14B b	W/C Shiny	Klebsiella (bact)				*bacteria (Klebsiella)*
F-15A	W/C Shiny	Candida glabrata ATCC 90030 VML	2.310	Candida glabrata ATCC 2001T THL	2.201	*C. glabrata*
F-15B	W/C Shiny	Candida glabrata DSM 11950 DSM	2.250	Candida glabrata ATCC 90030 VML	2.234	*C. glabrata*
F-16A	W/C Shiny	Candida parapsilosis ATCC 22019 THL	2.075	Candida parapsilosis DSM 70126 DSM	1.999	*C. parapsilosis*
F-16B	W/C Matt some Wrinkles	Candida parapsilosis MY924_09 ERL	2.133	Candida parapsilosis ATCC 22019 THL	2.111	*C. parapsilosis*
F-17A	W/C Matt Hyphae	Candida_krusei[ana]# (Issatchenkia_orientalis[teleo]) ATCC 6258 THL	2.115	Candida_krusei[ana]# (Issatchenkia_orientalis[teleo]) ATCC 14243 THL	2.096	*Issatchenkia orientalis*
F-17B	W/C Matt Hyphae	Candida_krusei[ana]# (Issatchenkia_orientalis[teleo]) ATCC 6258 THL	2.157	Pichia occidentalis, CBS 5459, Manual Entry	2.011	*Issatchenkia orientalis*
F-18A	W/C Matt Hyphae	Candida tropicalis ATCC 13803 THL	1.983	Candida tropicalis VML	1.959	*C. tropicalis*
F-18B	W/C Matt Hyphae	Candida tropicalis ATCC 13803 THL	1.867	Candida tropicalis DSM 9419 DSM	1.704	*C. tropicalis*
F-19A	W/C Matt Hyphae	Candida_krusei[ana]# (Issatchenkia_orientalis[teleo]) ATCC 14243 THL	2.161	Candida_krusei[ana]# (Issatchenkia_orientalis[teleo]) ATCC 6258 THL	2.143	*Issatchenkia orientalis*
F-19B	W/C Matt Hyphae	Candida_krusei[ana]# (Issatchenkia_orientalis[teleo]) ATCC 14243 THL	2.147	Candida_krusei[ana]# (Issatchenkia_orientalis[teleo]) ATCC 6258 THL	2.098	*Issatchenkia orientalis*
F-20A	W/C Matt Hyphae	Candida tropicalis RV_03 VML	1.898	Candida tropicalis VML	1.820	*C. tropicalis*
F-20B	W/C Shiny Hyphae	Candida tropicalis ATCC 13803 THL	2.036	Candida tropicalis VML	2.021	*C. tropicalis*
F-21A	W/C Matt Hyphae some Wrinkles	Candida tropicalis RV_03 VML	2.030	Candida tropicalis VML	1.973	*C. tropicalis*
F-21B	W/C Matt Hyphae some Wrinkles	Candida tropicalis RV_03 VML	1.998	Candida tropicalis ATCC 13803 THL	1.992	*C. tropicalis*
F-22A	W/C Shiny	Candida glabrata DSM 11950 DSM	2.213	Candida glabrata ATCC 90030 VML	2.103	*C. glabrata*
F-22B	W/C Shiny	Candida glabrata ATCC 90030 VML	2.366	Candida glabrata DSM 11950 DSM	2.324	*C. glabrata*
F-23A	W/C Matt Hyphae	Candida tropicalis ATCC 13803 THL	2.005	Candida tropicalis DSM 9419 DSM	1.901	*C. tropicalis*
F-23B	W/C Matt Hyphae	Candida tropicalis ATCC 13803 THL	2.123	Candida tropicalis DSM 9419 DSM	2.063	*C. tropicalis*
F-24A	W/C Shiny	Candida Albicans, CBS 1912, manual entry	2.208	Candida albicans ATCC 10231 THL	2.055	*C. albicans*
F-24B	W/C Shiny Hyphae	Candida Albicans, CBS 1912, manual entry	2.196	Candida maltosa, CBS 5612, Manual entry	2.023	*C. albicans*
F-25A	W/C Matt some Wrinkles	Candida parapsilosis DSM 4237 DSM	2.328	Candida parapsilosis DSM 70126 DSM	2.302	*C. parapsilosis*
F-25B	W/C Matt some Wrinkles	Candida parapsilosis ATCC 22019 THL	2.296	Candida parapsilosis DSM 70125 DSM	2.172	*C. parapsilosis*
F-26A1 a	W/C hite Shiny	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	2.352	Klebsiella pneumoniae RV_BA_03_B LBK	2.224	*bacteria (Klebsiella)*
F-26A1 b	Crème Slimey	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	2.251	Klebsiella pneumoniae 37585 PFM	2.182	*bacteria (Klebsiella)*
F-26A2	W/C Shiny	Candida tropicalis ATCC 13803 THL	2.098	Candida tropicalis DSM 9419 DSM	1.937	*C. tropicalis*
F-27A a	Crème Shiny	Klebsiella (bact)				*bacteria (Klebsiella)*
F-27A b	W/C Slimey	Klebsiella (bact)				*bacteria (Klebsiella)*
F-27B a	W/C Slimey	Enterobacter cloacae 13159_1 CHB	2.320	Enterobacter cloacae MB11506_1 CHB	2.219	*bacteria (Enterobacter)*
F-27B b	W/C Slimey	Enterobacter cloacae 13159_1 CHB	2.295	Enterobacter cloacae MB11506_1 CHB	2.245	*bacteria (Enterobacter)*
F-28A	W/C Shiny	Candida glabrata 10035463_101 USH	1.922	Candida glabrata ATCC 2001T THL	1.909	*C. glabrata*
F-28B	W/C Shiny	Candida glabrata CBS 5040 CBS	1.952	Candida glabrata ATCC 2001T THL	1.890	*C. glabrata*
F-29A a	W/C Matt	Candida tropicalis ATCC 13803 THL	2.121	Candida tropicalis DSM 9419 DSM	2.064	*C. tropicalis*
F-29A b	Crème Slimey	Klebsiella (bact)				*bacteria (Klebsiella)*
F-29B a	W/C Matt	Candida tropicalis ATCC 13803 THL	1.967	Candida tropicalis RV_03 VML	1.857	*C. tropicalis*
F-29B b	Crème Slimey	Klebsiella (bact)				*bacteria (Klebsiella)*
F-30A a	W/C Wrinkled/Ripled	Candida parapsilosis ATCC 22019 THL	2.119	Candida parapsilosis MY924_09 ERL	1.907	*C. parapsilosis*
F-30A b	W/C Matt	Candida parapsilosis DSM 70125 DSM	2.144	Candida parapsilosis ATCC 22019 THL		*C. parapsilosis*
F-30B a	W/C Wrinkled/Brainy	Candida parapsilosis ATCC 22019 THL	2.201	Candida parapsilosis MY924_09 ERL	1.926	*C. parapsilosis*
F-30B b	W/C Matt	Candida parapsilosis ATCC 22019 THL	2.207	Candida parapsilosis CBS 8181 CBS	1.971	*C. parapsilosis*
F-31A a	W/C Matt	Candida albicans ATCC 10231 THL	2.307	Candida albicans CBS 1893 CBS	2.238	*C. albicans*
F-31A b	Crème Slimey	Klebsiella pneumoniae RV_BA_03_B LBK	2.208	CHB	2.199	*bacteria (Klebsiella)*
F-31B a	W/C Matt	Candida albicans ATCC 10231 THL	2.363	Candida albicans DSM 1665 DSM	2.321	*C. albicans*
F-31B b	Crème Slimey	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	2.303	Klebsiella pneumoniae RV_BA_03_B LBK	2.189	*bacteria (Klebsiella)*

