ANTIBIOFILM EFFECTS OF PARTIALLY PURIFIED FRACTION OF ETHYLACETATE EXTRACT OF ACALYPHA WILKESIANA LEAVES ON CLINICAL ISOLATES OF CANDIDA ALBICANS I, II AND CANDIDA PARAPSILOSIS

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A THESISSUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA. IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY,
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DECLARATION

I declare that the work in this thesisentitled: **Antibiofilm Effects of Partially Purified Fraction of Ethylacetate Extract of *Acalyphawilkesiana* Leaves on Clinical Isolates of *Candida albicans* I, II and *Candida parapsilosis***has been carried out by me in the **Department of Biochemistry.**The information derived from the literature has been duly acknowledged in the text and a list of references provided.No part of this work has been presented for another degree or diploma at this or any other institution.

MaimunaZubairu _____ _____

Name of Student Signature Date

CERTIFICATION

This thesis work entitled ANTIBIOFILM EFFECTS OF PURIFIED FRACTION OF PARTIALLY PURIFIED FRACTION OF ETHYLACETATE EXTRACT OF *ACALYPHA WILKESIANA* LEAVES ON CLINICAL ISOLATES OF *CANDIDA ALBICANS* I, II AND *CANDIDA PARAPSILOSIS* byMAIMUNA ZUBAIRU meets the regulations governing the award of Ph.D. in Biochemistry of the Ahmadu Bello University Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to Almighty Allah, the most merciful of all those who show mercy. Praise be to Allah for granting me the opportunity to complete this study amidst all challenges of life.

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Praise be to Almighty Allah the Lord of mankind, the King on the day of judgment, by His will andbounteousness the accomplishment of this work is made possible, and may Hispeace and blessings be upon Prophet Mohammad (S.A.W).

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To all my friends and loved ones I say: thank you and God bless.

ABSTRACT

Candida infection is the most common hospital-acquired infection caused by fungal pathogen which is frequently involved in biofilm growth, exhibiting to increased resistance to antifungal drugs.This research is aimed at evaluating the biofilm eradication activity of purified fraction of ethylacetate extract of *Acalyphawilkesiana* leaveson biofilms of *C. albicans*I, II and *C. parapsilosis*. Column Chromatography-TLC was used to fractionate and partially purify the extract using a bioassay guided fractionation.The most potent fraction was partially purified using preparative TLC. The minimum inhibitory concentration (MIC), and median inhibitory concentration (IC_{50}) were carried out using microdilution method. Fungicidal activity (MFC) of the purified fraction was determined by agar well diffusion method. Adhered cells penetrability of the extracts was carried out by forming adhered cells on membrane polycarbonate filters, and determining the concentration of the antifungals that penetrated the adhered cells with time. Median adhered cells ($AdEC_{50}$) and median biofilm eradication concentration (BEC_{50}) were determined by microdilution method. Antibiofilm activity of the purified fraction on the morphology of the sessile cells of *Candida spp* was evaluated by scanning electron microscopy. The mode of action was predicted by the use of sorbitol and ergosterol to evaluate their effect on the IC_{50} of the antifungal and comparing it with the $IC_{50}$of caspofungin and Amphotericin B which are standard antifungals with known mode of actions. The result revealed that ethylacetate extract was the most potent with antifungal activities compared to n-hexane, methanol and aqueous extracts of *A. wilkesiana*leaves. The purified fraction of the ethylacetateextract had MIC of 1.25mg/ml on C. *albicans*I, II and *C. parapsilosis,* respectively. MFC of 2.5mg/ml on *C. albicans I* and *II* but hadno fungicidal activity on *C. parapsilosis*. The partially purified fraction had minimum values of IC_{50} and BEC_{50}compared to other fractions. There was reduction in clusters formed by proliferation of the sessile cells on the morphology of *C. albicans*I, II and C. *parapsilosis* in all treated plates compared to the control. There wasincrease in the $IC_{50}$of the purified fractionand caspofungin in the presence of sorbitol. In conclusion, the partially purified fraction of ethylacetate extract had antibiofilm activity against the three strains of *Candida* used. Increased in IC_{50} of the purified fraction and caspofungin implicated the fungal cell wall as the possible target of the partially purified fractionof ethylacetate extract of *A. wilkesiana* leaves.

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LIST OF ABBREVIATIONS

ABC	ATP Binding Cassette
ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
ATP	Adenosine Triphosphate
BEC_{50}	Median Biofilm Eradication Concentration
CDR	*Candida* Drug Resistance
$CDCl_3$	Deuterated chloroform
CFU	Coliform forming Unit
CV-I	Crystal Violet Iodine
DZI	Diameter Zone of Inhibition
GI	Gastro Intestinal
HREC	Health Research Ethics Committee
HIV	Human Immunodeficiency Virus
HVS	High Vaginal Swab
ICS	Intra Cervical Swab
LD_{50}	Median Lethal Dose
MDR	Multi Drug Resistance
MIC	Minimum Inhibitory Concentration
MFC	Minimum Fungicidal Concentration
NMR	Nuclear Magnetic Resonance
PL	phospholipases
PBS	Phosphate Buffered Saline
SAP	Secreted Aspartic proteinases
SDA	Sabouraud Dextrose Agar
TLC	Thin Layer Chromatography

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

*Candida*species are the cause of infections that range from non-life threatening mucocutaneous illness to invasive conditions which may result to destruction of vital organs (Umme *et al*., 2015).*Candida albicans* is the most common human fungal pathogen (Christina *et al*., 2016). It is a member of the healthy microbiota, asymptomatically colonizing the gastrointestinal (GI) tract, reproductive tract, oral cavity, and skin of most humans (Achkar and Fries, 2010: Kumamoto, 2011).

Candida species are now recognized as major agents of hospital-acquired infection. Their emergence as important nosocomial pathogens is related to specific risk factors associated with modern medical procedures, notably the use of immunosuppressive and cytotoxic drugs, powerful antibiotics that suppress the normal bacterial flora, and implanted devices of various kinds (Maki and Tambyah, 2000). *Candida* species are also frequently identified as agents of nosocomial pneumonias andurinary tract infections. Almost invariably, an implanted device such as an intravascular or urinary catheter, or endotracheal tube, is associated with these infections and a biofilm can be detected on the surface of the device (Crump and Collignon, 2000). Other devices totally implanted into the body, such as prosthetic heart valves, cardiac pacemakers and joint can also be contaminated by polymicrobial biofilms containing *Candida* species (Van der Mei *et al.*, 2000). Non-device-related infections, too, can involvebiofilms; these include *Candida* endocarditis and *Candida* vaginitis (Donlan and Costerton, 2002).

One of the main factors complicating antifungal therapy in an increasing number of patients is the ability of fungal cells to form biofilms (Ramage *et al*., 2006). *Candida albicans* produces highly structured biofilms composed of multiple cell types which include round, budding yeast-form cells; oval pseudohyphal cells; and elongated, cylindrical hyphal cells encased in an extracellular matrix (Ramage *et al*., 2005: Fox and Nobile, 2012).Biofilm is a structured microbial community attached to a surface and encased within a self-produced extracellular matrix (Donlan, 2002). It is well established that biofilms are the predominant form of microbial growth in nature, and during infection (Ramage *et al*., 2009). It is a community of adherent cells with properties that are distinct from those of free-floating (planktonic) cells (Bachmann *et al*., 2002). Among the pathogenic fungi, *Candida* spp. are the most frequently associated with biofilm formation (Ramage *et al*., 2005). Different types of biomaterials often used in the clinics support colonization and biofilm formation by *Candida*, most notably intravascular catheters (Crump and Collignon, 2000: Kojic and Darouiche, 2004).Candidiasis associated with intravenous lines and bioprosthetic devices are problematic, since these devices can act as substrates for biofilm growth (Donlan, 2002). The presence of biofilms can result in serious problems due to their resistance to antimicrobial agents(Kruppa, 2009). This resistance is developed by the presence of quorum-sensing molecules that play an important role in the biofilm formation and virulence, based on the local density of the fungal population present for the construction and dissolution of biofilm communities (Kruppa, 2009;Deveau and Hogan, 2011). The hyphae in biofilms contribute to the overall architectural stability, acting as a support scaffold for yeast

cells and other hyphae. Thus, the ability to form hyphae and the ability of these hyphae to adhere to one another and to yeast cells are critical for normal biofilm development and maintenance (Nobile and Mitchell, 2005).

There has been an alarming increase in the number of resistant pathogens due to indiscriminate use of antibiotics and inherent ability of microorganisms to evolve in order to subvert drug targets, induce immune skewness or form biofilm resistance (Ashutosh *et al*., 2017).

The primary molecular targets for antifungal agents are mostly lanosterole-14 α-demethylase and 3β-glucan synthase. Other targets include molecules involved in cell wall synthesis, plasma membrane synthesis, fungal DNA and protein synthesis, cellular function-related, and virulence factors(Friggeri *et al*., 2019).

Newer strategies combining the use of quorum inhibitors, nanoparticles, biofilm disrupters and antibiotics to simultaneously target various aspects of microbial physiology can possibly attenuate their survival (Zhang,2014). Drug treatment normally kills planktonic cells and the majority of biofilm cells. However, drug tolerant persisters repopulate the biofilm, disseminate into planktonic forms and start a new cycle of biofilm development (Keren *et al*., 2004; Lewis, 2010; Zhang, 2014). However, targeting biofilms could prove to be the masterstroke required for tackling a wide spectrum of microorganisms, particularly those involved in pathogenesis in humans. A multi-targeted strategy against biofilm formation could limit resilience of commonly observed hospital infections (Ashutosh *et al*., 2017).

It was estimated that 11.8% of the Nigerian population suffer from a serious fungal infection each year with over 960,000 estimated to be affected, with substantial mortality (Oladele and Denning, 2014).The high morbidity and mortality from fungal infection is mostly due to delayed diagnosis and treatment, the development of resistance and the severity of the disease (Silke *et al*., 2015).

Rita and Denning (2014) reported that studies on the epidemiology of fungal infection in Nigeria indicated about 1.5M Nigerian women with recurrent vaginal thrush and that *Tinea capitis* occurs in greater than 20% of school age children.

Plants have been a source of medicine in the past centuries and hence scientists and the general public recognize their value as a source of new or complimentary medicinal products (Premanath and Lakshmideri, 2010).There has been much concern on medicinal plants to reduce the adverse effects of various infections due to the resistance acquired by some microorganisms to some synthetic drugs and one of such plants is *Acalypha wilkesiana*(Ikewuchi *et al*., 2010).

Acalypha wilkesiana belongs to the Euphorbiaceae Family. Its common name is Copper leaf. ‘’Jan ganye ‘’ in Hausa, ‘’Jinwinini’’ in Igbo and ‘’kandiri’’ in Yoruba. Ithas been known to be used in most part of Nigeria to treat fungal and bacterial infections especially in the northern and western parts of the country (Oladele and Denning, 2014).Phytochemical screening of *Acalypha wilkesiana*showed the presence of some bioactive components including tannins and phenolics, saponins, alkaloids, phlobatannins and cardiac glycosides(Imaobong and Uwakmfon, 2019). Several plants which are rich in phenolic compounds and alkaloids have been shown to possess anti-

microbial activities against a number of microorganisms (Awe and Eme, 2014).The potent antifungal activity of *A. wilkesiana* extracts is due to presence of Ellagitannins especially corilagin and geranin (Mohamed *et al*., 2015).

Oyelami, *et al*. (2003) evaluated the safety and efficiency of *Acalypha wilkesiana* ointment against mycological infection. The ointment successfully controlled the mycosis in 73.3% of the affected patients. They concluded that *Acalypha wilkesiana* ointment can be used to treat superficial mycosis. Akinyemi, *et al*. (2006) evaluated crude extracts from six important medicinal plants which included *Acalypha wilkesiana* and *Ocimum gratissimum*, to find activity against methicillin resistant *Staphylococcus aureus* (MRSA). This study provided scientific support for the use of *Acalypha wilkesiana* and other leaves against MRSA based diseases.

Adesina *et al.*(2000) reported the presence of gallic acid, collagin, geranin, quercentin, 3-0-rutinoside and kaempferol in the leaves of *A. wilkesiana.* In another study, Iyekowa *et al*. (2016) reported thatthe aqueous extract of *A. wilkisiena* displayed activity against *S. aureus, Escherichia coli, P. aeruginosa and C. albican*s.

1.2 Statement of Research Problem

The increasing incidence of life-threatening fungal infections has driven the search for new, broad-spectrum fungicidal agents that can be used for treatment and prophylaxis in neonates and immunocompromised patients. Fungal infectionsare most often caused by

the pathogenic *Candida* species, particularly C. *albicans*.Majority of fungal infections are associated with biofilm formation (Ramage *et al*., 2009). The presence of biofilms can result in serious problems due to their resistance to antimicrobial agents and it has been shown that the cells that detach from the biofilm have a greater association with mortality than equivalent planktonic yeasts (Gordon *et al*., 2012).Antifungal treatments are limited due to host toxicity and fungal resistance (Nathan, 2017).

1.3 Justification

Studies on fungal biofilms provide new opportunities for development of novel antifungal agents.The ability of fungi to form hyphae and biofilms are important virulence factors since they enhance tissue penetration and restrict antibiotics penetrability to tissues. *Acalypha wilkesiana* has been shown to inhibit the growth of microorganisms even those that were resistant to some synthetic antibiotics such as *A. niger*.The associated increase in fungal infections intensified the search for new, safer, and more efficacious agents to combat serious fungal infections.

The isolation of corilagin from *Acalyphawilkesiana* as antimycotic components on planktonic cells has been reported but to the best of our knowledge, no work has been reported on its antibiofilm effects and mode of action.

1.4 Aim and Objectives

1.4.1 Aim

The aim of this research was to determine the antibiofilm effects of partially purified fractions of ethylacetate extract of *Acalypha wilkesiana* leaves on *Candida albicans*I, II and *C. parapsilosis.*

1.4.2 Specific Objectives

The specific objectives were to:

i. determine the median inhibitory concentration (IC_{50}), minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of n-hexane, ethylacetate, methanol and aqueous crude extracts of leaves of *Acalypha wilkesiana* on the planktonic cells of the clinical isolates of*Candida* spp.

ii. determine the penetrability concentration and median adhered cells eradication concentration of n-hexane, ethylacetate, methanol and aqueous crude extracts of leaves of *Acalypha wilkesiana* on the adhered cells of the *Candida* spp.

iii. use bioassay guided fractionation to isolate and partially purify the most potent extract

iv. examine the antibiofilm effect of the purified component on morphology of the *Candida albicans* biofilm using scanning electron microscopy**.**

v. determine the mode of action ofthe purified antibiofilm componenton cell wall and membraneof*Candida albicans.*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Candida*Species

Candida species are frequently found in the normal microbiota of humans; this facilitates their encounter with most implanted biomaterials and host surfaces. The major complication associated with the use of medical implants such as catheters or prostheses is infection. By 1963, approximately five medically important species of *Candida*had been described. The species were C. *albicans,* C. *stellatoidea* (which is nowconsidered synonymous with C. *albicans),* C. *parapsilosis,* C. *tropicalis,* and C.*guilliermondii* (Emmons *et al.,* 1963). However, the appearance in the 1960s ofnew modalities to treat cancer, increasing use of central venous catheters, increasesin average life expectancy, and other developments in medicine resulted in aparallel increase in the number of serious *Candida* infections. There are now atleast 17 species of *Candida* that have been shown to cause disease in humans(Rinaldi, 1993).

However, pathogenic *Candida* species are opportunistic pathogens capable ofinflicting disease in immunocompromised persons. The principal pathogen of the genus, C. *albicans,* can grow either asoval, budding yeasts or as continous septate hyphae, and both morphological formsare usually seen in infected tissues. Disease states include; common, superficialinfections, especially those of the mouth and vaginal, to rarer, but frequently fatal,deep-seated infections (Adam *et al*., 2002).

2.1.1 *Candida albicans*

*Candida albicans*is a fungus which may develop into a number of differentmorphological forms, including yeasts, hyphae and pseudohyphae. This changedepends on environmental conditions. The ability to form hyphae is generallyconsidered to be an important virulence factor since hyphae are able to penetratetissue more easily. A widely distributed yeast species thatis closely related to *C. albicans*has been identified(Coleman *et al.,* 1997). This newly describedspecies was isolated from oropharyngeal lesions in HIV -positive patients in Dublin. Infact, C. *dubliniensis* was initially difficult to distinguish from C. *albicans* and was often misidentified as such in standard clinical laboratory examinationsbecause both species are closely related and share phenotypic and genotypiccharacteristics (Odds *et al.,* 1998; Sullivan and Coleman, 1998; McCullough *et al.*,1999).The pseudohyphal form of *Candida albicans* is the developmental state that has series of conjoined elongated yeast cell that have obvious constrictions at septal sites.Yeast cells are the default cell morphology of *Candida albicans*. In this form they are round or oval, have a unicellular morphology and can be involved in biofilm formation. They can be toxic or remain symbiotic in blood, and maintain symbiosis in the oral cavity, skin, and vagina (Antinori , 2016).

2.1.2 *Candidaglabrata*

C. *glabrata* was considered a relatively non-pathogenic saprophyte of thenormal flora of healthy persons, rarely causing serious infection in humans (Haley,1961; Stenderup and Pedersen, 1962). However, following the widespread andincreased use of immunosuppressive drugs, together with broad-spectrumantimycotic therapy, the frequency of infections caused by C. *glabrata*increased significantly (Komshian *et*

al., 1989; Hitchcock *et al.*, 1993; Knoke *etal.*, 1997). C. *glabrata* is now the second or third most common cause of candidosis after C. *albicans* (Wingard, 1995). It is a non-dimorphic yeast that existsas small blastoconidia (yeasts) under all environmental conditions. Infact, C.*glabrata* is the only *Candida* species that does not form pseudohyphae attemperatures above 37°C.

2.1.3 *Candidakrusei*

Candidakrusei is an emerging *non-albicansCandida* species, with a particular predilection for neutropenic adult cancer patients(Wingard, 1995).*C. krusei* has been recognized as a potentially multidrug-resistant (MDR) fungal pathogen, because to its intrinsic fluconazole resistance and decreased susceptibility to both flucytosine and amphotericin B.*C. krusei* structural polysaccharides chitin and β-1,3-glucan are localized underneath other cell wall components like seen in other *Candida* species this impairs the proper sensing of these polysaccharides by the host immunity (Navarro *et al*., 2019).Unlike *C. glabrata, Candidakrusei* forms pseudohyphae with elongated blastoconidia, creating acrossed matchsticks or treelike appearance.

2.1.4 *Candida parapsilosis*

C. *parapsilosis* forms blastoconidia, singly or in small clusters, which areseen under a microscope along the pseudohyphae. This organism is identifiable bythe crooked or curved appearance of relatively short pseudohyphae and theoccasional presence of large hyphal elements called giant cells (Davis *et al*., 2007). Theavailable data suggest that the role of C. *parapsilosis* as an exogenous pathogen results from its introduction through contaminated intravenous fluids or biomaterials.The numbers of infections due to this organism are accordingly on the increase(Weems, 1992; Branchini *et al.*, 1994; Price *et al.*, 1994).

2.1.5 *Candida tropicalis*

C. *tropicalis* forms blastoconidia singly, or in very small groups, all alongthe pseudohyphae. True hyphae may also be present. Also, a few teardrop-shapedchlamydospores maybe produced irregularly (Davis *et al*., 2007). C. *tropicalis* is one ofthe three most commonly isolated *non-albicans Candida* species. It accounts for 4to 25% of all *Candida* species isolated and a higher proportion (approximately 20-45%) of *non-albicans Candida* species isolated from patients with candidemia(Fraser *et al.,*1992; Viscoli *et al.*, 1999).

2.2 Antifungal Agents

There are three classes of antifungal agents namely azoles, polyenes and echinocandins. They constitute the mainstay of antifungal therapy for patients with life-threatening invasive fungal infection (Odds *et al*., 2003).

2.2.1 Polyenes

Polyenes particularly Amphotericin B, remained the 'gold standard' of antifungal therapy for decades (Min *et al*., 2017). The efficacy of polyenes is severely limited by their intrinsic toxicity, particularly nephrotoxicity, but also infusion-related toxicity. These amphipathic compounds bind ergosterol, the principal sterol in the fungal membrane, and create pores that compromise membrane integrity causing leakage of cellular contents and death (Ostrosky *et al*., 2010).

2.2.2 Azoles

Azoles were first developed in the 1980s, and are the largest class of antifungal agents used today in clinical medicine (Kaila and Andrew, 2016). They include not only some topical agents but also systemic triazoles. Azoles are fungistatic drugs that inhibit ergosterol biosynthesis by targeting 14-α-lanosterol demethylase, a

cytochrome P-450 enzyme which catalyzes a key step in the ergosterol biosynthetic pathway (Odds *et al*., 2003: Ostrosky *et al*., 2010). In contrast to fluconazole, whose activity is mostly limited to yeasts, itraconazole, voriconazole and posaconazole display good activity against filamentous fungi (Pfaller, 2012). The improved safety profile compared to Amphotericin B impelled the use of azoles for a variety of therapeutic indications. However, the major problem of azoles is the emergence of resistance (White *et al*., 1998: Sanglard and Odds, 2002).

2.2.3 Echinocandins

The echinocandins (caspofungin, micafungin and anidulafungin) are actually the first class of antifungal drugs that act against a specific component of the fungal organisms which is the cell wall not present in mammalian (host) cells (Odds *et al*., 2003).These are a group of semisynthetic lipopeptide antibiotics whose mechanism of action is specific and act as non-competitive inhibitor of 1,3 - D-glucan synthase complex. This enzyme is critical for the synthesis of the key structural glucan polymers of the fungal cell wall, and its inhibition leads to depletion of cell wall glucan and lysis of fungal cells (Perlin, 2011).Echinocandins display excellent fungicidal activity against most *Candida* spp. They are considered fungistatic against *Aspergillus* spp. and some other moulds, for which voriconazole and Amphotericin B are generally used as preferred therapeutic options(Pfaller, 2012). Echinocandins represent a welcome addition to the antifungal arsenal due to their excellent safety profile, through emergence of resistance has been observed despite their relatively recent introduction into the clinics(Wiederhold, 2008: Perlin, 2011)

2.2.4 Antimetabolites

Flucytosine is an antimetabolite; it enters fungal cells via cytosine permease where it is converted intracellularly to fluorouracil (5-FU), which acts as a false nucleoside, inhibiting synthesis of DNA and RNA.It is usually administered in combination with other antifungals such as amphotericin B or fluconazole due to the occurrence of resistance when used as alone (Pappas *et al*., 2009). It should be noted that mutations in the enzymes, which are responsible for the metabolism of flucytosine to the toxic molecules 5-fluorouracil and 5-fluorouridine monophosphate can also promote resistance to this antifungal drug (Nett and Andes, 2016).

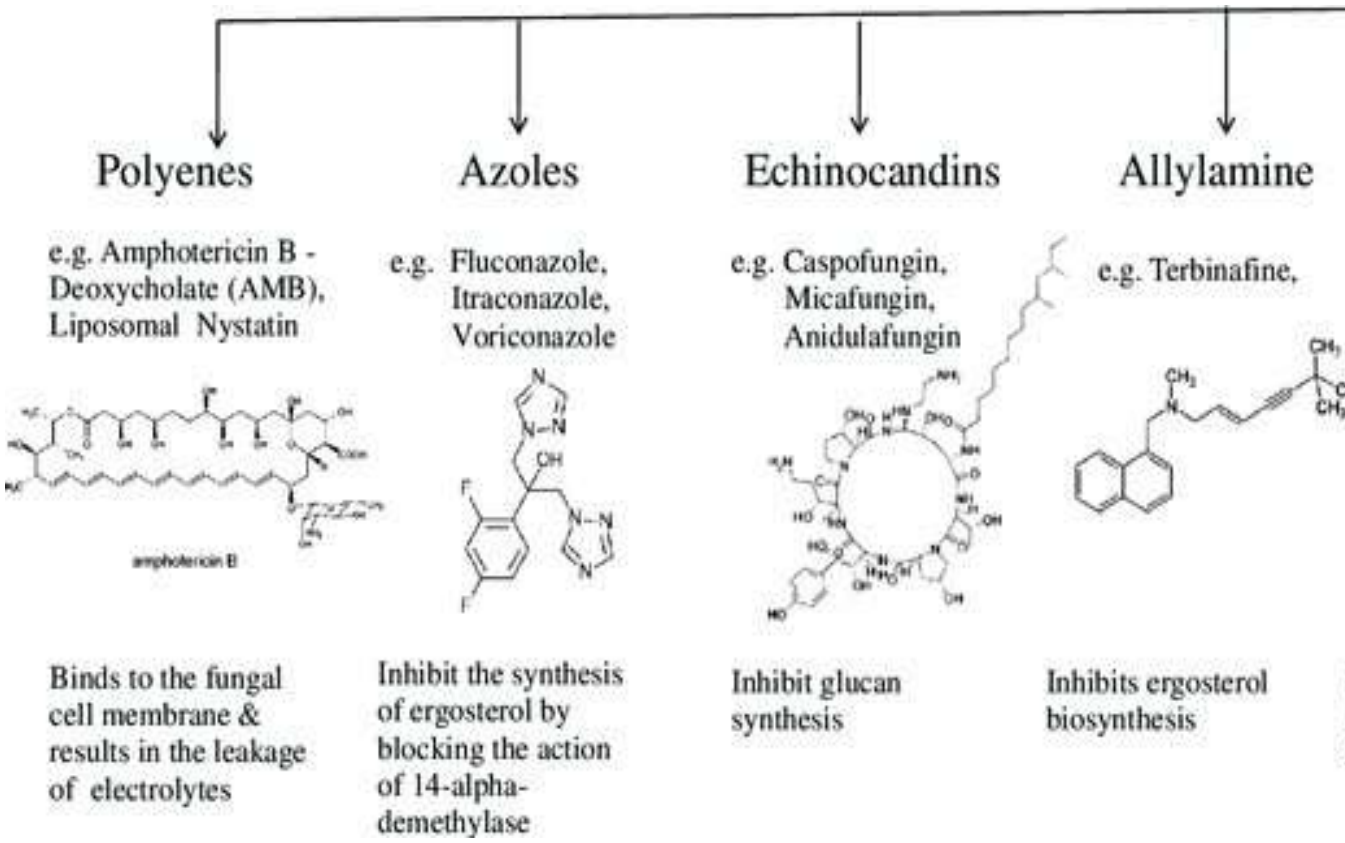


Figure 2.1: Antifungal Drugs and their Mode of Action

(Preetida *et al*., 2011).

2.3 Biosynthesis of Fungal Cell wall

Fungal cell walls are dynamic structures that are important for cell viability, morphogenesis, and pathogenesis. The wall is much more than the outer layer of the fungus. It is also a dynamic organelle whose components greatly affect the ecology of the fungus and whose composition is highly controlled in response to environmental conditions and imposed stresses (Latge, 2007). Cell walls are built to be both malleable and mechanically robust. The high total solute concentration inside fungal cells results in the osmotic uptake of water and the pressing of the cell membrane onto the wall. The cell wall is a valuable source of most diagnostic antigens that are used to detect human fungal diseases, and also a rich source of unique targets for chemotherapeutic treatment of pathogens (Neil *et al*.,2017).

2.3.1 Core polysaccharides: chitin and β-(1,3) glucan

The major synthases that make chitin and glucans reside in the plasma membrane (PM) and use UDP-sugars as the substrate for the formation of the nascent polysaccharide that is extruded into the fungal cell wall. In the cell wall, polysaccharides can then bond with hydrogen or be cross-linked by enzymes that reside in the cell wall (Figure 2.1) The pathway of cell wall synthesis therefore comprises biosynthetic reactions that take place inside the cell in the Golgi, at the PM, and in the cell wall itself.UDP-*N*-acetylglucosamine is the substrate for chitin biosynthesis. Chitin is composed of linear chains of β-(1,4) *N*-acetylglucosamine and represents the most ancestral structural polysaccharide in the fungal cell wall (Schorr*et al*., 2001).The cell wall is composed almost exclusively of molecules that are not represented in the human body yet are important or essential for fungal

pathogenesity as such, the cell wall is an ideal target for the design of antifungal drugs for clinical use. Nikkomycins and polyoxins are specific chitin synthase inhibitors of chitin synthases, and although they often potently inhibit enzyme activity in *in vitro* assays, they are not efficiently taken up *in vivo* and consequently are often not effective antifungals.

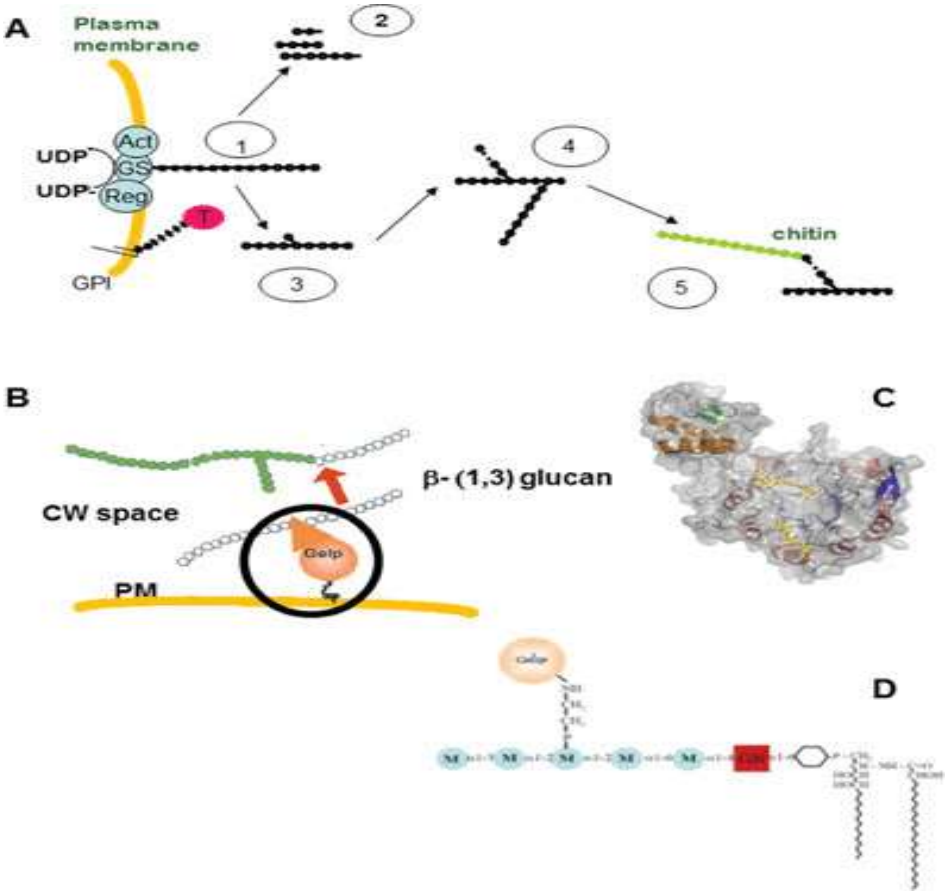


Figure 2.2: Biosynthesis of Fungal β-(1,3) Glucan

Source: Neil *et al.*, (2017).

2.4 Biofilms

Biofilms are multicellular structures formed by the attachment and aggregation of microorganisms followed by coating with a polysaccharide-rich extracellular matrix (Garrett *et al*., 2008). There are many advantages to an organism forming a biofilm, since these structures are more resistant to adverse environmental conditions compared to planktonic cells.

A biofilm is a community of adherent cells with properties that are distinct from those of free-floating (planktonic) cells (Krueger *et al*., 2004: Kolter and Greenerg, 2006). Although biofilms are often attached to solid surfaces, they can also be formedin other environments, for example, liquid to air interfaces. A near-universal characteristic of biofilms, compared to their free-floating counterparts, is the greater resistance of their cells to chemical and physical agents (Costerton, 1999).

2.4.1 Model biofilm systems

Various model systems have been used to characterize *Candida* biofilms. They include catheter disks, acrylic strips, microtitre plates, cylindrical cellulose filters, and the perfused biofilm fermenter (Baillie and Douglas, 1999; Douglas, 2003). Almost all of these model systems have been adapted from methods reported previously for bacteria. The simplest method, and the first to be described, involves growing adherent populations on the surfaces of small disks cut from catheters (Hawser and Douglas, 1994; Baillie and Douglas, 1999). A similar model system has been used to study the formation of biofilms on strips of denture acrylic (Nikawa *et al.,* 1996).