F-32A	W/C Shiny	Candida Albicans, CBS 1912, manual entry	2.175	Candida albicans DSM 11945 DSM	2.174	*C. albicans*
F-32B	W/C Shiny	Candida Albicans, CBS 1912, manual entry	2.314	Candida albicans ATCC 10231 THL	2.244	*C. albicans*
F-33A a	W/C Matt	Candida_krusei[ana]# (Issatchenkia_orientalis[teleo]) ATCC 14243 THL	2.052	Candida_krusei[ana]# (Issatchenkia_orientalis[teleo]) ATCC 6258 THL	2.036	*Issatchenkia orientalis*
F-33A b	Crème Slimey	Klebsiella (bact)				*bacteria (Klebsiella)*
F-33B a	W/C Matt	Candida albicans ATCC 10231 THL	2.297	Candida albicans CBS 1893 CBS	2.233	*C. albicans*
F-33B b	Crème Slimey	Klebsiella (bact)				*bacteria (Klebsiella)*
F-34A	W/C Shiny	Candida parapsilosis ATCC 22019 THL	2.155	Candida parapsilosis MY924_09 ERL	1.928	*C. parapsilosis*
F-34B	W/C Shiny	Candida parapsilosis ATCC 22019 THL	2.238	Candida parapsilosis MY924_09 ERL	2.009	*C. parapsilosis*
F-35A a	W/C Slimey	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	1.793	Klebsiella pneumoniae RV_BA_03_B LBK	1.762	*bacteria (Klebsiella)*
F-35A b	Crème Slimey	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	2.125	Klebsiella pneumoniae RV_BA_03_B LBK	2.055	*bacteria (Klebsiella)*
F-35B a	W/C Shiny	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	2.281	Klebsiella pneumoniae RV_BA_03_B LBK	2.086	*bacteria (Klebsiella)*
F-35B b	Crème Slimey	Klebsiella pneumoniae ssp pneumoniae 9295_1 CHB	2.137	Klebsiella pneumoniae RV_BA_03_B LBK	2.038	*bacteria (Klebsiella)*
F-36A a	W/C no Hyphae	Candida tropicalis ATCC 13803 THL	1.747	Kandleria vitulina DSM 20405T DSM	1.545	*C. tropicalis*
F-36A b	W/C Hyphae	Candida tropicalis RV_03 VML	2.107	Candida tropicalis ATCC 13803 THL	2.093	*C. tropicalis*
F-36B a	W/C no Hyphae	Candida tropicalis ATCC 13803 THL	2.032	Candida tropicalis DSM 9419 DSM	1.987	*C. tropicalis*
F-36B b	W/C Hyphae	Candida tropicalis ATCC 13803 THL	1.987	Candida tropicalis VML	1.970	*C. tropicalis*
F-36B c	W/C Shiny Stripes	Candida parapsilosis ATCC 22019 THL	2.049	Candida parapsilosis VML	1.916	*C. parapsilosis*
F-37A a	W/C Clear	Candida tropicalis ATCC 13803 THL	2.121	Candida tropicalis CBS 94 CBS	1.880	*C. tropicalis*
F-37A b	W/C Matt	Candida tropicalis ATCC 13803 THL	2.097	Candida tropicalis CBS 94 CBS	1.954	*C. tropicalis*
F-37A c	W/C Pointy Hyphae	Candida tropicalis RV_03 VML	1.919	Candida tropicalis ATCC 13803 THL	1.834	*C. tropicalis*
F-37B a	W/C Matt	Candida tropicalis ATCC 13803 THL	2.023	Candida tropicalis DSM 9419 DSM	2.018	*C. tropicalis*
F-37B b	W/C Clear	Candida tropicalis ATCC 13803 THL	2.027	Candida tropicalis CBS 94 CBS	1.954	*C. tropicalis*
F-37B c	W/C Pointy Hyphae	Candida tropicalis ATCC 13803 THL	2.194	Candida tropicalis DSM 9419 DSM	1.968	*C. tropicalis*
F-38A	W/C Matt Hyphae	Candida_krusei[ana]# (Issatchenkia_orientalis[teleo]) 36 PSB	1.986	Candida_krusei[ana]# (Issatchenkia_orientalis[teleo]) ATCC 14243 THL	1.884	*Issatchenkia orientalis*
F-38B	W/C Matt Hyphae	Candida_krusei[ana]# (Issatchenkia_orientalis[teleo]) ATCC 14243 THL	1.967	Candida_krusei[ana]# (Issatchenkia_orientalis[teleo]) ATCC 6258 THL	1.886	*Issatchenkia orientalis*
F-39A	W/C Matt Hyphae	Candida tropicalis ATCC 13803 THL	2.258	Candida tropicalis DSM 9419 DSM	2.100	*C. tropicalis*
F-39B	W/C Matt Hyphae	Candida tropicalis ATCC 13803 THL	2.185	Candida tropicalis DSM 9419 DSM	2.102	*C. tropicalis*
F-40A a	W/C Hyphae	Candida Albicans, CBS 1912, manual entry	2.300	Candida albicans ATCC 10231 THL	2.094	*C. albicans*
F-40A b	W/C no Hyphae	Candida albicans ATCC 10231 THL	2.148	Candida albicans CBS 1893 CBS	2.073	*C. albicans*
F-40B a	W/C Hyphae	Candida albicans DSM 1665 DSM	2.160	Candida albicans ATCC 10231 THL	2.094	*C. albicans*
F-40B b	W/C Hyphae	Candida albicans ATCC 10231 THL	1.981	Candida albicans CBS 1893 CBS	1.950	*C. albicans*
F-41A	W/C Shiny Hyphae	Candida Albicans, CBS 1912, manual entry	2.183	Candida maltosa, CBS 5612, Manual entry	2.080	*C. albicans*
F-41B	W/C Shiny Hyphae	Candida albicans ATCC 10231 THL	2.210	Candida Albicans, CBS 1912, manual entry	2.175	*C. albicans*
F-42A	W/C Shiny Hyphae	Candida albicans ATCC 10231 THL	2.143	Candida albicans VA_17248_07 04 UKE	2.048	*C. albicans*
F-42B	No Growth					