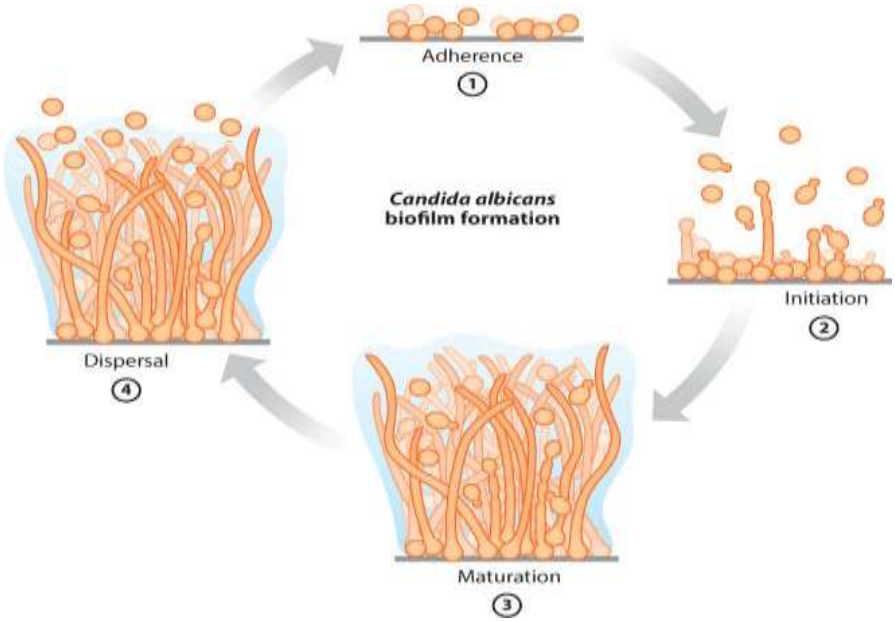


Figure 2.3:Stages of *Candida albicans* Biofilm Formation

Key

① Adherence of yeast-form cells to a surface. ② Initiation of cell proliferation, forming a basal layer of anchoring cells. ③ Maturation, including growth of hyphae concomitant with the production of extracellular matrix material. ④Dispersal of yeast-form cells from the biofilm to seed new sites.

Source: Clarissa and Alexander (2015).

2.4.2 Mechanisms of biofilm drug resistance

The mechanisms of biofilm resistance to antimicrobial agents are not fullyunderstood. At least four possible explanations for increased resistance of biofilmshave been proposed. These include: (l) phenotypic changes resulting from adecreased growth rate or nutrient limitation; (2) differential gene expression(induction of biofilm phenotype) including drug efflux pumps; (3) delayed orrestricted penetration of drug through the biofilm matrix; and (4) the existence of asmall number of 'persisters'

cells. It is now thought likely that a combination ofthese mechanisms operates in both bacterial and fungal biofilms(Chandra *et al.,* 2001).

2.4.2.1 Slow Growth Rate or Nutrient Limitation

Phenotypic changes conferring drug resistance could be induced by a slow growth rate or by nutrient limitation within the biofilm. Both growth rate and nutrient limitation are known to affect the cell surface composition ofmicroorganisms and hence perhaps their susceptibility to antimicrobial agents.Growth rate could therefore be an important modulator of drug activity in biofilms.Using a method of cell culture designed to study the effect of growth rateseparately from other biofilm processes, Evans *et al.* (1990a) found that the slowestgrowing *E. coli* cells in biofilm culture were the most resistant to the drug,cetrimide. However, biofilm and planktonic cells were equally susceptible to thedrug when exposed at growth rates higher than 0.3 generation per hour (Evans *etal.*, 1990a). Another study by the same group showed that drug susceptibilityincreased for both S. *epidermidis* biofilm and planktonic cultures with increases in growth rate(Evans *etal.,* 1990a). A separate study using the cylindrical cellulose filter model system (Baillieand Douglas, 1998) demonstrated that glucose-limited and iron-limited biofilmsof C. *albicans* grown at the same low rate were equally resistant to Amphotericin B.

However, daughter cells from iron-limited biofilms were significantly more susceptible to the drug than those from glucose-limited biofilms. An acute disseminated infection produced by the release of such cells from an implant biofilm might therefore respond rapidly to Amphotericin B but the biofilm would be unaffected.

2.4.2.2 Differential Gene Expression (Induction of Biofilm Phenotype)

A second possible explanation for biofilm drug resistance is that when microorganisms attach to a surface and form a biofilm there is an upregulation of genes which affect antimicrobial susceptibility. Altered gene expression by organisms within the biofilm can result in a phenotype with reduced susceptibility to an antimicrobial agent. Upregulation of genes coding for multi-drug efflux pumps would result in amultidrug-resistant phenotype. C. *albicans* possesses two different types of effluxpump: ATP-binding cassette (ABC) transporters and major facilitators, which areencoded by *Candida* drug resustance (CDR) and multi drug resistance (MDR) genes, respectively. A recent study demonstrated thatgenes encoding both types of efflux pump are up regulated during biofilm formationand development. However, mutants carrying single or double deletion mutationsin some of these genes were highly susceptible to fluconazole when growingplanktonically but still retained the resistant phenotype during biofilm growth(Ramage *et al.,* 2002).

Subsequent studies by Mukherjee *et al.* (2003) determined the antifungalsusceptibilities of biofilms developed by C. *albicans* mutants carrying single,double, or triple deletion mutations of the CDR and MDRI genes. These resultsshowed that at an early phase of biofilm development these mutants were moresusceptible to fluconazole than the wild-type strain. Interestingly, at later timepoints (12 and 48 h), all the strains became resistant to fluconazole. Theseobservations indicate a lack of involvement of efflux pumps in resistance at latestages of biofilm formation and suggest that multicomponent, phase-

specificmechanisms are effective in antifungal resistance of *Candida* biofilms (Mukherjee*et al., 2003).*

2.4.2.3 Delayed Penetration of Drug through the Biofilm Matrix

The production of matrix material, or glycocalyx, is one of thedistinguishing characteristics of biofilms. Undoubtedly, one of the most importantfunctions of the matrix is to provide the structural complexity and mechanicalstability of the biofilm. A number of other functions have been ascribed to thebiofilm matrix which includesprotection of the enveloped cells from antimicrobialagents (Allison, 2003). Baillie and Douglas (2000) compared the susceptibility of C.*albicans* biofilms grown statically (which have minimal matrix material) with thoseof biofilms incubated with gentle shaking (which produce much more matrixmaterial). The results of the study showed that the extent of matrix formation in*Candida* biofilms grown with or without shaking did not significantly affectbiofilm susceptibility to any of the clinically important antifungal agents tested,including Amphotericin B, flucytosine, and fluconazole (Baillie and Douglas,2000). However, other investigations with biofilms developed under flowconditions suggested that matrix material might play a minor role in biofilmresistance, since resuspended cells (which presumably had lost most of their matrixmaterial) were 20% less resistant to Amphotericin B than intact C. *albicans* biofilms(Baillie and Douglas, 1998a; Baillie and Douglas, 1998b). These findings withresuspended biofilm cells were subsequently confirmed elsewhere (Ramage *et al.*,2002b).Mixed species fungal-bacterial biofilms consisting of C. *albicans* and S.*epidermidis* have been investigated by Adam *et al.* (2002). Drugsusceptibility studies suggested that fungal cells can modulate the action

ofantibiotics, and that bacteria can affect antifungal activity in these mixed fungalbacterialbiofilms, possibily as a result of increased viscosity of the matrix. Overall,these results seem to indicate that the matrix material plays a partial role in fungalbiofilm resistance, but other factors are also likely to be involved.

2.4.2.4 Persister Cells

One of the newest hypotheses for the reduced susceptibility of biofilms to antimicrobials is the formation of a special class of protected subpopulation cells termed 'persisters' (Spoering and Lewis, 2001). Little is known about persisters, no doubt because of the technical difficulties of working with a small fraction of cells (usually 10^{-6}-10^{-4} of the population) expressing a temporary phenotype of uncertain functional significance (Lewis, 2001) and Brown *et al.* (2003) studied a dose-response killing of *P. aeruginosa* biofilms by the quinolones, ofloxacin and ciprofloxacin. The results proved that the majority of cells were effectively killed by low, clinically achievable concentrations of drug akin to the concentrations which are lethal to planktonic cultures.These experiments indicated that the presence of a small fraction ofpersister cells was primarily responsible for the very high level of resistance of thebiofilms (Brown *et aI.,* 2003). It has been suggested that persisters, and biofilmsthat contain them, display tolerance, which means that cells do not grow in the presence of antimicrobial agents; however, they do not die either. This capability toavoid being killed is one of the defining features of persisters (Keren *et al., 2004;*Cogan, 2006).

LaFleur *et al.* (2006) reported that biofilms formed by C. *albicans*exhibited a strikingly biphasic killing pattern in response to two antifungal agents.The results

indicated that a subpopulation of highly tolerant cells existed.Interestingly, surviving C. *albicans* persisters was only observed in biofilms andnot in planktonic exponentially growing, or stationary, populations. The authorsconcluded that C. *albicans* persisters are not mutants, but phenotypic variants ofthe wild type which switch on "persister" genes in response to the biofilm mode ofgrowth (LaFleur *et aI.*, 2006). Another recent study by Khot *et al.*(2006)demonstrated that C. *albicans* biofilms cultured in a tubular flow cell systemharboured a subpopulation of yeast cells that were significantly more resistant toAmphotericin B than planktonic populations. This subpopulation of yeast cellsformed a basal layer that was tenaciously attached to the surface of the tubing(Khot *et al., 2006).*

2.5 Fungal-bacterial Biofilms

Bacteria are often found with *Candida* species in polymicrobial biofilms *invivo,* and it is likely that extensive interspecies interactions take place in theseadherent populations (Adam *et al.,* 2002). In the oral cavity, *Candida* or other yeast species always co-exist with commensal bacteria within a biofilm matrix. *Invitro,* Larnfon *et al.* (2005) used denture acrylic (polymethylmethacrylate) discs to co-culture fungal-bacterial biofilms derived from denture plaque from denture stomatitis patients. They found that *Actinomyces, Lactobacillus, Streptococcus* and *Candida* species grew readily in the biofilm mode *in vitro* and suggested that the system could be used to assess the susceptibility of *Candida* species within these biofilms to antifungal agents (Larnfon *et al.,* 2005).

2.6 Virulence Factors for *Candida albicans* Infections

Generally, the organism most often responsible for *Candida* infections is *C.albicans*, and this is due to a number of virulence factors that contribute topathogenesis. These factors include morphogenesis (the reversible transitionbetween unicellular yeast cells and filamentous growth forms), adhesion (the hostrecognition biomolecules), and secreted aspartyl proteases and phospholipases(Calderone and Fonzi, 2001). Exactly and simply defined, a virulence factor is anyattribute that a fungus possesses which increases its virulence in the host orescalates binding of the organism to host cells.

2.6.1 Morphogenesis

The yeast-to-hyphal transition has been shown to be an important virulence attribute that enables C. *albicans* to invade human tissues (Brown and Gow, 1999). Many fungal pathogens of humans are dimorphic and undergo reversible morphogenetic transitions between budding, pseudohyphal and hyphal growth forms. *Candida* pseudohyphae range from relatively short to extended cells, and are microscopically distinguishable from true hyphae only by conspicuous constrictions of the pseudohyphae at septal junctions (Merson-Davies and Odds, 1989). Morphogenesis can be activated by a wide range of factors and signals, many of which connect signalling pathways within fungal cells. (Gow *et al.,* 2002*).* For example,akinase named CRK1 (in the Cdc2 subfamily of kinases) has also been shown to be essential for conversion of yeast to filamentous growth on serum-containing agar media (Chen *et al.,* 2000*).*

2.6.2 Adhesion

Microbial adherence to biomaterial surfaces or to host cells is seen as anessential early step and one of the most important determinants of pathogenesis.*Candida*

species can adhere to the surfaces of medical devices, particularlycatheters, and form biofilms which results in increased resistance to antifungaldrugs compared with planktonic *Candida* cells, and an increase in *Candidaemia*related to catheter insertion (Hawser and Douglas, 1994; Chandra *et al.*, 2001).

There is a correlation between the virulence of different *Candida* species and theability to form biofilms (Hawser and Douglas, 1994).Many studies have shown that several factors influence adhesion *in vitro.*For example, the composition of the growth medium can affect adhesion; yeastsgrown in medium containing 500mM galactose or glucose are significantly moreadherent to denture acrylic or to buccal epithelial cells than organisms grown in a low concentration (50mM) of glucose (McCourtie and Douglas, 1981; McCourtienand Douglas, 1984). Increased adhesion appears to be due to enhanced production of a surface mannoprotein that binds to fucose-containing glycolipids in epithelial cell membranes (Cameron and Douglas, 1996).

2.6.3 Hydrolytic enzymes

To assist in the invasion of host tissues, many pathogenic microbes possess constitutive and inducible hydrolytic enzymes that destroy, alter, or damage membrane integrity, leading to dysfunction or disruption of host cells. Pathogenic species of *Candida* produce a large variety of secreted hydrolases, among which are the secreted aspartic proteinases (Sap) and phospholipases (PL) have beenintensively investigated (Calderone and Ponzi, 2001; Haynes, 2001)

2.6.3.1 Aspartic Proteinases

Medically important yeasts of the genus *Candida* have the ability to secreteaspartic proteinases (Saps) extracellularly, which are of particular interest asvirulence factors. This characteristic is shared by C. *albicans*, C. *dubliniesis*, C.*tropicalis* and C. *parapsilosis.* The enzymes produced by these yeasts are allcarboxyl proteinases capable of degrading human proteins such as albumin,hemoglobin, keratin, and secretory immunoglobulin A (Hube *et al.,* 1998*).*There are at least ten proteins that comprise the Sap family.*In vivo* studies show that SAPgenes 1, 2 and 3 are expressed by yeast cellsonly, whereas SAP 4, 5 and 6 expressions have been detected in *C.albicans*undergoing a transition from yeast to hyphae at neutral pH (Schaller *et al.*, 1999).The expression of SAP 7 has never been detected under any growth conditions.SAP 8 transcripts have been detected in yeast cells grown at 25°C in a definedmedium and SAP 9 is preferentially expressed in later growth phases (Monod *et al.,*1998). Like C. *albicans* and C. *tropicalis* secrete*in vitro* Sap activity in a mediumcontaining bovine serum albumin as the only source of nitrogen (Symersky *et al.,* 1997).

2.6.3.2 Phospholipases and Lipases

The role of extracellular phospholipase (PL) as a potential virulence factor has been investigated in several pathogenic fungi, including *C.albicans,Cryptococcus neoformans,* and *Aspergillus fumigatus* (Ghannoum, 2000). According to the different and specific ester bond cleaved, these enzymes have been classified into four PLs so far (A, B, C and D). Only PLB activity has been demonstrated in *C. albicans.* This is

an 84-kDa glycoprotein that has both hydrolase (fatty acid release) and lysophospholipase trans acylase activities (Mukherjee *et al*., 2003). PL activity has also been observed with *C. glabrata, C. parasilosis, C. tropicalis, C. krusei* and *C. lusitaniae* (Ghannoum, 2000). Studies by Dagdeviren *et al.* (2005) demonstrated a correlation between adherence-phospholipase and adherence-aspartic proteinase properties of *C.parasilosis* strains; PL production appeared to be an important virulence factor in bloodstream infections caused by *C. parasilosis.* In a more recent study, 61 isolates of *Candida* recovered from HIV and cancer patients were investigated for PL Sap activity. This was more obvious in *C. albicans* isolates, with 100% PL and 94.1 % Sap activity. In contrast, non-*C. albicans* species showed only 29.6% PL and 70.3% Sap activity, indicating a relationship between other virulence determinants in these yeasts with colonization and disease (Kumar *et al.,* 2006). Secreted hydrolytic lipases and esterases are also thought to be important as virulence factors in bacteria and fungi. These enzymes are characterized by their ability to catalyse both the hydrolysis and synthesis of ester bonds of mono-, di and triacylglycerols or even phospholipids (Schaller *et al.,* 2005).

2.7 Biofilm Matrix and its Composition

A characteristic feature of biofilms is the production of an extracellular matrix that envelops the attached cells. This is generally composed of water and microbial macromolecules and provides a complex group of micro environments surrounding the microorganisms (Sutherland, 2001b). Inaddition, the matrix contains a range of enzymic and regulatory activities. The matrix architecture is based upon a combination of intrinsic factors such as the genotype of the attachedcells and a

number of extrinsic factors, including fluctuations in nutrient and gaseous levels and fluid shear. Together, these intrinsic and extrinsic factors combine to produce a dynamic, heterogeneous microenvironment for the attached and enveloped cells (Allison, 2003). Microbial polymers such as exopolysaccharides, proteins, nucleic acids and various other components are invariably in biofilms. However, water is by far the main component of the biofilm matrix, accounting for up to 97% of the mass (Sutherland, 2001a). Some polysaccharides carry hydrophobic groups which may be involved in hydrophobic interactions when adhering to hydrophobic interfaces (Neu *et al.,* 1992). Additionally, many matrix materials possess backbone structures that contain sequences of 1,3- or l,4-p-linked hexose residues that are likely to be more rigid in structure, and in some cases less soluble or even insoluble (Sutherland, 2001). Moreover, in the presence of ions, the extracellular polymeric material shows increased viscosity or gelation (Loaec *et al.,* 1997). Generally, matrix polymer synthesis is dependent on the availability of nutrients such as carbon, nitrogen, potassium, or phosphate.

2.8 Human Infections Involving *Candida* Biofilms

This involves common diseases associated with biofilm colonization which include biofilms attached to tissues in different organs of the human body and device-associated infections where the biofilm is attached to some sort of prosthesis placed within the body.

2.8.1Native valve endocarditis

Native valve endocarditis is a condition that results from the interaction between the vascular endothelium, generally of the mitral, aortic, tricuspid, and pulmonic valves of the heart, and bacteria or fungi circulating in the bloodstream (Livomese and

Korzeniowski, 1992). An early study by Tunkel and Mandell (1992) noted that of 2,300 cases of infective endocarditis, 56% were caused by streptococci (including viridans streptococci, enterococci, pneumococci, and S. *bovis),* 25% by staphylococci, and the balance by Gram-negative bacteria and fungi including *Candida* and *Aspergillus* species. These organisms gain access to the bloodstream, primarily via the oropharynx, gastrointestinal tract, and genitourinary tract. Fungal endocarditis is a serious condition that affects particular groups of patients and is associated with considerable morbidity and mortality. The incidence of this disease has increased during the past 2 decades, and fungi now account for 1 -10% of organisms isolated in patients with infective endocarditis (Giamarellou, 2002).

2.8.2 Implant-associated infections

Medical devices are responsible for a large proportion of nosocomialinfections. Device-associated infections can cause major medical and economicsequelae. Moreover, the pathogenesis of device-related infection results from thecomplicated interaction of microorganism, device, and host factors. Microbialfactors are probably the most important in the pathogenesis of infection, whereasdevice factors are the most amenable to modification with the objective ofpreventing infection (Bums, 2000; Darouiche, 2001; Donlan, 2001). Biofilmsformed by bacteria on various medical devices have been studied extensively overthe last 20 years. Until recently, less attention has been focused on the formation offungal biofilms. However, *Candida* species are now recognized as importantnosocomial pathogens, and an

implanted device with a detectable biofilm isfrequently associated with these infections (Douglas, 2003).

2.8.3 Central venous catheters

It was noted by Maki (1994) that central venous catheters pose a greater risk ofdevice-related infection than does any other indwelling medical device, withinfection rates of 3 to 5%. Colonization and biofilm formation may occur within three (3)days of catheterization (Anaissie *et al.,* 1995). Analysis of National NosocomialInfections Surveillance data shows that 87% of primary bloodstream infectionsoccur in patients with a central line (Mermel *et al.,* 2001). A separate study byRichards *et al.* (2000) found that a total of 72 to 87% of bloodstream infections,including *Candidaemia,* are catheter related in intensive care unit patients. *C.albicans* accounts for up to 63% of all cases of *Candidaemias,* followed by *C.glabrata* or, in some hospitals, C. *tropicalis* (Fraser *et al.,* 1992). A recent study (Yapar *et al.,* 2006) has demonstrated a progressive increase in the frequency of nosocomial *Candidaemia,* especially among critically ill or immunocompromised patients. The results showed that *C. albicans* was the most common species (57.7%) and *non-albicans* species accounted for (42.3%) of all episodes. The most common *non-albicans Candida* species isolated was *C.tropicalis* (20.2%) followed by *C. parapsilosis* (12.5%).

2.8.4 Joint prostheses

In modem medicine, the most commonly implanted joint prostheses are hip and knee prostheses. The incidence of infection is low: 1 % in primary cases and up to 3% in

secondary procedures. However, *Candida* accounts for less than 1 % of all cases ofinfections of joint prostheses (Stocks and Janssen, 2000).

2.8.5 Dialysis access

The number of persons with a dialysis access continues to rise. Infection is the second most common cause of death in patients with end-stage renal disease. Additionally, infection is the most frequent cause of hospitalization and is the leading cause of morbidity and mortality in patients requiring dialysis (Cheung and Wong, 2001). Fungal infections of hemodialysis access sites are rare. However, current reports indicate that *Candida* accounts for 2.6 to 7% of peritoneal dialysis related infections (Lew and Kaveh, 2000; Vas and Oreopoulos, 2001). Moreover, *non-albicans Candida* species account for up to two-thirds of *Candida* isolates. C.*parapsilosis* seems to be the most prevalent non-C. *albicans* species. This infectious complication is associated with a high mortality and morbidity, including prolonged hospital stay and recourse to hemodialysis (Asim *et al.,* 1999; Prasad and Gupta, 2005).

2.8.6 Central nervous system devices

Most recently used ventriculoperitoneal shunts are produced from siliconepolymers. Obstruction and infection are the two most common complicationsoccurring in patients exposed to these devices. *Candida* is the causative agent in 1 % ofsuch infections and the mortality rate was estimated to be 9%. Central nervous system shunt infection is a cause of significant morbidity, causing malfunction and chronic ill health, and a few cases may even turn fatal for example, nosocomial meningitis can occur in association with central nervous system (CNS) devices such as cerebrospinal

shunts or drains, intrathecal pumps, and deep brain stimulators(Sanchez-portocarrero *etal.,* 1994*).*

2.9 Metabolic Impacts of*Candida* Immunogenicity

Metabolism is integral to the pathogenicity of *Candida albicans*.It provides the platform for nutrient assimilation and growth in diverse host niches, metabolic adaptation affects the susceptibility of *C. albicans* to host-imposed stresses and antifungal drugs, the expression of key virulence factors, and fungal vulnerability to innate immune defences. These effects are driven by complex regulatory networks linking metabolism, morphogenesis, stress adaptation and cell wall remodeling.

2.9.1 Adaptation of *Candida albicans* to the host

The need for fungal pathogens to survive, multiply and assimilate nutrients require the flexibility to adapt to environmental change. In the long term this has depended on the evolution of mechanisms that permit this flexibility. The outcome for the host, although being of importance to thatindividual, is of secondary importance to the fungal pathogen. Following dissemination to a new host, a fungal cell try to assimilate local nutrients, counter any local environmental stresses, and, if possible, evade any local host defences. Infection outcome depends on the physiological robustness of the fungal pathogen within host niches as well as on the efficacy of host defences in these niches (Alistair*et al*., 2014).

2.9.2 Fungal stress adaptation

Metabolism also promotes the virulence of *C. albicans* indirectly by enhancing stress adaptation. Stress resistance is required for *C. albicans* virulence: it increases the survival of fungal cells in host niches by reducing their vulnerability to local environmental stresses and to phagocytic killing. Metabolism contributes to stress

adaptation by generating molecules such as the osmolyte glycerol, antioxidants such as glutathione, and the stressprotectant trehalose (Gow and Hube, 2012). Therefore, the ability of *C. albicans* cells to respond to environmental stress is likely to depend upon the preadapted metabolic state of these cells, and hence upon available nutrients in host microenvironments.

The analysis of *C. albicans* stress responses has been carried out on cells cultured on rich, glucose-containing media that differ significantly from host microenvironments which are often glucose-limited. Significantly, recent data indicated that changes in carbon source exert dramatic effects upon the stress resistance of *C. albicans*(Gow and Hube, 2012).

Carbon adaptation affects resistance to osmotic stress and antifungal drugs (Ene, 2012). These effects are mediated partly throughPKA signaling and may relate to the effects of metabolic adaptation upon the cellular abundances of osmolytes such as glycerol, and antioxidants such as glutathione and trehalose. Glycerol and trehalose are synthesised via short metabolic branches off the glycolytic pathway. However, these effects also involve *C. albicans* cell wall remodelling (Alistair*et al*., 2014).

2.9.3 Modulation by endogenous tryptophan

Metabolic modulation of immune responses is provided by endogenous tryptophan catabolism in the GI mucosa, which promotes IL-22 production by innate lymphoid cells, this in turn enhances intestinal immunity and protection against *C. albicans*. Therefore, the local metabolic environment of the host contributes, together with the metabolic adaptation of *C. albicans*, to the efficacy or failure of local immune surveillance mechanisms (Zelante, 2013).

Recent studies have shown that glycolysis and succinate play key roles in modulating the capacity of the innate immune system to mount a proper inflammatory response (Tannahill, 2013).

2.10 Selected African Plants with Antifungal Properties

The medicinal and antimicrobial activities of extracts from plants are gaining attention of researchers worldwide.Medinal plants are rich sourcesof antimicrobial agents. They are a source of powerful potent drugs (Srivastava *et al.*, 1996). Plants are known to contain numerous phytochemicals which are defined as secondary, or specialized, metabolites(Zhao*et al*., 2013). Among the best-known classes of plant secondary metabolites are terpenoids, phenylpropanoids and alkaloids (Chen *et al.*, 200: Zhao *et al*., 2013). Plant secondary metabolites have numerous biological and ecological functions which include defense against insect herbivores and pathogenic microorganisms and for establishment of mutualistic interactions (Griffins *et al.*, 2015).

With increasing number of fungal resistant to various antifungal drugs, there has been several attempts to use plants with antifungal potentials against resistance microbial strain that are of clinical importance (Jeff-Agboola and Awe, 2016).

2.10.1 *Allium sativum* (Garlic)

Garlic is a plant with known antimicrobial, anti-inflammatory, anti-thrombotic and antitumor activities. Previous *in vitro* studies indicated the effects of garlic extract on the inhibition of the growth in a large number of yeasts including *Candida* spp., some fungi such as *Coccidioides immitis*, and also dermato-phytic fungi *T. rubrum*, *T.*

mentagrophytes, T. verrucosum, Microsporum canis and *Epidermophyton flocossum*(Aala *et al*., 2010).

A sulphur-containing compound in garlic, known as di-allyl thiosulfinate (allicin), is the active component in inhibition of the growth of fungi and bacteria. Fresh aqueous extract of garlic showed antifungal activity specifically against some *Aspergillus* spp. including *A.fumigates, A.terreus, A. nidulans, and A. niger*(Pai and Patt, 1995).The inhibitory effects of allicin against *Trichophyton* spp were more pronounced than those of the essential oils isolated from other plants (Pyun and Shin, 2006).

2.10.2 *Moringa oleifera* (Drumstick Tree)

Moringa oleifera known as the is a medicina plant that has been reported to be safer and more compatible alternative to synthetic fungicides.All parts of the plant including roots, flowers, bark, stem, leaves, seeds andessential oils possess antimicrobial properties and are therefore being used for medicinal and other purposes (Dwivedi and Enespa, 2012). Chemical analysis of these

Morphological parts showed a profile of important minerals, vitamins, protein and a large quantity of phytochemical compounds that have biological activity and can potentially be used to retard the effects of microorganisms (*Arora et al*., 2013). Recent reports on phytochemical analysis of the *M. oleifera* leaf revealed the presence of various antioxidant compounds like ascorbic acid, fatty acids and phenolic acids .Other compounds reported present in M. oleifera includes quercetin, zeatin, β-sitosterol, kaempferol, caffeoylquinic acid (Ahmadu*et al*.,2021).

2.10.3 *Magnifera indica* (Mango)

Mango is known to possess antioxidant, antiallergenic, and anti-inflammatory properties (Ajila *et al*., 2010). Phytochemical isolated from mango (*Mangifera indica L.*) leaves, at various concentrations, have been reported to have significant growth suppression on five fungal namely *Alternaria alternata (Fr.)* , *Keisslers Aspergillus, fumigatus Fresenius, Aspergillus niger, van Tieghem,* Macrophomina phaseolina and Penicillium citrii(Hussain et al., 2010). Five flavonoids, namely (−)-epicatechin-3-O-β-glucopyranoside,5-hydroxy-3-pyrano, taxifolin-7-O-β-D-glucoside, quercetin-3-O-α-glucopyranosyl-(1 → 2)-β-D-glucopyranoside , epicatechin and 4-dihydro-2H-chromene-3,5,7-triol were isolated from the leaves of mango (*Mangifera indica* L.) and the evaluation of these compounds against fungal cells indicated significant antifungal activities (Qudsia *et al*., 2010).

2.10.4 *Acalypha wilkesiana*(Copper Leaf)

Acalypha wilkesiana is commonly known as copper leaf, Joseph coat, fire dragon and beef steak. It is found all over the world most especially in the tropics of Africa, America and Asia. *Acalypha wilkesiana* is often used in traditional medicine either exclusively or as a main constituent of many herbal preparations for the management of hypertension (WHO, 1991).The expressed juice or boiled decoction is used for the management of gastrointestinal disorders and fungal infections such as *Cadida intetrigo, Tinea vericolor*and *Tinea Pedis.*In southern *Nigeria,* the leaves of *A. wilkesiana* are eaten as vegetables in the management of hypertension (Ikewuchi *et al*., 2010).Previous studies on *Acalypha wilkesiana* revealed that it has antifungal activity (Oyelami *et al*., 2003).

Phytochemical analysis of the ethanol leaf extracts of *A. wilkesiana* revealed a high presence of tannins and glycoside, a moderate presence of saponin, flavonoids, Phylobatanins and glycosides (reducing sugar) and slight presence of alkaloids and cardiac glycosides (Awe *et al*., 2013). *Acalypha wilkesiana* juice or boiled decoction was reported to be used in the treatment of gastrointestinal disorders, hypertension, malaria and fungal skin infection such as *pityriasis versicolar, impetigo contagiosa, candida intetrigo, Tinea versicolor, Tinea corporis* and *Tinea pedis*(Imaobong and Uwakmfon, 2019).

Plate I: *Acalypha wilkesiana* Leaves

Source: (Iyekowa *et al*, 2016).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemical and Reagents

Morphiline sulphonic acid, Ergosterol, Sorbitol, Caspofungin, Amphotericin B, Voriconazole were purchased from Sigma Aldrich. Potato Dextrose Agar (PDA), Sabaraud Dextrose broth, Chloramphenicol Capsules, Sample Bottles, Human Serum, Lactophenol Cotton Blue, API 20 C Aux Kit (Biomerieux SA France), McFarland Standard Scales 0.5 and 2, apiweb^{TM} Identification software (Ref. 40 011).Paper concentration discs (6mm). All other chemicals used were of analytical grade except where otherwise stated.

3.1.2 Collection and authentication of plant material

Fresh leaves of *Acalypha wilkesiana*were collected from the garden of National Ear Care Center, Golf Course Road, City Centre, Kaduna at Latitude: 10^{o} $31^{/}10.73^{//}$and Longitude: $7^{o}26^{/}$ $25.85^{//}$ (Geoview.info).Samples of *A. wilkesiana*were authenticated in the herbarium section of the Department of Botany, Faculty of Life Sciences,

Ahmadu Bello University, Zaria. The voucher number (02805) of the plant was deposited.

3.1.3 Clinical isolates: Four(4)clinicalisolates of *Candida species*wereobtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital (ABUTH), Shika.

3.1.4 Ethical clearance: Ethical clearance certificate was granted by the Health Research Ethics Committee (HREC) of Ahmadu Bello University Teaching Hospital Shika with an assigned number of ABUTHZ/ HREC/AO1/2017 dated 30th April, 2018.

3.2 Methods

3.2.1 Determination of Minimum Inhibitory Concentration (MIC), Minimum Fungicidal Concentration (MFC) and Median Inhibitory Concentration (IC_{50}), of Crude Extracts of *A.wilkesiana* Leaves against Planktonic Cells of Clinical Isolates of *Candida* spp.