Organism	**Number of strains**
C. albicans	20
C. glabrata	6
C. parapsilosis	21
C. tropicalis	34
Issatchenkia orientalis	7
bacteria	28
no growth	1

Plate 8a: Light green colonies of *Candida albicans* after growth on Chromogenic *Candida* Agar

Plate 8b: Blue Colonies of *Candida tropicalis* after growth on Chromogenic *Candida*

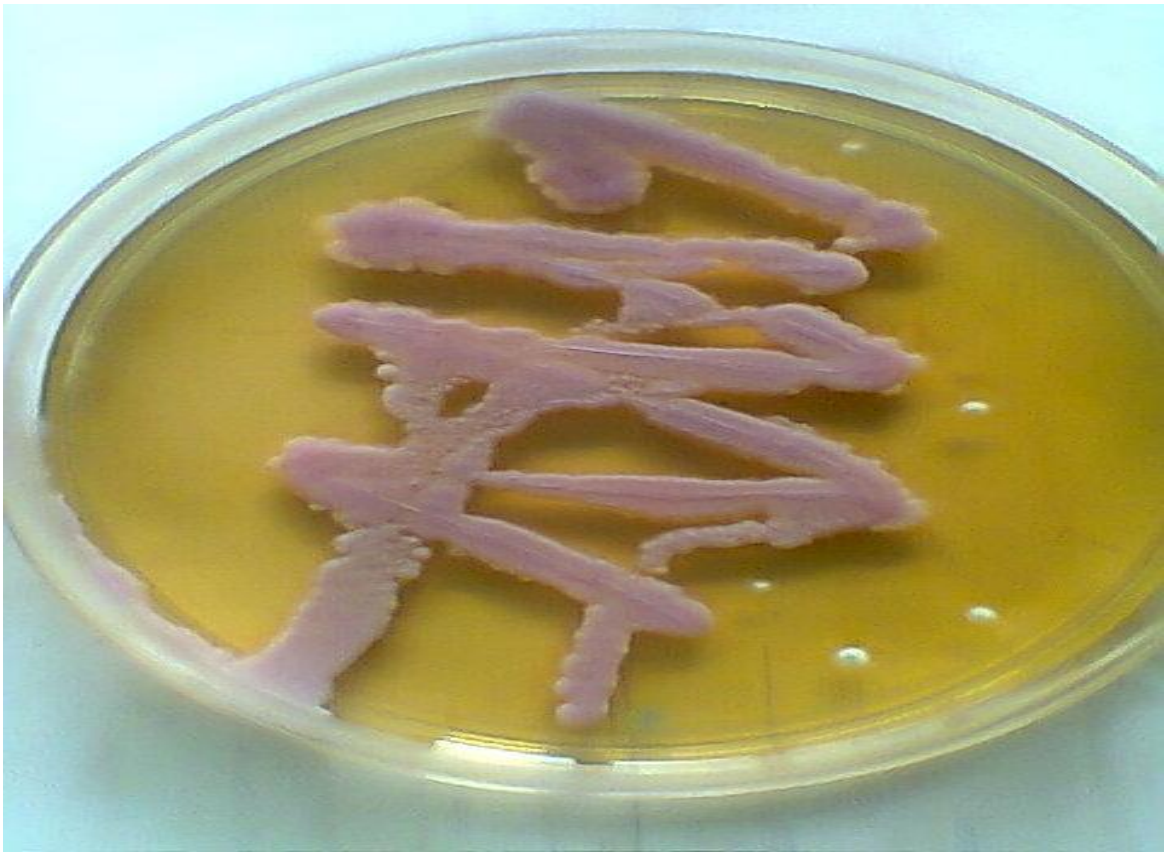

Plate 8c: Large, Flat, Dry, Spreading, Rose-pink Colonies of *Candida krusei* after Growth on Chromogenic *Candida* Agar