3.2.1.1 Preparation of crude extracts

The leaves of *Acalayphawilkesiana*were air dried and pulverized into uniform powder using mortar and pestle. Four (4) solvents with increasing polarity (n- Hexane, ethylacetate, methanol and water) were used for extracting the phytochemicals from the leaves of *A. wilkesiana.* Successive extraction using cold maceration method was used as described by Handa *et al*., (2008). Two Hundred grams (200g) of the pulverized leaves of *A. wilkesiana*was soaked in 1.5 L of n-hexane for 48hours in a conical flask after which the mixture was filtered through Whatman No. 1 filter paper. The filtrate was concentrated at 40°C using rotary evaporator to obtain constant dry weight of the extract. The remaining plant residue from n-hexane was air dried and soaked in 1500ml of ethylacetate, the same procedure for n-hexane was

applied for the ethylacetate, methanol and aqueous extraction. Percentage yield of each extract was calculated using the following formular,

Yield (%) = $W_1 / W_2 \times 100$

Where: W_1 = weight of extract after evaporation of solvent and W_2 = dry weight of sample (powder).

3.2.1.2 Qualitative phytochemical screening of extracts of Acalypha wilkesiana leaves

Test for flavonoids and tannins were carried out as described byTrease and Evans, (2002). Test for Alkaloids was carried out according to the method of Sofowora (1993) while phlobotannins and saponins were determined as described byEdeoga (2005).

Test for the presence of phenolicswas determined as described by Tamilselvi *et al*. (2012). Test for the presence of cardiac glycoside was determined as described by Ayoola *et al*. (2008).

3.2.1.3 Quantitative assays for phenolics, flavonoids and alkaloids

Total phenolic content was determined using Folin-Ciocalteu (FC) reagent (Mallick and Singh, 1980). The plant extract (0.5 mg) was mixed with 0.5 ml of FC reagent (1:1 diluted with distilled water) followed by addition of 2 ml of 20% Na_2CO_3after five (5) minutes.After ninety (90) minutes, the absorbance of the mixture was measured at 650 nm. The total phenolic content (mg equivalent of garlic acid) was calculated using gallic acid as standard.

Total flavonoid content was determined using a modified colorimetric method described by Vabkova and Neugebauerova (2012). The assay mixture consisting of

0.5 mg of the plant extract, 0.5 ml distilled water, and 0.3 ml of 5% $NaNO_2$ was incubated for 5 min at 25°C. This was followed by addition of 0.3 ml of 10% $AlCl_3$. Two milliliters (2ml) of 1 M NaOH was then added to the reaction mixture, and absorbance measured at 510 nm. Quercetin was used as a standard.

Total alkaloid content was determined according to UV-Spectrophotometric method described by Manjunath*et al*., (2012). This method is based on the reaction between alkaloid and bromocresol green. The plant extract (0.5 mg) was dissolved in 2 ml of 2 N HCl and then filtered. Exactly 1.0 ml of this solution was transferred to a separatory funnel and washed with 10 ml chloroform. The pH of phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. One ml (1ml) of this solution was transferred to a separating funnel and then 5 ml of bromocresol solution along with 5 ml of phosphate buffer (7.0) was added. The mixture was shaken and the complex formed was fractioned with chloroform by vigorous shaking. The fractions were collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. Atropine was used as standard.

3.2.1.4 Collection andidentification of clinical isolates of Candida

Collection of Candida Isolates

Four (4) isolates were collected, three (3) of which were of High Vaginal Swab (HVS) and one (1) was of Intra cervical swab (ICS). Sterilized wire loop was used to pick different colonies from four (4) prepared plates that were obtained from different patients and streaked on their respective sabaraud dextrose agar (SDA) media.

Identification of Clinical Isolates of Candida

(i) **Gram staining**

The cell wall of yeast don't contain lipid layer unlike bacteria. When a decolorizer is added, the crystal violet taken up by yeast cells is retained. Therefore, Yeast cells give a positive gram's reaction.

Procedure

Gram staining was carried out according to the method described by Moyes *et al.*, 2009. A drop of sterilized distilled water was added on the surface of the slides and, a colony of clinical isolate of *candida* was fixed to the surface of the microscope slide using sterilized wire loop. The slide was washed with 2% crystal violet which stained all cells blue / purple and then rinsed with distilled water. Iodine solution (mordant) was added and allowed for 1 minute to form a crystal violet iodine (CV-I) complex and rinsed with distilled water. The slide was the washed with acetone and allowed to stay for 2 seconds and then rinsed with distilled water. The slide was then rinsed with red dye safranin stain which decolorized gram-negative cells red/pink while gram-positive cells remain blue. The slides were viewed using light microscope (x100).

(ii) Germ tube Test

Germ tube is one of the virulence factors test for *Candida albicans.* Germ tube test is a rapid test for the identification of *C. albicans.*

Principle: Formation of germ tube is associated with increased synthesis of protein and ribonucleic acid. Protrusion of germ tube indicate positive test for the presence of *Candida* specie.

Procedure

Exactly 0.5 ml of human serum was pipetted into a small tube using a Pasteur pipette, a colony of yeast was gently emulsified in the serum. The tube was incubated at 37°C for 2 hours. A drop of the serum was transferred to a slide which was covered with cover slip for examination. The slides were examined microscopically under low (×40) and high-power objectives (×100).Short hyphal (filamentous) extension arising laterally from a yeast cell, with no constriction at the point of origin indicates a positive test for*Candida albicans* and *Candida dubliniensiswhile*no hyphal (filamentous) extension arising from a yeast cell or a short hyphal extension constricted at the point of origin indicate positive test for *non albican speciesincluding C. parapsilosis.*

(iii) Biochemical Characterization of the Clinical Isolates

Principle

The API 20 C AUX strip consists of 20 cupules containing dehydrated substrates which enable the performance of 19 assimilation tests.The API 20 C Aux is based on 19 carbohydrate assimilation tests plus a negative control, read by assessing cupules for turbidity. The cupules are inoculated with a semi-solid minimal medium. The yeast will only grow if they are capable of utilizing each substrate as the sole carbon source. The reactions are read by comparing them to growth controls. Identification is obtained by referring to the analytical profile index or using the identification software (API web CANDIDA version 2.1)

Procedure

The incubation box (tray and lid) was prepared and 5ml of distilled water was distributed into the honeycombed wells of the tray to create a humid atmosphere. The strips were placed in the incubation tray. A sterilized wire loop was used to pick up a portion of the *Candida* colony from 24 hours culture by successive touches into 2ml of 0.85% NaCl in a sterilized bottle, the suspension was prepared with turbidity equal to 2 McFarland. An ampoule of API Medium was opened and 100µl of the previous suspension was transferred into it and gently mixed with the pipette tip.

The cupules were filled with the suspension obtained in the ampule of API C medium. To avoid the formation of bubbles, the tip of the pipette was placed against the side of the cupule. The lid was placed on the tray and incubated at room temperature for 48 and 72 hours. The incubation box was observed for changes in turbidity and a 7 code profile index was generated for each sample using the result sheet provided in the API C Aux kit at 48 hours and 72 hours incubation, respectively. The characterization was achieved by inserting the 7 code profile generated on the APIweb Identification software.

3.2.1.5 Determination of minimum inhibitory concentration (MIC)

Minimum Inhibitory Concentration (MIC) was determined by broth dilution method as described by Takahagi *et al*. (2009). Ethylacetate, n-hexane extracts, voriconazole, Amphotericin B and Caspofunginwere reconstituted with dimethyl sulfoxide, while methanol and aqueous extracts were reconstituted with sterile water. Sterile plastic microtiter plates containing flat-bottomed wells were utilized (Corning Costar). In each well, 100 µL of saboraud dextrose broth was added,two-fold serial dilutions of the extracts and antifungal drugs were added. Drug-free medium wells were

employed to provide sterility and growth controls. McFarland (0.5) of *C.albicans* (I and II) and *C.parapsilosis* were prepared. A 100-µL aliquot of each was added to each well of the microdilution plate. Final concentrations of Corilagin, Voriconazole,Amphotericin B, and Caspofungin were serially diluted from 0.01mg/ml to 0.00125mg/ml and100 µL were added to the corresponding wells. Extracts were also serially diluted from 100mg/ml to 12.56mg/ml and 100µL were also added to the corresponding wells. The microdilution plates were incubated at ambient temperature for 24 hours and absorbance was taken after the incubation period at 630nm using microtiter plate reader.

3.2.1.6 Determination of minimum fungicidal concentration (MFC)

Minimum Fungicidal Concentrationof the crude extracts was determined as described by Russell and Furr (1977). This method involves sub culturing from plates that showed no growth. These agar portions were transferred into petridishes containing freshly prepared Sabouraud Dextrose Agar (SDA). The plates were incubated at 25-27°C for 2days and observed daily for growth. The absence of growth at the end of incubation period signifies total cell death. The minimum concentration of the plant extracts that produces total cell death is taken as the MFC (Siddiqui *et al*., 2013).

3.2.1.7 Determination of median inhibitory concentration (IC_{50})

Median Inhibitory concentration was determined by microdilution method.The procedure is the same with that for MIC.

The following equation was used for calculations of cell inhibition IC_{50} as described by Jessica *et al*., 2013

% cell inhibition = 100- [(At- Ab)/ (Ac- Ab)] ×100 Where,

At=Absorbance value of test compound

Ab= Absorbance value of blank

Ac= Absorbance value of control.

3.2.2 Median adhered cells eradication concentration and adhered cells penetrability of n-hexane, ethylacetate, methanol and aqueous crude extracts of leaves of *Acalypha wilkesiana*on the biofilms of the isolated *Candida spp*.

3.2.2.1Quantification of Adhered Cells formed from the characterized clinical isolates of Candida albicans I, II and C. parapsilosis

Quantificationof adhered cells formed by the three (3) *Candida* strains wascarried out by microdilution method as described by Ramage *et al*. (2005). For each strain, 100µl fungal suspension of 0.5 McFarland standard (1×10^8cfu/ml) serially diluted to 1×10^2cfu/ml was inoculated into individual wells of polystyrene 96 well plates (flat bottom) containing 100µl of sabaraud dextrose (SD) broth containing 0.05M glucose and 1% bovine serum albumin. The process was performed three (3) times for each strain. The plates were incubated at 37^oC for 90 minutes (adhesion period). Supernatant including planktonic cells and liquid medium was then discarded and wells were gently washed twice with phosphate buffered saline (PBS) to get rid of any non-adherent cells and then incubated for 24, 48 and 72 hours.After the required incubation time for each well, an aliquot of 100 µl of 2% crystal violet was added to each well and incubated for 20 minutes at 37^oC. Then, 150µl 95% ethanol was added to dissolve the dyed adhered cells and 100 µl of each mixture was transferred to a

new 96 well microtitre plate. The absorbance for each well was determined using microplate reader at 630nm. Wells containing only SD broth without the fungal cells were used as negative control.

Calculations

Adhq= At – Ac

where;

Adhq= quantity of adhered cells formed

At= Absorbance of test

Ac= Absorbance of control (negative control, containing no cells)

3.2.2.2 Determination of Median Adhered CellsEradication Concentration of the Extracts and Drugs on C. albicans I, II and C. parapsilosis

Median adhered cells eradicationconcentrationwas determinedby microdilution method as described by Djordjevic*et al*. (2002). A 100 µl aliquot of SD broth was added to each welland 100 µl cell suspension (0.5 McFarland standards) of each of the three strains of *Candida* was added to the corresponding wellsand incubated for 2 h at 37^{o}C for initial adherence. After incubation, supernatant was discarded and wells washed twice with 200µl PB andfollowed by addition 100µl of nitrogen base broth (6.7 g / L) for biofilm growth. Exactly 100 µl of the extracts corresponding to MIC, 2 MIC and 4 MIC were added to the corresponding plates. The plates were, incubated for 48 h at 37^{o}C. For biofilm quantification, the wells were washed twice with PBS, air-dried for 45 min, and dyed with 0.4% crystal violet aqueous solution. Absorbance values were read at 630 nm using a micro plate reader. Corilagin, Amphotericin B,Voriconazole and caspofungin were used as positive controls and untreated biofilm served as the growth control.

Calculation :

Percentage Adhered Cells Eradication = {A630 nm experimental well / A630nm control well} x 100] (Jadhav *et al.*, 2013).

3.2.2.3 Determination ofPenetrability of n-hexane, Ethylacetate, Methanol and Aqueous Extracts of Acalypha wilkesianaLeaves on Adhered Cells of Candida albicans

Polycarbonate membrane filters (diameter, 25 mm; pore size 0.2 mm; Whatman) were sterilized by exposure to UV radiation for 15 min on both sides prior to inoculation with cell suspension and then placed on the surface of Sabouraud DextroseAgar containing 0.1% Bovine serum albumin. Exactly 50 µl of standardized cell suspension (0.5 Mcfarland Standard) was applied to the surface of each sterile membrane. All plates were incubated at 37°C for 24 h. The membrane-supported adhered cells were then transferred to fresh nitrogen base agar for a further 24 h, giving a total incubation time of 48 h (Mohammed and Julia, 2004).The crude extracts were reconstituted immediately before use and then added tomolten culture medium at 50°C by use of a Pasteur pipette tocreate antifungal agent-supplemented agar for the adhered cells experiments. Corillagin, voriconazole, caspofungin and amphotericin B were used as reference drugs. The medium was buffered to pH 7 with 0.165 Mmorpholinepropanesulfonic acid buffer (Sigma). Penetration of antifungal agents through adhered cellswas assessed by filter disk technique described for bacterial biofilms ((Ander *et al*., 2002).After formation of adhered cells on membrane filters, smaller polycarbonate membranefilters (diameter, 13 mm; pore size, 0.2 m:Whatman) weresterilized by exposureto UV radiation for 15 min on both sides and then carefully placed ontop of the 48 hours old adhered cells. Paper concentration

disks (diameter, 6 mm; BectonDickinson) was also sterilized by exposure to UV radiation for 15 min per sideand then moistened with growth medium prior to placement ontop of the 13-mm-diameter membranes. Adhered cells sandwichedbetween the membranes and a moistened disk was transferred to antifungalagent-containing agar medium. All plates were incubated for specified exposuretimes (60, 90, 120, 180, 240 and 360 min).The amount of antifungal agent which had penetrated each adhered cell and whichhad reached the concentration disk was determined by using the disk in astandard drug diffusion assay.

Plates of SDA containing 200 mM glucosewas seeded with 150µL of standardized suspension of planktonic *C. albicans*(as an indicator organism andwas adjusted to an optical densityat 520 nm of 1.0). After the appropriate exposure time, concentration disks wereremoved from the biofilm "sandwiches" and placed on the seeded plates, which wasthen incubated at 37°C for 24 h. The zones of growth inhibition weremeasured and used to determine the concentration of active antifungal agent inthe disks by reference to a standard curve prepared by using extracts and drug solutions ofdifferent concentrations but fixed volumes. All drug penetration assays werecarried out in duplicate on at least two separate occasions. In control assays,concentration diskswere placed on the two-membrane system to which no cellshad been added, that is the unit without the adhered cells. The drug concentration thatpenetrated the adhered cells(C)was divided by the drug concentration determinedfor the controls (Co) to provide a normalized penetration curve (Ander *et al*., 2002).

Equations

Diameter zone of inhibition (DZI) plotted against known concentration of the drugs or extracts to give a standard curve.

C/Co plotted against the specified exposure time (60, 90, 120, 180, 240 and 360 min) gives the concentration of the drugs and extracts that penetrated through the biofilms.

The concentration (C) is calculated from the regression equation of the standard curve.

3.2.3 Fractionation and partialpurification of the most potent extract of *Acalypha wilkesiana* leaves with antibiofilm activity

Two (2) solvent systemswere used to fractionate the ethylacetate extract as the most potent extract using column chromatography as described by Davies and Johnson (2007). Silica gel was used as the adsorbent. Exactly 50g of silica was made into slurry with ethylacetateand placed in a cylindrical tube that is plugged at the bottom by a piece of glass wool using a funnel.The ethylacetate extract was reconstituted in ethylacetate. A pipette was used to add the sample to the top of the column. The stopcork was opened and the solvent was allowed to drain.Avery small amount of solvent was used to wash down any sample that may have clung to the sides of the column. This additional solvent was also drained. To elute the sample through the column: a pipette was used to add 4 ml of theethylacetate. Afunnel was placed at the top of the column and the remainder of the column was filled with solvent. The stopcork was opened and the solvent was allowed to drain through the column. The mobile phase was collected as it drained from the column into test tubes. Additional solvent with increasing polarity starting from absolute n-hexane (100%), n-hexane: Ethylacetate (4:1, 3:2, 2:3,1:4), and absolute ethylacetate (100%) were added to the top of the column as needed until all the desired compounds have eluted from the

column.The purity of the compounds was verified using thin layer chromatography (TLC).The fractions were evaporated to dryness by using rotary evaporator at low temperature of 39°C and kept in air tight containers for further analysis. Each fraction was tested for antifungal activities and biofilm penetrability concentration(Ander *et al*., 2002: Djordjevic*et al*.,2002). The fraction with the best antibiofilm activitywas further purified using column chromatography and TLCin a two solvent system with increase in polarity starting from absolute chloroform (100%), chloroform : ethylacetate (4:1, 3:2, 2:3, 1:4)and absolute ethylacetate (100%). Pooled fractions were tested for antibiofilm activities. The fraction with the best antibiofilmeffect was also tested forantifungal activity on different isolates.

The formular used for calculating Retiontion factor (Rf) is given below:

Rf = Distance traveled by solute / Distance traveled by solvent

Preparative TLC was used to partially purify and isolate the most potent fraction in terms of antifungal activity and biofilm eradication.Preparative TLC revealed only one (1) band of G7 developed in ethylacetate: methanol (7:3).The TLC glass was scraped and the purified fraction was collected in a beaker and reconstituted using 98% ethylacetate, the silver gel was filtered and the solvent was evaporated and the purified fraction was isolated.

However, we attempted to identifythe isolated antifungal component using NMR spectroscopyat School of Chemistry and Physics, University of KwaZulu-Natal, Westville campus, Durban, South Africabut, perhaps we got an artifact.

3.2.3.1Determination of Minimum Inhibitory Concentration (MIC) And Minimum Fungicidal Concentration (MFC) of the Purified Antibiofilm Component using Microdilution Technique

The MIC is defined as the lowest concentration of the test substance inhibiting microbial growth. The Minimum Inhibitory Concentration (MIC) of the purified antifungal component againstclinical isolates of *Candida albicans*and *Candida parapsilosis*wasdetermined using the method described by Larissa *et al*. (2017).Microtiter plates with 96 U-bottom wells wereused and serial dilutions of the test substance, culture medium and fungal inoculum (2.5×10^3 CFU/ml, 630 nm, abs 0.08–0.1) wereadded to the plates. The plates were incubated for 24 h at 37 ºC, and the results were read by visual observation of cell aggregates at the bottom of the wells and taking readings by using microtitre plate reader at 600nm. Corillagin, Amphotericin B, voriconazole and caspofungin were used as positive control in the assays at concentrationsranging from (0.00125 to 0.01mg / ml).The Minimum Fungicidal Concentration (MFC) is defined as the lowest concentration inhibiting visible growth on solid medium (Rasooli and Abyaneh, 2004). The Minimum Fungicidal Concentration (MFC) of the extract with the best inhibitory activity was determined using the method of Russell and Furr (1977). This method involves sub culturing portions of the agar from plates that showed no growth in the tests for determination of MICs. These agar portionswere transferred into petridishes containing freshly prepared Sabouraud Dextrose Agar (SDA). These plates were incubated at 25-27°C for 2 days and observed daily for growth. The absence of growth at the end of incubation period signified total cell death. The minimum concentration of the plant extracts that produced total cell death was taken as the MFC (Siddiqui *et al*., 2013).

3.2.4 Microscopic Examination of Antibiofilm Activity of the Purified Antifungal Component using Scanning Electron Microscopy

Antibiofilm activity of the purified ethyl acetate extract of *Acalyphawilkesiana* leaveswas observedby scanning electron microscopy as described by Shafreen *et al*. (2014). Biofilms were formed on 24 well microtitre plates as earlier described (Larissa *et al*. (2017) and then the discs were fixed with 4.0% formaldehyde plus 2.0% glutaraldehyde. Then swab sticks were used to transfer the biofilms to glass slides from the 24-well microtitre plates and thesamples weredehydrated with a series of ethanol, coated with 0.05mM gold and examined on a JOEL-JSM5300 scanning electron microscope.

3.2.5 Determination of mode of action of purified antibiofilm principleon *Candida albicans*

*3.2.5.1*Sorbitol Assay

Microdilution technique was used to determine the mode of action of the antibiofilm principle on *C. albicans* cell wall as described byPerlin, (2011) and Pierce *et al*. (2013) in the presence of sorbitol (D-sorbitol anhydrous), an osmotic protector. In this assay, the inoculum was prepared with sorbitol at a concentration of 0.8 M. The plates wereincubated at 37ºC, and readings were performed 24 h and 48 h after incubation (Escalante*et al*., 2008;Lima *et al*., 2013; Freires *et al*., 2014). Caspofungin was used as a positive control in this assay at an initial concentration of 0.05 mg/mL due to its known activity on the yeast cell wall.The IC_{50}in the presence of sorbitolis

defined as the concentration where the response is reduced by half. $IC_{50}$was carried out as described by Jessica *et al*. (2013).

The following equation was used for calculations of cell inhibition:

% cell inhibition = 100- [(At- Ab)/ (Ac- Ab)] ×100

Where: At=Absorbance value of test compound

Ab= Absorbance value of blank

Ac= Absorbance value of control.

3.2.5.2 Ergosterol Assay

The assay was performed using the microdilution technique, as earlier described, in the presence of exogenous ergosterol at concentration 200 mg/mL. The plates were incubated at 37ºC, and readings were carried out after (24 and 48) h (Escalante *et al*., 2008;Lima *et al*., 2013; Freires *et al*., 2014). Amphotericin B was used as a positive control due to its known activity on the yeast cell membranes by binding to membrane sterols and thus changing membrane permeability (Perlin, 2011; Pierce *et al*., 2013). A control with 96% ethanol and Tween 80, which was used for preparation of ergosterol solutions, was also included. The IC_{50} in the presence of ergosterol isdefined as the concentration where the response is reduced by half.

The following equation was used for calculations of cell inhibition IC_{50} as described by Jessica *et al*., 2013

% cell inhibition = 100- [(At- Ab)/ (Ac- Ab)] ×100

Where: At=Absorbance value of test compound

Ab= Absorbance value of blank

Ac= Absorbance value of control.

3.3 Statistical Analysis

The results were expressed as mean± SD. Tests were performed in triplicate. Significant differences were analyzed between groups using one-way analysis of variance (ANOVA). Post-hoc mean separations was performed by Turkey- Kramer Multiple Test atp<0.05.

CHAPTER FOUR

4.0 RESULTS

4.1 Minimum Inhibitory Concentration (MIC), Minimum Fungicidal Concentration (MFC) and Median Inhibitory Concentration (IC_{50}), of the Extractson Planktonic Cells of the Clinical Isolates of *Candida* spp.

4.1.1 Some phytochemicals and their concentrations in aqueous, methanol, ethylacetate and n- hexane crude extracts of *Acalypha wilkesiana* leaves

Table 4.1 shows that the n-hexane, ethylacetate, methanol and aqueous extracts of *Acalypha wilkesiana* leaves contain flavonoids, tannins, alkaloids, phlobotannins, phenolics and saponins while all the extracts showed negative results for cardiac glycoside. Ethylacetate extract of *Acalypha wilkesiana*leaves contains more phlobatannins than the other extracts. Table 4.2 shows that methanol extract had higher amount of total phenolic content (255.5 $\pm$0.51mg equivalent of garlic acid) which was significantly different ($p \leq 0.05$) from the n-hexane, ethylacetateaqueous

extractsof *Acalypha wilkesiana* leaves. The flavonoid content of methanol extract was 398.0 ± 0.25 and aqueous extract had 300.5 ± 0.08 mg equivalent of quercetin. The flavonoid content of n-hexane was 169.0±0.40and ethylacetate had 398±0.25mg equivalent of quarcetine. The methanol extract was shown (Table 2) to have the highest flavonoid content 175.5±0.20mg equivalent of quarcetine. The alkaloidscontent in methanol extract was 333.6 ± 0.20, n-hexane was 86.0 ±0.21, ethylacetate had 151.1 ± 0.03, and the aqueous extract had 259.6 ± 0.03mg equivalent of atropine.

4.1.2 Identification of clinical Isolates of *Candida*

Colonies were formed which retained the crystal violet indicating the presence of *candida*spp. This confirms that all the samples as shown in plates II, IV, VI and VIII were positive to gram staining. Plates III, V and VII were positive togerm tube test as shown to have short hyphal (filamentous) extensions arising laterally from the yeast cell with no constriction at the point of origin, while plate IX showed less sprouting. Table 4.3 shows the biochemical characterization of the *Candida* isolates. The two (2) High vagina swab samples were identified to be and *C. albicans* II (88.1%) and *C.albicans* I (98.1%).The intracervical swab sample was identified to be *Candida parapsilosis*(98.2%).

Table 4.1: Some Phytochemicals Detected in the Aqueous, Methanol, Ethyl Acetate and n- Hexane Extracts of *Acalypha wilkesiana* Leaves

Extract	Flavonoids	Tannins	Alkaloids	Phlobatannins	Phenolics	Cardiac glycoside	Saponins
n-Hexane	+	+	+	+	+	-	+
Ethylacetate	+	+	+	++	+ +	-	+
Methanol	+	+	+ +	+	+ +	-	+
Aqueous	+	+	+ +	+	+	-	+

+ and++ show presence of phytochemicals but ++ are in higher concentration while - shows absence of phytochemical.

Table 4. 2 :Concentrations of some Phytochemicals in Aqueous, Methanol, Ethyl Acetate and n- Hexane Extracts of *Acalypha wilkesiana* Leaves

Extract	**% Yield**	**Total Phenol** mg/ Equivalent of galic acid	**Total Flavonoids** mg/ Equivalent of quercetin	**Total Alkaloids** mg/ Equivalent of atropine
n-Hexane	2.01	114.9 ± 0.31^{a}	169.0 ± 0.40^{a}	86.0 ± 0.2^{a}
Ethylacetate	1.70	159.8 ± 0.02^{b}	175.5 ± 0.20^{a}	151.1 ± 0.03^{b}
Methanol	9.10	255.5 ± 0.51^{c}	398.0 ± 0.25^{b}	333.6 ± 0.20^{c}
Aqueous	13.45	186.5 ± 0.68^{b}	300.5 ± 0.08^{b}	259.6 ± 0.03^{d}

Values are means ± SD of Triplicate Determination. Different superscripts down the columns are significantly different ($p \leq 0.05$).

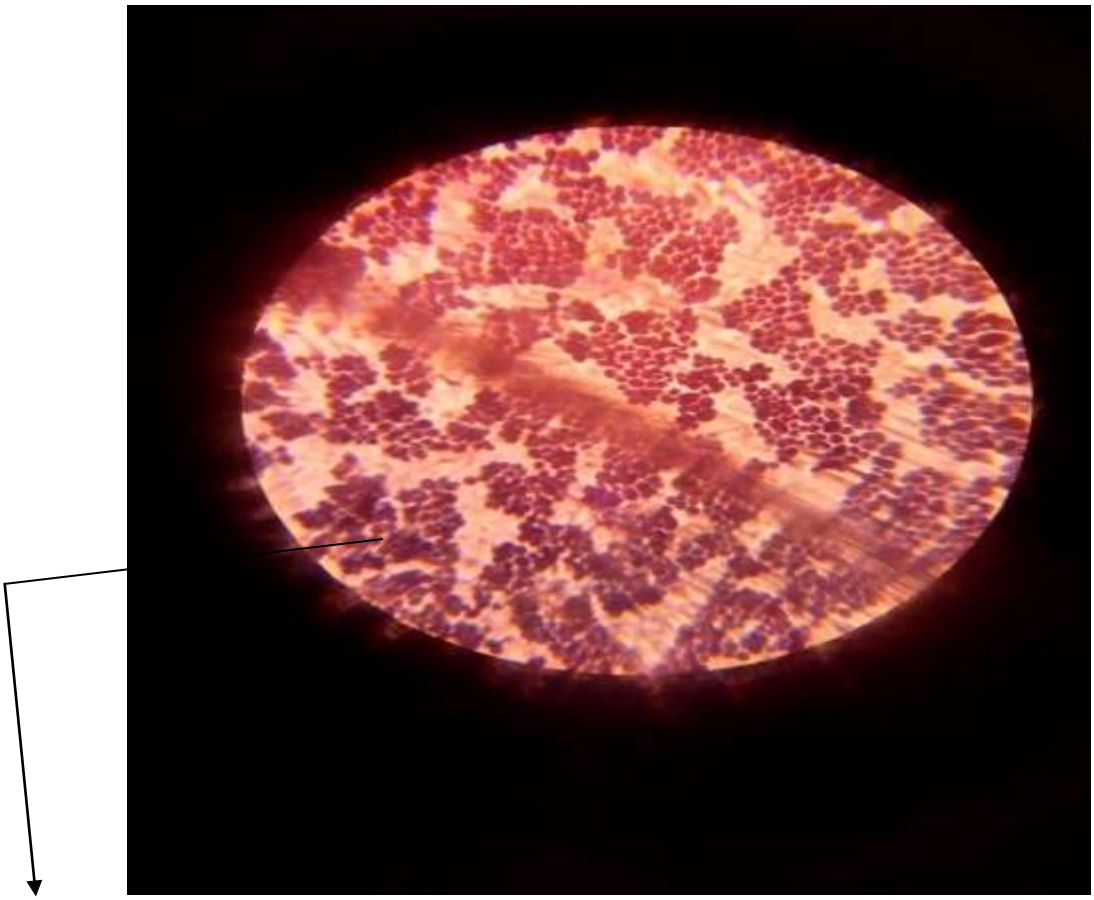
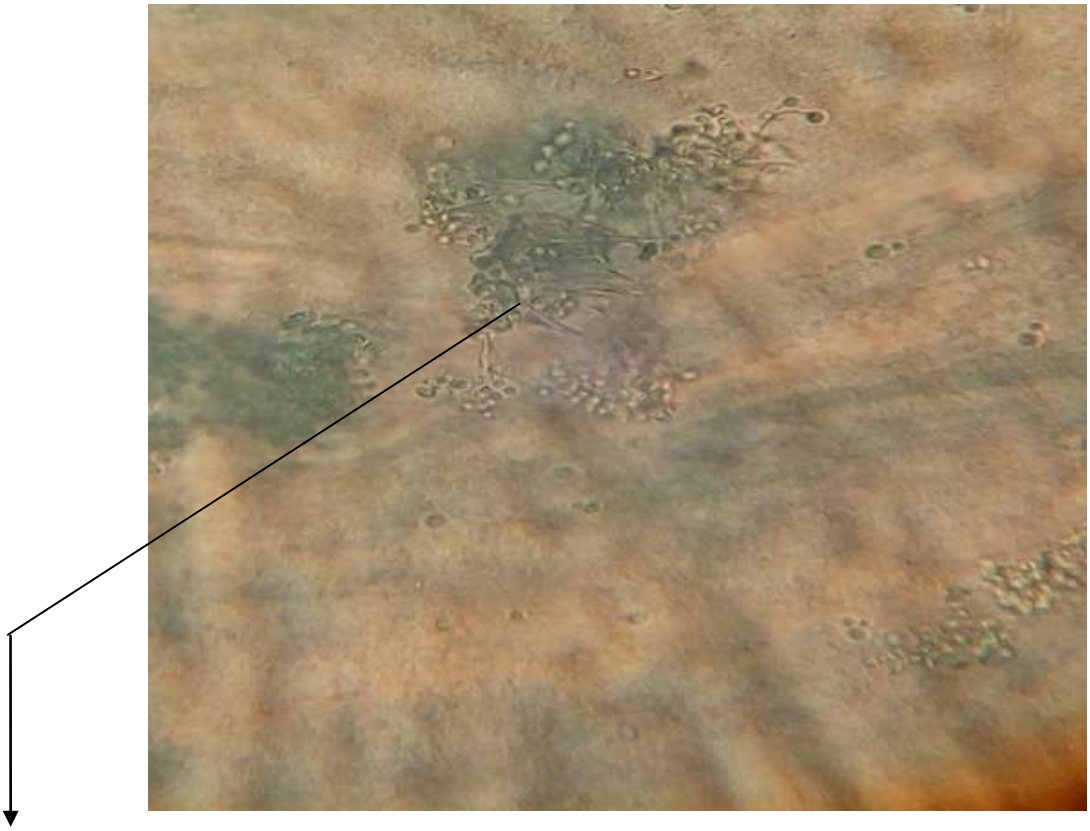


Plate II: Micrograph of Gram stained Colonies of *Candida albicans*



hyphal extension

Plate III: Micrograph of Germ Tube Formed by the Yeast Cells.