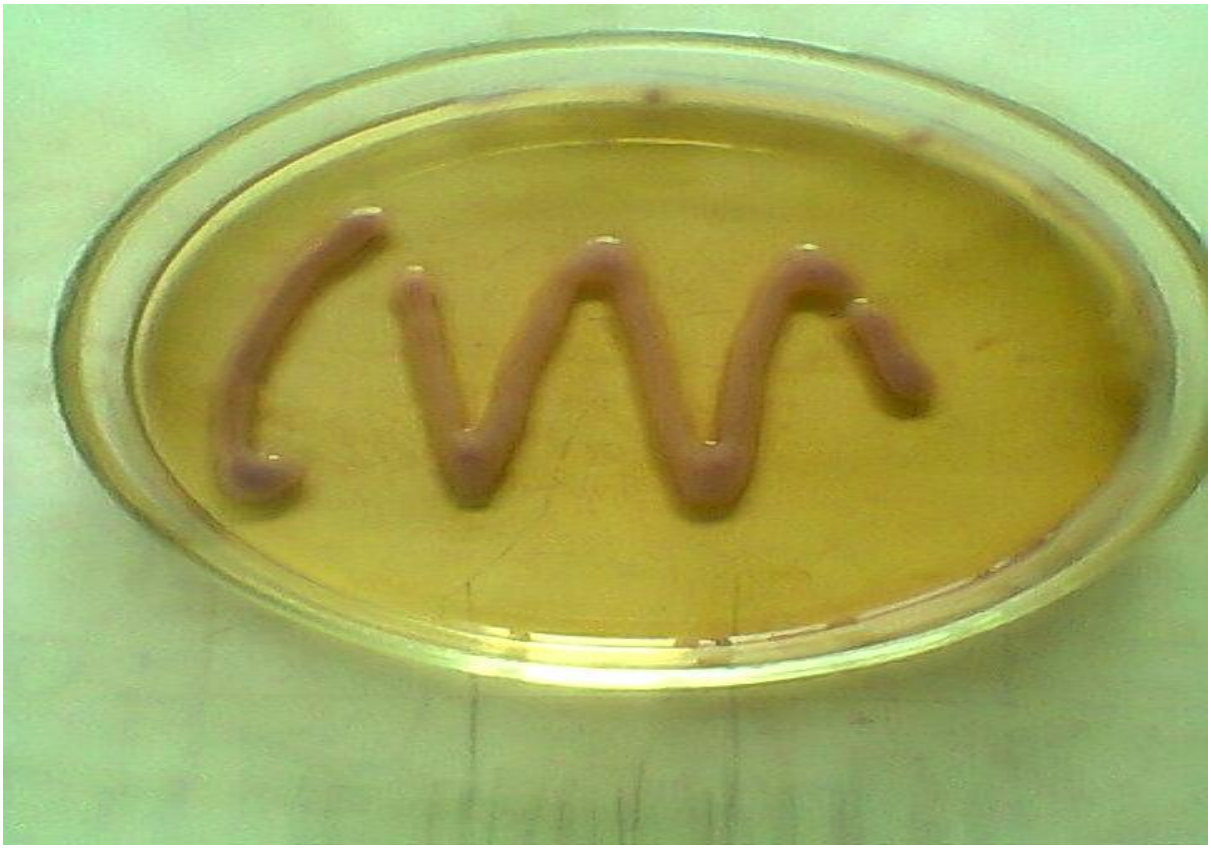

Plate 8d: Large, Glossy, Pink to Violet Colonies of *Candida glabrata* after growth on Chromogenic *Candida* Agar

Plate 8e: Off-White Colonies of *Candida parapsilopsis* after growth on Chromogenic *Candida* Agar

APPENDIX B

RESEARCH STUDY QUESTIONNAIRE ON VAGINAL DISCHARGE WITH EMPHASIS ON THE KNOWLEDGE, ATTITUDE AND PRACTICE OF RESPONDENTS.

INSTRUCTIONS: Please circle your responses to the questions. Some questions may also require you to fill in your responses. There is no right or wrong answer.

About you:

1. What is your age in years as at last birthday?
   (a) 18 – 25 (b) 26 – 33 (c) 34 – 41 (d) 42 – 49 (e) >50

2. What is your last menstrual period (LMP)? ----------

3. What is your parity (number of pregnancies conceived)? ----------

4. Are you currently pregnant?

   (a)Yes (b) No (c) Not sure

5. If yes, what trimester?
   (a) 1st trimester (0 – 3 months) (b) 2nd trimester (above 3 – 6 months) (c) 3rd trimester (above 6 – 9 months).

6. Marital status?

   (a) Single (b) Married (c) Divorced

7. What religion do you practice?

   (a) Christianity (b) Muslim (c) Other ----------

About your education and occupation:

7. What is your highest level of education?

   (a) Secondary (b) National Diploma (c) University Degree

8. If a graduate, what course did you study? ----------

9. What work do you do at present? ----------

10. How long have you been doing this job?

    (a) < 5 years (b) 5-10 years (c) 11-15 years (d) 16-20 years (e) > 20 years

About your knowledge on vaginal discharge:

11. Do you have vaginal discharge?
    (a) Yes (b) No (c) Not sure

12. If yes to the above, what is the colour like?

    (a) White (b) Yellow (c) Green (d) Not sure

13. If yes to the above, is it malodorous?

(a) Yes (b) No (c) Not sure

14. If yes to the above, how copious is it?

(a) Sparingly (b) Copious (c) Excessive

15. What is the relationship of the discharge to your menstrual period?

(a) Before period (b) During period (c) After period

16. Do you have any of these associated symptoms?

(a) Burning (b) Itching (c) Dysuria (painful urination) (d) Dispareuria (painful intercourse)

17. Have you had similar symptoms before?

(a) Yes (b) No (c) Not sure

18. Knowledge of previous diagnosis?

(a) Vaginal candidiasis (b) Cytolytic vaginosis

(c) Bacterial vaginosis (d) Others ----------

About your practices:

19. Do you routinely use sanitary pads during your period?

(a) Yes (b) No (c) Not sure

20. If no to the above, please specify? ----------

21. Do you poke your fingers and soap into your genitals while bathing?

(a) Yes (b) No (c) Not sure

22. How often do you douche?

(a) Weekly (b) Fortnightly (c) Monthly (d) Never

23. Are you presently taking any antibiotics/antifungals (eg erythromycin, ketoconazole, tetracycline, etc)?

(a) Yes (b) No

24. If yes, which antibiotics? ----------

25. Would you like to have the result of this study if it shows you have an infection?

(a) Yes (b) No

UNIVERSITY OF NIGERIA TEACHING HOSPITAL

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Our Ref: UNTH/CSA/329/OL.5

Date: 6th March, 2015.

NHREC/05/01/2008B-FWA00002458-1RB00002323

ETHICAL CLEARANCE CERTIFICATE

TOPIC: ANTIFUNGAL RESISTANCE PATTERN AMONG CLINICAL ISOLATES OF CANDIDA SPECIES AND THEIR SUSCEPTIBILITY TO SOME PLANT EXTRACTS USING ALBINO MICE MODEL.

BY: ***EZEADILA JOACHIM .O.***

FOR A PH.D THESIS IN THE DEPARTMENT OF APPLIED MICROBIOLOGY AND BREWING, FACULTY BIOSCIENCES, NNAMDI AZIKIWE UNIVERSITY, AWKA.

This research project on the above topic was reviewed and approved by the University of Nigeria Teaching Hospital Health Research Ethics Committee. This certificate is valid for **one year** from date of issue. Please note that the Committee Reserves the Right to monitor the Conduct of the study at any time for strict Compliance to the Protocol.

Prof. R.E. Umeh
Chairman, Health Research Ethics Committee

Date: 12/03/15

APPENDIX D: Statistical Analysis

I. Chi-square Test for Prevalence of Vaginal *Candida* Colonization According to Age Group

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Respondents age * Microbial Growth	336	98.8%	4	1.2%	340	100.0%

Respondents age * Microbial Growth Crosstabulation

			Microbial Growth		
			no growth	growth	Total
Respondents age	18-25	Count	95	40	135
		% within Respondents age	70.4%	29.6%	100.0%
		% within Microbial Growth	37.5%	48.2%	40.2%
	26-33	Count	82	19	101
		% within Respondents age	81.2%	18.8%	100.0%
		% within Microbial Growth	32.4%	22.9%	30.1%
	34-41	Count	50	12	62
		% within Respondents age	80.6%	19.4%	100.0%
		% within Microbial Growth	19.8%	14.5%	18.5%

	42-49	Count	20	6	26
		% within Respondents age	76.9%	23.1%	100.0%
		% within Microbial Growth	7.9%	7.2%	7.7%
	>50	Count	6	6	12
		% within Respondents age	50.0%	50.0%	100.0%
		% within Microbial Growth	2.4%	7.2%	3.6%
	Total	Count	253	83	336
		% within Respondents age	75.3%	24.7%	100.0%
		% within Microbial Growth	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	8.765[a]	4	.067
Likelihood Ratio	8.284	4	.082
Linear-by-Linear Association	.038	1	.845
N of Valid Cases	336		

a. 1 cells (10.0%) have expected count less than 5. The minimum expected count is 2.96.