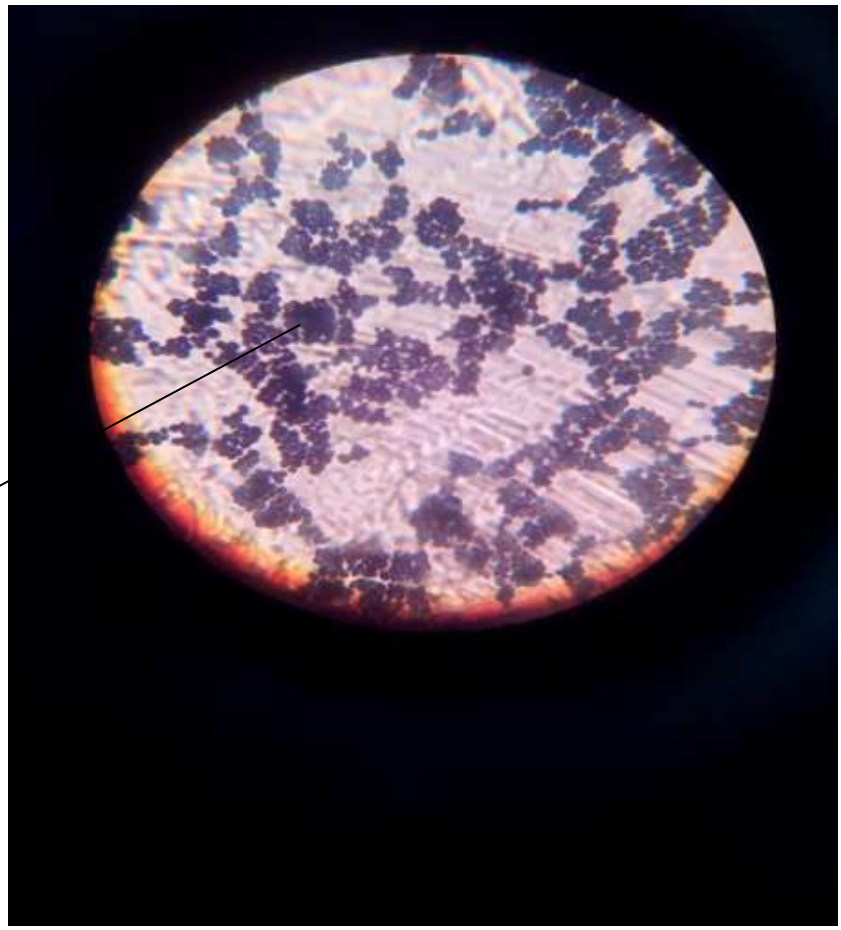
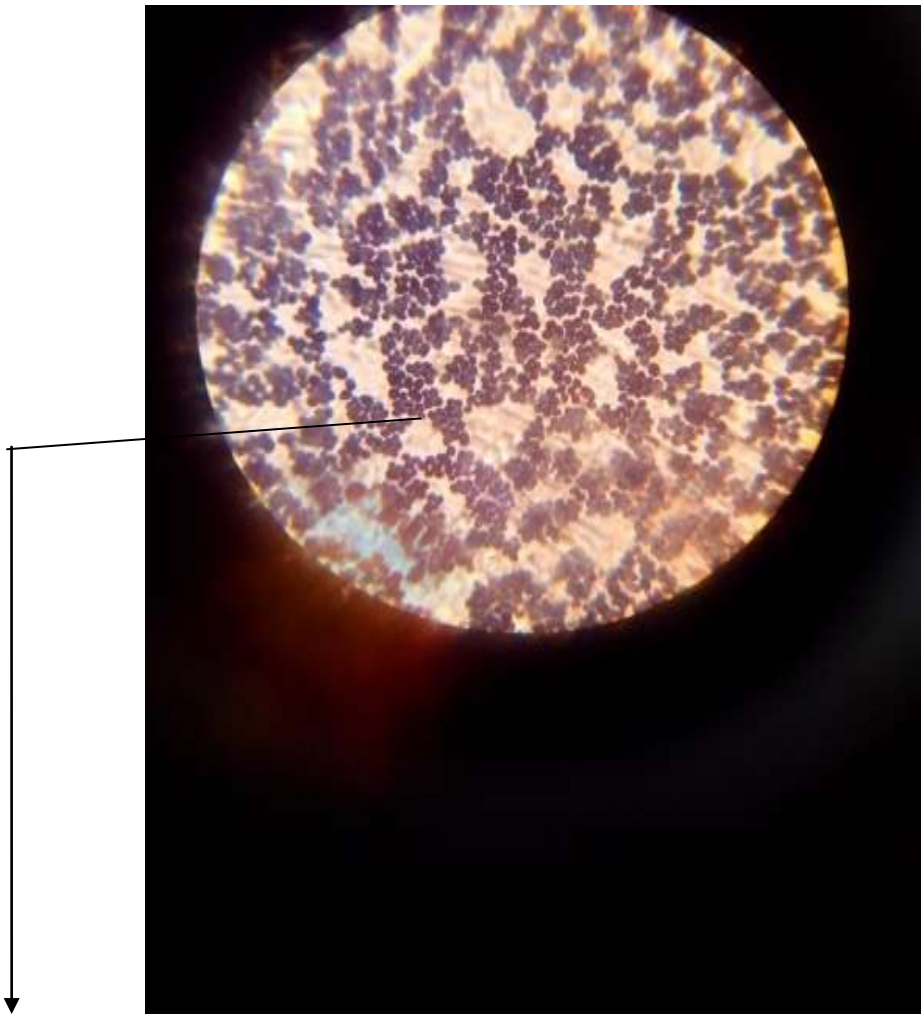


Plate IV: Micrograph of Gram stained Colonies of *Candida albicans*

Plate V: Micrograph of Germ Tube Formed by the Yeast Cells.



Colonies

Plate VI: Micrograph of Gram stained Colonies of *Candida albicans*

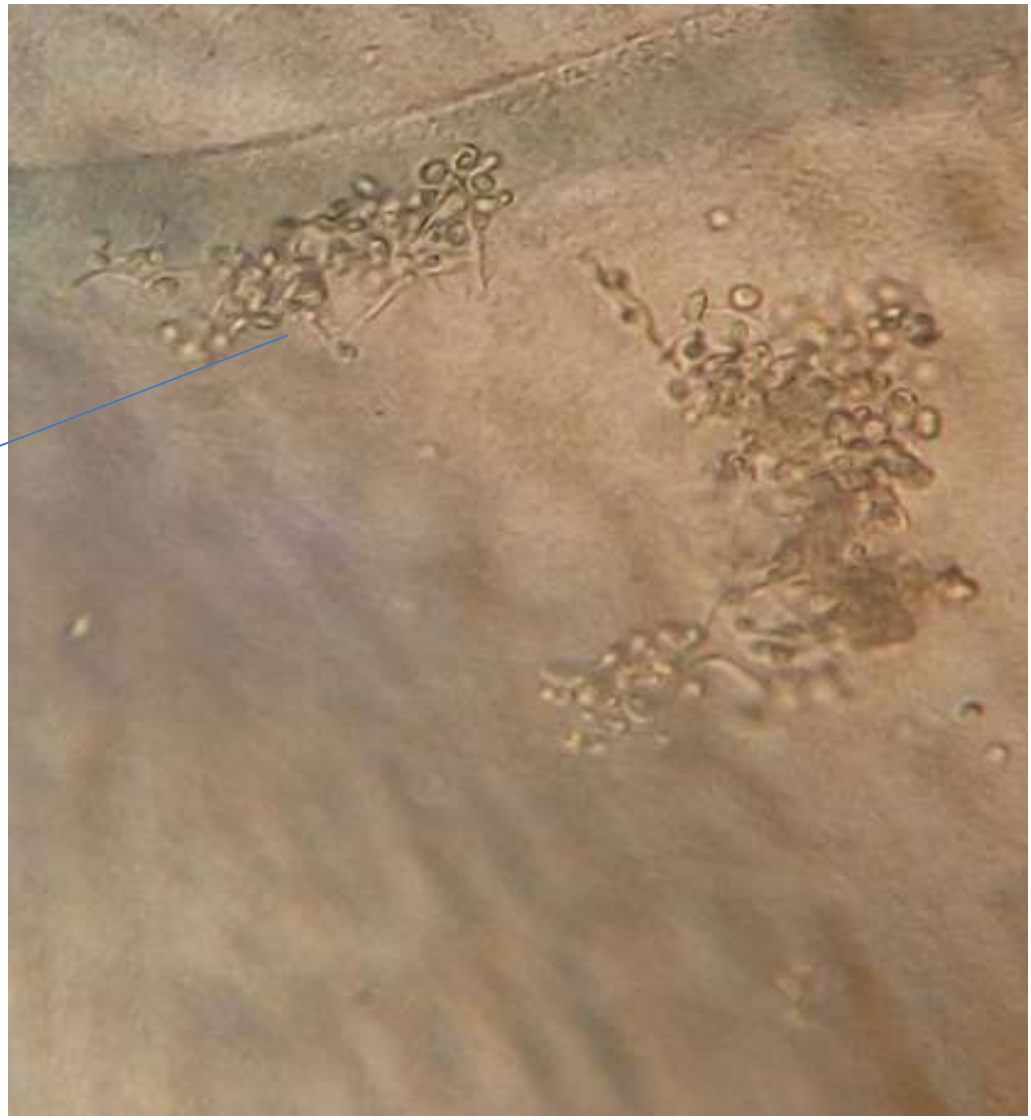


Plate VII: Micrograph of Germ Tube Formed by the Yeast Cells.

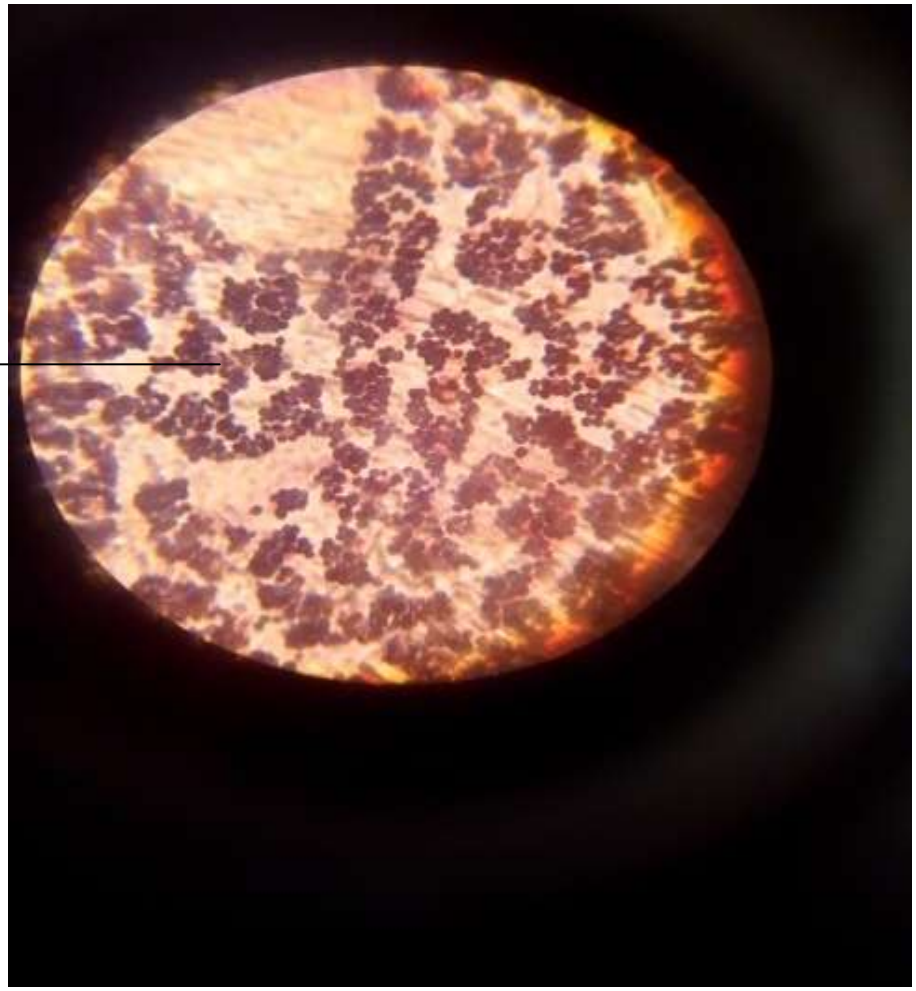


Plate VIII: Micrograph of Gram stained Colonies of *Candida albicans*

Colonies

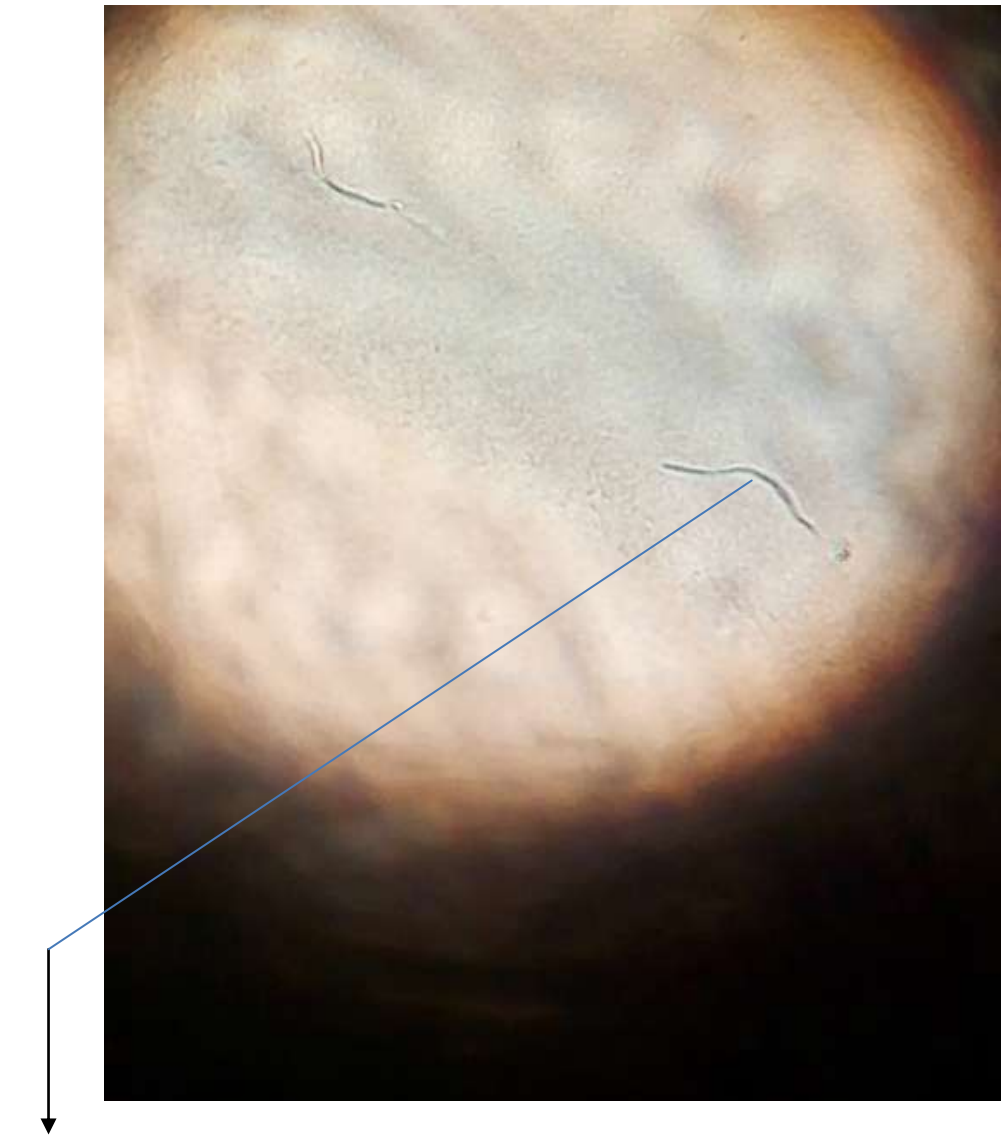


Plate IX: Micrograph of Germ Tube Formed by the Yeast Cells.