II: Chi-square Test for Prevalence of Vaginal *Candida* Colonization amongst the Women in Relation to Pregnancy

Crosstab

			Microbial Growth		
			no growth	growth	Total
Pregnant	no	Count	157	63	220
		% within Pregnant	71.4%	28.6%	100.0%
		% within Microbial Growth	62.1%	75.9%	65.5%
	yes	Count	84	16	100
		% within Pregnant	84.0%	16.0%	100.0%
		% within Microbial Growth	33.2%	19.3%	29.8%
	Not sure	Count	3	0	3
		% within Pregnant	100.0%	.0%	100.0%
		% within Microbial Growth	1.2%	.0%	.9%
	no response	Count	9	4	13
		% within Pregnant	69.2%	30.8%	100.0%
		% within Microbial Growth	3.6%	4.8%	3.9%
	Total	Count	253	83	336
		% within Pregnant	75.3%	24.7%	100.0%
		% within Microbial Growth	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	7.143[a]	3	.067
Likelihood Ratio	8.194	3	.042
Linear-by-Linear Association	.076	1	.782
N of Valid Cases	336		

a. 3 cells (37.5%) have expected count less than 5. The minimum expected count is .74.

III. Prevalence of Vaginal *Candida* Colonization amongst the Women According to Trimester

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Microbial Growth * Trimester	336	98.8%	4	1.2%	340	100.0%

Microbial Growth * Trimester Crosstabulation

			Trimester				
			Not pregnant	first	second	third	Total
Microbial Growth	no growth	Count	169	33	32	19	253
		% within Microbial Growth	66.8%	13.0%	12.6%	7.5%	100.0%
		% within Trimester	71.6%	75.0%	88.9%	95.0%	75.3%
	growth	Count	67	11	4	1	83
		% within Microbial Growth	80.7%	13.3%	4.8%	1.2%	100.0%
		% within Trimester	28.4%	25.0%	11.1%	5.0%	24.7%
Total		Count	236	44	36	20	336
		% within Microbial Growth	70.2%	13.1%	10.7%	6.0%	100.0%
		% within Trimester	100.0%	100.0%	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	9.476	3	.024
Likelihood Ratio	11.540	3	.009
Linear-by-Linear Association	8.964	1	.003
N of Valid Cases	336		

IV. Prevalence of Vaginal *Candida* Colonization amongst the Women in Relation to Marital Status

Crosstab

			Microbial Growth		
			no growth	growth	Total
Marital status	single	Count	108	38	146
		% within Marital status	74.0%	26.0%	100.0%
		% within Microbial Growth	42.7%	45.8%	43.5%
	married	Count	138	43	181
		% within Marital status	76.2%	23.8%	100.0%
		% within Microbial Growth	54.5%	51.8%	53.9%
	Divorced	Count	5	0	5
		% within Marital status	100.0%	.0%	100.0%
		% within Microbial Growth	2.0%	.0%	1.5%
	no response	Count	2	2	4
		% within Marital status	50.0%	50.0%	100.0%
		% within Microbial Growth	.8%	2.4%	1.2%

Total	Count	253	83	336
	% within Marital status	75.3%	24.7%	100.0%
	% within Microbial Growth	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.241[a]	3	.356
Likelihood Ratio	4.244	3	.236
Linear-by-Linear Association	.350	1	.554
N of Valid Cases	336		

a. 4 cells (50.0%) have expected count less than 5. The minimum expected count is .99.

V. Prevalence of Vaginal *Candida* Colonization in Symptomatic and Asymptomatic Women

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Microbial Growth * symptoms presented	336	98.8%	4	1.2%	340	100.0%

Microbial Growth * symptoms presented Crosstabulation

		symptoms presented						Total		
		Symptomatic			Assymptomatic					
		Count	% within Microbial Growth	% within symptoms presented	Count	% within Microbial Growth	% within symptoms presented	Count	% within Microbial Growth	% within symptoms presented
Microbial Growth	no growth	194	76.7%	76.7%	59	23.3%	71.1%	253	100.0%	75.3%
	growth	59	71.1%	23.3%	24	28.9%	28.9%	83	100.0%	24.7%
Total		253	75.3%	100.0%	83	24.7%	100.0%	336	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)

Pearson Chi-Square	1.052	1	.305		
Continuity Correction	.773	1	.379		
Likelihood Ratio	1.028	1	.311		
Fisher's Exact Test				.308	.189
Linear-by-Linear Association	1.049	1	.306		
N of Valid Cases	336				

VI. Prevalence of Vaginal Candida Colonization amongst the Women in Relation to the Different Symptoms

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Microbial Growth * symptoms	254	74.7%	86	25.3%	340	100.0%

Microbial Growth * sympotoms Crosstabulation

		Microbial Growth						Total		
		no growth			growth					
		Count	% within Microbial Growth	% within symptoms	Count	% within Microbial Growth	% within symptoms	Count	% within Microbial Growth	% within symptoms
symptoms	v	69	35.4%	63.3%	40	67.8%	36.7%	109	42.9%	100.0%
	b	4	2.1%	100.0%	0	0.0%	0.0%	4	1.6%	100.0%

	i	32	16.4%	88.9%	4	6.8%	11.1%	36	14.2%	100.0%
	d	4	2.1%	80.0%	1	1.7%	20.0%	5	2.0%	100.0%
	dp	1	0.5%	100.0%	0	0.0%	0.0%	1	0.4%	100.0%
	vb	9	4.6%	81.8%	2	3.4%	18.2%	11	4.3%	100.0%
	vi	50	25.6%	87.7%	7	11.9%	12.3%	57	22.4%	100.0%
	vd	14	7.2%	87.5%	2	3.4%	12.5%	16	6.3%	100.0%
	vdp	4	2.1%	57.1%	3	5.1%	42.9%	7	2.8%	100.0%
	vid	2	1.0%	100.0%	0	0.0%	0.0%	2	0.8%	100.0%
	vbi	1	0.5%	100.0%	0	0.0%	0.0%	1	0.4%	100.0%
	id	2	1.0%	100.0%	0	0.0%	0.0%	2	0.8%	100.0%
	bi	1	0.5%	100.0%	0	0.0%	0.0%	1	0.4%	100.0%
	bd	1	0.5%	100.0%	0	0.0%	0.0%	1	0.4%	100.0%
	vbid	1	0.5%	100.0%	0	0.0%	0.0%	1	0.4%	100.0%
Total		195	100.0%	76.8%	59	100.0%	23.2%	254	100.0%	100.0%

Key: v = vaginal discharge; b = burning; i = itching; d = dysuria; dp = dispareuria

Chi-square Test

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	24.548	14	.039
Likelihood Ratio	27.419	14	.017
Linear-by-Linear Association	11.316	1	.001
N of Valid Cases	254		

VII: Chi-square Test for Prevalence of Vaginal *Candida* Colonization amongst the Women in Relation to Antimicrobial Therapy

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Antimicrobial Therapy * Microbial Growth	336	98.8%	4	1.2%	340	100.0%

Antimicrobial Therapy * Microbial Growth Crosstabulation

			Microbial Growth		
			no growth	growth	Total
Antimicrobial Therapy	no	Count	209	81	290
		% within Antimicrobial Therapy	72.1%	27.9%	100.0%
		% within Microbial Growth	82.6%	97.6%	86.3%
	yes	Count	44	2	46
		% within Antimicrobial Therapy	95.7%	4.3%	100.0%
		% within Microbial Growth	17.4%	2.4%	13.7%
Total		Count	253	83	336
		% within Antimicrobial Therapy	75.3%	24.7%	100.0%
		% within Microbial Growth	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	11.871[a]	1	.001		
Continuity Correction[b]	10.637	1	.001		
Likelihood Ratio	15.688	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	11.836	1	.001		
N of Valid Cases	336				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 11.36.

b. Computed only for a 2x2 table

APPENDIX E: Statistical Analysis Using ANOVA

I. Inhibition Zone Diameter of the Ethanolic Plant Extracts against *Candida albicans*[1]

Case processing summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
Inhibition zone diameter * Plant Extract * Concentration(mg/ml) * Isolate	45	100.0%	0	.0%	45	100.0%

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)		
		200mg/ml		100mg/ml
		Mean	Std. Deviation	Mean
C. albicans[1]	*Moringa*	19.00	.000	17.33
	Vernonia	15.33	.577	18.67
	Ocimum	.00	.000	.00
	Total	11.44	8.734	12.00
Total	*Moringa*	19.00	.000	17.33
	Vernonia	15.33	.577	18.67
	Ocimum	.00	.000	.00

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)		
		200mg/ml		100mg/ml
		Mean	Std. Deviation	Mean
C. albicans[1]	*Moringa*	19.00	.000	17.33
	Vernonia	15.33	.577	18.67
	Ocimum	.00	.000	.00
	Total	11.44	8.734	12.00
Total	*Moringa*	19.00	.000	17.33
	Vernonia	15.33	.577	18.67
	Ocimum	.00	.000	.00
	Total	11.44	8.734	12.00

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)				
		100mg/ml	50mg/ml		25mg/ml	
		Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
C. albicans[1]	*Moringa*	.577	15.67	.577	15.00	.000
	Vernonia	.577	17.00	1.000	15.00	.000
	Ocimum	.000	.00	.000	.00	.000
	Total	9.028	10.89	8.207	10.00	7.500

Total	*Moringa*	.577	15.67	.577	15.00	.000
	Vernonia	.577	17.00	1.000	15.00	.000
	Ocimum	.000	.00	.000	.00	.000
	Total	9.028	10.89	8.207	10.00	7.500

Report

Inhibition zone diameter

		Concentration(mg/ml)			
		12.25mg/ml		Total	
Isolate	Plant Extract	Mean	Std. Deviation	Mean	Std. Deviation
C. albicans[1]	*Moringa*	14.33	.577	16.27	1.792
	Vernonia	13.00	.000	15.80	2.042
	Ocimum	.00	.000	.00	.000
	Total	9.11	6.864	10.69	7.798
Total	*Moringa*	14.33	.577	16.27	1.792
	Vernonia	13.00	.000	15.80	2.042
	Ocimum	.00	.000	.00	.000
	Total	9.11	6.864	10.69	7.798

ANOVA Table

			Sum of Squares	df
Inhibition zone diameter * Plant Extract	Between Groups	(Combined)	2572.311	2
		Within Groups	103.333	42
		Total	2675.644	44

ANOVA Table

			Mean Square	F	Sig.
Inhibition zone diameter * Plant Extract	Between Groups	(Combined)	1286.156	522.760	.000
		Within Groups	2.460		

Measures of Association

	Eta	Eta Squared
Inhibition zone diameter * Plant Extract	.980	.961

II. Inhibition Zone Diameter of the Ethanolic Plant Extracts against *Candida parapsilosis*

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
Inhibition zone diameter * Plant Extract * Concentration(mg/ml) * Isolate	45	100.0%	0	.0%	45	100.0%

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)		
		200mg/ml		100mg/ml
		Mean	Std. Deviation	Mean
Candida parapsilosis	*Moringa*	19.00	.000	17.33
	Vernonia	19.00	1.000	17.67
	Ocimum	.00	.000	.00
	Total	12.67	9.513	11.67
Total	*Moringa*	19.00	.000	17.33
	Vernonia	19.00	1.000	17.67
	Ocimum	.00	.000	.00

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)		
		200mg/ml		100mg/ml
		Mean	Std. Deviation	Mean
Candida parapsilosis	*Moringa*	19.00	.000	17.33
	Vernonia	19.00	1.000	17.67
	Ocimum	.00	.000	.00
	Total	12.67	9.513	11.67
Total	*Moringa*	19.00	.000	17.33
	Vernonia	19.00	1.000	17.67
	Ocimum	.00	.000	.00
	Total	12.67	9.513	11.67

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)				
		100mg/ml	50mg/ml		25mg/ml	
		Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
Candida parapsilosis	*Moringa*	.577	15.67	.577	15.00	.000
	Vernonia	.577	15.33	1.528	13.33	1.528
	Ocimum	.000	.00	.000	.00	.000
	Total	8.761	10.33	7.794	9.44	7.161

Total	*Moringa*	.577	15.67	.577	15.00	.000
	Vernonia	.577	15.33	1.528	13.33	1.528
	Ocimum	.000	.00	.000	.00	.000
	Total	8.761	10.33	7.794	9.44	7.161

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)			
		12.25mg/ml		Total	
		Mean	Std. Deviation	Mean	Std. Deviation
Candida parapsilosis	*Moringa*	14.33	.577	16.27	1.792
	Vernonia	12.33	.577	15.53	2.774
	Ocimum	.00	.000	.00	.000
	Total	8.89	6.735	10.60	7.811
Total	*Moringa*	14.33	.577	16.27	1.792
	Vernonia	12.33	.577	15.53	2.774
	Ocimum	.00	.000	.00	.000
	Total	8.89	6.735	10.60	7.811

ANOVA Table

			Sum of Squares	df
Inhibition zone diameter * Plant Extract	Between Groups	(Combined)	2532.133	2
		Within Groups	152.667	42
		Total	2684.800	44

ANOVA Table

			Mean Square	F	Sig.
Inhibition zone diameter * Plant Extract	Between Groups	(Combined)	1266.067	348.307	.000
		Within Groups	3.635		

Measures of Association

	Eta	Eta Squared
Inhibition zone diameter * Plant Extract	.971	.943

III. Table 4.11c: Inhibition Zone Diameter of the Ethanolic Plant Extracts against *Candida tropicalis*[1]

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
Inhibition zone diameter * Plant Extract * Concentration(mg/ml) * Isolate	45	100.0%	0	.0%	45	100.0%

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)		
		200mg/ml		100mg/ml
		Mean	Std. Deviation	Mean
Candida tropicalis[1]	*Moringa*	17.00	.000	15.67
	Vernonia	17.00	.000	15.67
	Ocimum	15.00	.000	13.67
	Total	16.33	1.000	15.00
Total	*Moringa*	17.00	.000	15.67
	Vernonia	17.00	.000	15.67
	Ocimum	15.00	.000	13.67

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)		
		200mg/ml		100mg/ml
		Mean	Std. Deviation	Mean
Candida tropicalis[1]	*Moringa*	17.00	.000	15.67
	Vernonia	17.00	.000	15.67
	Ocimum	15.00	.000	13.67
	Total	16.33	1.000	15.00
Total	*Moringa*	17.00	.000	15.67
	Vernonia	17.00	.000	15.67
	Ocimum	15.00	.000	13.67
	Total	16.33	1.000	15.00

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)				
		100mg/ml	50mg/ml		25mg/ml	
		Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
Candida tropicalis[1]	*Moringa*	.577	15.00	.000	14.67	1.528
	Vernonia	.577	15.00	.000	14.67	1.528
	Ocimum	.577	13.00	.000	11.67	.577
	Total	1.118	14.33	1.000	13.67	1.871

Total	Moringa	.577	15.00	.000	14.67	1.528
	Vernonia	.577	15.00	.000	14.67	1.528
	Ocimum	.577	13.00	.000	11.67	.577
	Total	1.118	14.33	1.000	13.67	1.871

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)			
		12.25mg/ml		Total	
		Mean	Std. Deviation	Mean	Std. Deviation
C. tropicalis[1]	*Moringa*	14.00	.000	15.27	1.223
	Vernonia	14.00	.000	15.27	1.223
	Ocimum	11.33	.577	12.93	1.438
	Total	13.11	1.364	14.49	1.687
Total	*Moringa*	14.00	.000	15.27	1.223
	Vernonia	14.00	.000	15.27	1.223
	Ocimum	11.33	.577	12.93	1.438
	Total	13.11	1.364	14.49	1.687

ANOVA Table

			Sum of Squares	df
Inhibition zone diameter * Plant Extract	Between Groups	(Combined)	54.444	2
		Within Groups	70.800	42
		Total	125.244	44

ANOVA Table

			Mean Square	F	Sig.
Inhibition zone diameter * Plant Extract	Between Groups	(Combined)	27.222	16.149	.000
		Within Groups	1.686		

Measures of Association

	Eta	Eta Squared
Inhibition zone diameter * Plant Extract	.659	.435

IV. Inhibition Zone Diameter of the Ethanolic Plant Extracts against *Candida tropicalis*[2]

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
Inhibition zone diameter * Plant Extract * Concentration(mg/ml) * Isolate	45	100.0%	0	.0%	45	100.0%

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)		
		200mg/ml		100mg/ml
		Mean	Std. Deviation	Mean
Candida tropicalis[2]	*Moringa*	.00	.000	17.00
	Vernonia	17.67	.577	16.33
	Ocimum	17.00	.000	16.00
	Total	11.56	8.676	16.44
Total	*Moringa*	.00	.000	17.00
	Vernonia	17.67	.577	16.33
	Ocimum	17.00	.000	16.00

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)		
		200mg/ml		100mg/ml
		Mean	Std. Deviation	Mean
Candida tropicalis[2]	*Moringa*	.00	.000	17.00
	Vernonia	17.67	.577	16.33
	Ocimum	17.00	.000	16.00
	Total	11.56	8.676	16.44
Total	*Moringa*	.00	.000	17.00
	Vernonia	17.67	.577	16.33
	Ocimum	17.00	.000	16.00
	Total	11.56	8.676	16.44

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)				
		100mg/ml	50mg/ml		25mg/ml	
		Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
Candida tropicalis[2]	*Moringa*	.000	15.33	.577	14.00	1.732
	Vernonia	.577	16.00	.000	15.00	.000
	Ocimum	.000	14.00	1.000	13.67	.577
	Total	.527	15.11	1.054	14.22	1.093

Total	*Moringa*	.000	15.33	.577	14.00	1.732
	Vernonia	.577	16.00	.000	15.00	.000
	Ocimum	.000	14.00	1.000	13.67	.577
	Total	.527	15.11	1.054	14.22	1.093

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)			
		12.25mg/ml		Total	
		Mean	Std. Deviation	Mean	Std. Deviation
Candida tropicalis[2]	*Moringa*	11.67	1.155	11.60	6.322
	Vernonia	13.00	.000	15.60	1.639
	Ocimum	12.33	.577	14.60	1.805
	Total	12.33	.866	13.93	4.191
Total	*Moringa*	11.67	1.155	11.60	6.322
	Vernonia	13.00	.000	15.60	1.639
	Ocimum	12.33	.577	14.60	1.805
	Total	12.33	.866	13.93	4.191

ANOVA Table

			Sum of Squares	df
Inhibition zone diameter * Plant Extract	Between Groups	(Combined)	130.000	2
		Within Groups	642.800	42
		Total	772.800	44

ANOVA Table

			Mean Square	F	Sig.
Inhibition zone diameter * Plant Extract	Between Groups	(Combined)	65.000	4.247	.021
		Within Groups	15.305		

Measures of Association

	Eta	Eta Squared
Inhibition zone diameter * Plant Extract	.410	.168

V. Inhibition Zone Diameter of the Ethanolic Plant Extracts against *Candida albicans*[2]

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
Inhibition zone diameter * Plant Extract * Concentration(mg/ml) * Isolate	45	100.0%	0	.0%	45	100.0%

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)		
		200mg/ml		100mg/ml
		Mean	Std. Deviation	Mean
Candida albicans[2]	*Moringa*	15.00	.000	16.33
	Vernonia	.00	.000	.00
	Ocimum	18.00	.000	15.67
	Total	11.00	8.352	10.67
Total	*Moringa*	15.00	.000	16.33
	Vernonia	.00	.000	.00
	Ocimum	18.00	.000	15.67

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)		
		200mg/ml		100mg/ml
		Mean	Std. Deviation	Mean
Candida albicans[2]	*Moringa*	15.00	.000	16.33
	Vernonia	.00	.000	.00
	Ocimum	18.00	.000	15.67
	Total	11.00	8.352	10.67
Total	*Moringa*	15.00	.000	16.33
	Vernonia	.00	.000	.00
	Ocimum	18.00	.000	15.67
	Total	11.00	8.352	10.67

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)				
		100mg/ml	50mg/ml		25mg/ml	
		Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
Candida albicans[2]	*Moringa*	.577	14.67	.577	14.00	.000
	Vernonia	.000	18.33	.577	17.00	.000
	Ocimum	.577	14.00	.000	.00	.000
	Total	8.016	15.67	2.062	10.33	7.858

Total	*Moringa*	.577	14.67	.577	14.00	.000
	Vernonia	.000	18.33	.577	17.00	.000
	Ocimum	.577	14.00	.000	.00	.000
	Total	8.016	15.67	2.062	10.33	7.858

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)			
		12.25mg/ml		Total	
		Mean	Std. Deviation	Mean	Std. Deviation
Candida albicans[2]	*Moringa*	11.67	.577	14.33	1.633
	Vernonia	15.33	.577	10.13	8.626
	Ocimum	.00	.000	9.53	8.167
	Total	9.00	6.946	11.33	7.100
Total	*Moringa*	11.67	.577	14.33	1.633
	Vernonia	15.33	.577	10.13	8.626
	Ocimum	.00	.000	9.53	8.167
	Total	9.00	6.946	11.33	7.100

ANOVA Table

			Sum of Squares	df
Inhibition zone diameter * Plant Extract	Between Groups	(Combined)	205.200	2
		Within Groups	2012.800	42
		Total	2218.000	44

ANOVA Table

			Mean Square	F	Sig.
Inhibition zone diameter * Plant Extract	Between Groups	(Combined)	102.600	2.141	.130
		Within Groups	47.924		

Measures of Association

	Eta	Eta Squared
Inhibition zone diameter * Plant Extract	.304	.093

VI. Inhibition Zone Diameter of the Ethanolic Plant Extracts against *Candida krusei*

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
Inhibition zone diameter * Plant Extract * Concentration(mg/ml) * Isolate	45	100.0%	0	.0%	45	100.0%

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)		
		200mg/ml		100mg/ml
		Mean	Std. Deviation	Mean
Candida krusei	*Moringa*	.00	.000	14.33
	Vernonia	.00	.000	18.00
	Ocimum	.00	.000	16.00
	Total	.00	.000	16.11
Total	*Moringa*	.00	.000	14.33
	Vernonia	.00	.000	18.00
	Ocimum	.00	.000	16.00

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)		
		200mg/ml		100mg/ml
		Mean	Std. Deviation	Mean
Candida krusei	*Moringa*	.00	.000	14.33
	Vernonia	.00	.000	18.00
	Ocimum	.00	.000	16.00
	Total	.00	.000	16.11
Total	*Moringa*	.00	.000	14.33
	Vernonia	.00	.000	18.00
	Ocimum	.00	.000	16.00
	Total	.00	.000	16.11

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)				
		100mg/ml	50mg/ml		25mg/ml	
		Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
Candida krusei	*Moringa*	.577	13.00	.000	12.00	.000
	Vernonia	.000	15.00	.000	14.00	.000
	Ocimum	.000	14.00	.000	13.00	.000
	Total	1.616	14.00	.866	13.00	.866

Total	*Moringa*	.577	13.00	.000	12.00	.000
	Vernonia	.000	15.00	.000	14.00	.000
	Ocimum	.000	14.00	.000	13.00	.000
	Total	1.616	14.00	.866	13.00	.866

Report

Inhibition zone diameter

Isolate	Plant Extract	Concentration(mg/ml)			
		12.25mg/ml		Total	
		Mean	Std. Deviation	Mean	Std. Deviation
C. krusei	*Moringa*	12.67	2.082	10.40	5.501
	Vernonia	13.00	.000	12.00	6.448
	Ocimum	.00	.000	8.60	7.337
	Total	8.56	6.502	10.33	6.477
Total	*Moringa*	12.67	2.082	10.40	5.501
	Vernonia	13.00	.000	12.00	6.448
	Ocimum	.00	.000	8.60	7.337
	Total	8.56	6.502	10.33	6.477

ANOVA Table

			Sum of Squares	df
Inhibition zone diameter * Plant Extract	Between Groups	(Combined)	86.800	2
		Within Groups	1759.200	42
		Total	1846.000	44

ANOVA Table

			Mean Square	F	Sig.
Inhibition zone diameter * Plant Extract	Between Groups	(Combined)	43.400	1.036	.364
		Within Groups	41.886		

Measures of Association

	Eta	Eta Squared
Inhibition zone diameter * Plant Extract	.217	.047

Key:

Moringa = Moringa oleifera, *Vernonia = Vernonia amygdalina*, *Ocimum = Ocimum gratissimum*

APPENDIX F: Protocol for Sequencing
PCR products were cleaned using ExoSAP Protocol as follows:

1. Prepare the Exo/SAP master mix by adding the following to a 0.6ml micro-centrifuge tube:

a. Exonuclease I (Catalogue No. NEB M0293L) 20 U/ul 50 µl

b. Shrimp Alkaline Phosphatase (Catalogue No. NEB M0371) 1 U/ul 200 µl

2. Prepare the following reaction mixture:

Amplified PCR Product 10 µl
ExoSAP Mix (step 1) 2.5 µl
3. Mix well and incubate at **37°C** for **15 min**

4. Stop the reaction by heating the mixture at **80°C** for 1**5 min**

Fragments were sequenced using the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 according to manufacturer's instructions:

https://www.nimagen.com/products/Sequencing/Capillary-Electrophoresis/BrilliantDye-Terminator-Cycle-Sequencing-Kit/

The labelled products are then cleaned with the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053):
http://www.zymoresearch.com/downloads/dl/file/id/52/d4052i.pdf
The cleaned products are injected on the Applied Biosystems ABI 3500XL Genetic Analyser with a 50cm array, using POP7:
https://www.thermofisher.com/order/catalog/product/4406016
Sequence chromatogram analysis is performed using FinchTV analysis software:

https://www.softpedia.com/get/Science-CAD/FinchTV.shtml