Table4. 3: Biochemical Characterization of Clinical Isolates of *Candida*

Sample Source	Profile	Significant taxa	Next Taxon	% Identity of Significant taxa	% Identity of Next Taxon
HVS	6540134	*Candida albicans* II	*Candida dubliniensis*	88.1	8.4
HVS	6576174	*Candida albicans* I	*Candida tropicalis*	98.1	1.7
ICS	6748174	*Candida parapsilosis*	*Candida albicans i*	98.2	1.4

HVS: high vaginal swap
ICS: intra cervical swab

4.1.3 Minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and IC_{50} of n-Hexane, ethylacetate, methanol and aqueous extracts of leaves of *Acalypha wilkesiana* on the planktonic cells of *Candida* spp

Table 4.4 shows the Minimum Inhibitory and Fungicidal Concentration (MIC/ MFC mg/ml)of the crude extracts, corilagin, voriconazole, Amphotericin B. and caspofungin on planktonic cells of *C. albicans (*I and II*)* and *Candida parapsilosis.* The ethylacetate extract had MIC of 25mg/ml and MFC of 50 mg/ml for both*C.albicans* I and II but MIC of 25mg/ml and MFC of 100 mg/ml on C.*parapsilosis* while methanol extract had MIC of 100mg/mland MFC 100mg/ml on *C. albicans I* and *II* but MIC of 50mg/ml in *C. parapsilosis* and no total cell death was observed with *C. parapsilosis*. Corilagin and other reference drugs except voriconazole showed total cell death for the three *Candida* strains at low concentrations compared to that for the extracts.

Table 4.5 shows themedian inhibitory concentration (IC_{50} mg/ml) of the crude extracts, corilagin, voriconazole, Amphotericin B. and caspofungin on planktonic cells of *C. albicans (*I and II*)* and *Candida parapsilosis.* The ethylacetate extract was shown to have the lowest IC_{50}(46 mg/ml ,48 mg/ml and 50 mg/ml) against*C. albicans* I and II and *C. parapsilosis* when compared to the n-hexane, methanol and aqueous extract of *Acalypha wilkesiana* leaves.Corilagin and other reference drugs used in this research had lessIC_{50}compared to the $IC_{50}$of the crude extracts of *A. wilkesiana* leaves. There was significant difference ($p \leq 0.05$) among the IC_{50}ofthecrude extracts and corilagin and all the reference drugs.

Table 4.4:Minimum Inhibitory / Fungicidal Concentrations (MIC/ MFC) (mg/ml) of the Extracts and Reference Drugs on *Candida albicans (I and II) and Candida Parapsilosis*

ract / Drugs	***C.albicans I***	***C.albicans II***	***C. parapsilosis***
lexane	100 / NTCD	100/ NTCD	NA / NTCD
ylacetate	25 / 50	25 / 50	25 / 100
thanol	100 / 100	100 / 100	50 / NTCD
ueous	100/NTCD	100 /NTCD	100 / NTCD
rilagin	4×10^{-2}/ 4×10^{-2}	4×10^{-2}/ 4×10^{-2}	4×10^{-2}/ 4×10^{-2}
riconazole	12.5×10^{-6}/NTCD	12.5×10^{-6}/NTCD	12.5×10^{-6}/NTCD
photericin B	12.5×10^{-6}/ 25×10^{-4}	12.5×10^{-6}/ 25×10^{-4}	12.5×10^{-6}/ 25×10^{-4}
spofungin	12.5×10^{-6} / 25×10^{-4}	12.5×10^{-6}/ 25×10^{-4}	12.5×10^{-6}/ 25×10^{-4}

NA :No Activity
NTCD : No Total Cell Death

Table 4.5 Median Inhibitory Concentration (IC_{50}) of the Extracts and Reference Drugs on *Candida albicans (*I and II*)and Candida parapsilosis*

Extract / Drugs	***C. albicans I*** **mg/ml**	***C. albicans II*** **mg /ml**	***C. parapsilosis*** **mg/ml**
n-Hexane	71.0	72.0	90.0
Ethylacetate	46.0	48.0	50.0
Methanol	50.0	58.0	68.0
Aqueous	68.0	70.0	76.0
Corilagin	8.8×10^{-2}	12.8×10^{-2}	10.0×10^{-2}
Voriconazole	$65.0.0 \times 10^{-5}$	67×10^{-5}	70.0×10^{-5}
Amphotericin B	70.0×10^{-5}	69.0×10^{-5}	69.0×10^{-5}
Caspofungin	69.0×10^{-5}	69.0×10^{-5}	67.0×10^{-5}

4.2 Adhered Cells Penetrability of Ethylacetate and Methanol extracts of*Acalypha wilkesiana*leaves,Corilagin Voriconazole, Amphotericin B and Caspofunjin on *Candida albicans* I, II*and C. parapsilosis*

4.2.1 Quantification of adhered cells formed by the clinical isolates of*Candida albicans* I, IIand *C. parapsilosis*

Figure 4.2 showed adhered cells formed by the clinical isolates of *C. albicans* I and II and *C. parapsilosis* over 72 hours incubation. The adhered cells formed by *C. albicans* I within 24 and 72 hours were 8.85 ± 0.67 and 9.89 ± 0.3 which was not significantly ($p \geq 0.05$) different from the adhered cells formed by *C.* albicans II and C. parapsilosis within the same ours of incubation.

4.2.2 Adhered Cells penetrability concentration of ethylacetate and methanol extracts of*Acalypha wilkesiana*leaves,corilagin voriconazole, Amphotericin B and caspofungin on *Candida albicans* I, II*and C. parapsilosis*

Figure 4.4 showedadhered cells penetrability of ethylacetate and methanol extracts, and the reference drugs on *C.albicans* I. There was no diffusion of ethylacetate extract through the adhered cells of *C. albicans* Iat 60, 120 and 180mins but the extract penetrated through the adhered cells at 240 to 360 mins. The methanol extract only penetrated through the adhered cells of *C. albicans* I at 300 to 360minutes not significantly ($p > 0.05$) different from that of ethylacetate extract.

Significant ($p < 0.001$) level of corilagin diffused through the *C.albicans*Iadhered cells when compared to ethylacetate and methanol extracts and other reference drugs (Voriconazole, Amphotericin B and Caspofungin). The Penetrability Concentration increased with time with significant ($p<0.001$) difference observed between the ethylacetate concentrations that penetrated at 180 minutes in *C. albicans* I compared to that in *C. albicans* II at same time duration. Figure 4.5 and appendice v showed

that higher concentration of ethylacetate extract (0.51 mg/ml) penetrated compared to the methanol extract (0.03 mg/ml) on the adhered cells of *C. albicans* IIat 300 minutes. There was no diffusion of the ethylacetate extract at 60 to 180mins but at 300 to 360mins the penetrability concentrations ranged from 0.51±0.02 to 0.7±0.03mg/ml, respectively. Methanol Extract penetrated through the *C. albicans* IIadhered cells at 240 to 360 mins with 0.02 ± 0.01 to 0.04 ± 0.01mg/ml.Corilagin, voriconazole and Amphotericin B penetrated at 180 to 360 mins with corilagin having a significantly ($p \leq 0.05$) higher penetrability concentration of 0.56 ± 0.02 to 0.89 ± 0.07 mg/ml. Caspofungin diffused the *C. albicans*IIadhered cells at 120 mins and had a lower penetrability concentration than that of corilagin at 180 minutes with no significant difference ($p > 0.05$) as presented in Figure 4.5.

Figure 4.6 shows the penetrability concentration of ethylacetate, methanol extracts, corilagin, voriconazole, Amphotericin B and caspofungin on the adhered cells of *C.parapsilosis.* The ethylacetate and methanol extracts diffused through the biofilm at 240 to 360 minutes with increase in penetrability concentration with time in ethylacetate extract but the methanol extract penetrability concentration increased at 300 mins (0.25 ±0.01mg/ml) and decreased at 360 minutes (0.14 ±0.02mg/ml). Penetrability concentration of corilagin was significantly lower in *C.parapsilosis* compared to *C. albicans* Iand IIat same time duration.Corilagin, voriconazole, Amphotericin B and caspofungin all had increase in penetrability concentration with timeas shown in figures4.4, 4.5and 4.6.

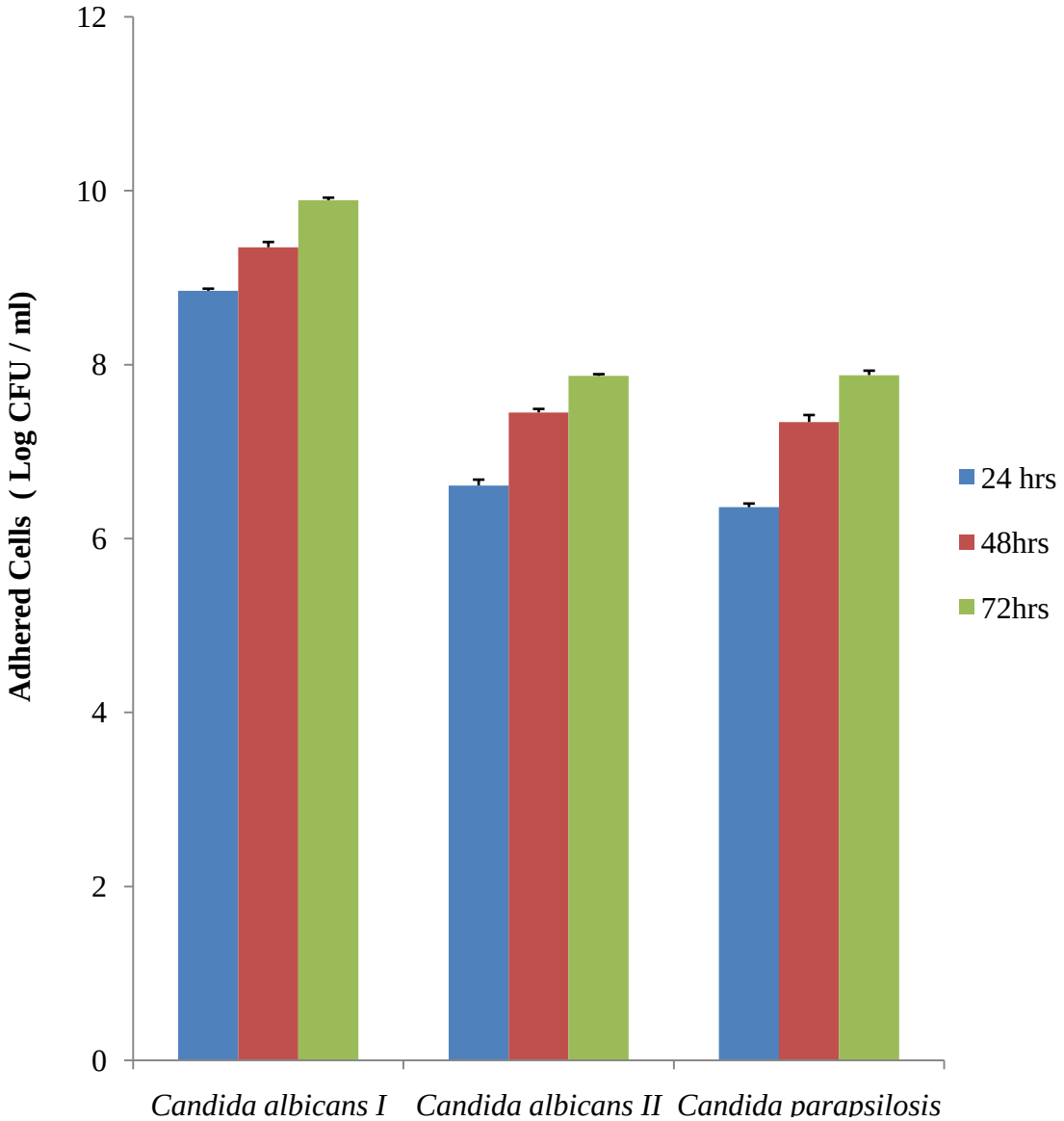


Figure 4.1:Adhered Cells Formed by Clinical Isolatesof *Candida albicans* (IandII) and *Candida parapsilosis* over a Period of 72 hours

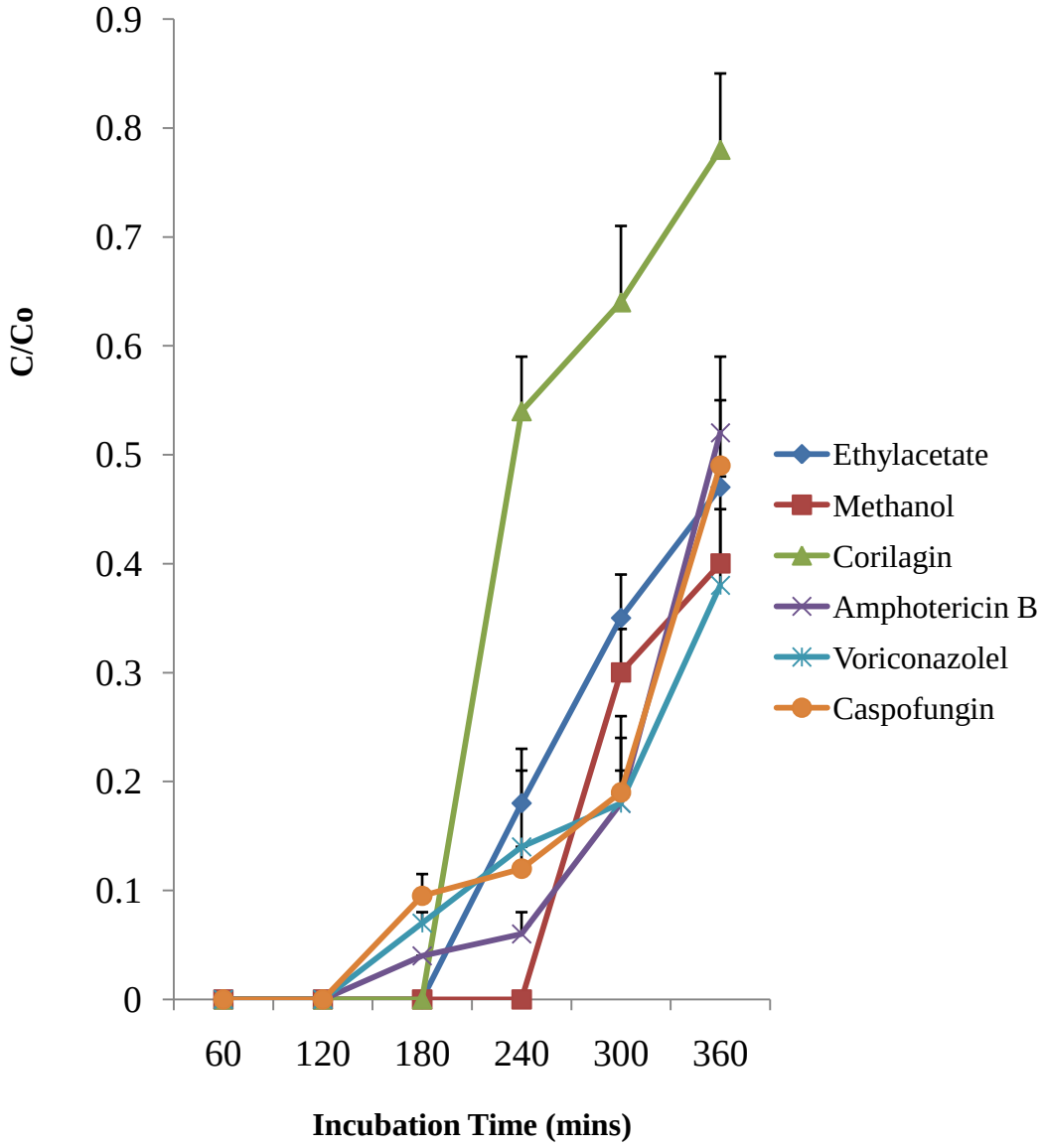


Figure 4.2:Penetrability Concentration of Ethylacetate and Methanol Extracts of *Acalypha wilkesiana*, Corilagin, Voriconazole, Amphotericin B and Caspofungin on Adhered Cells of *Candida albicans* I

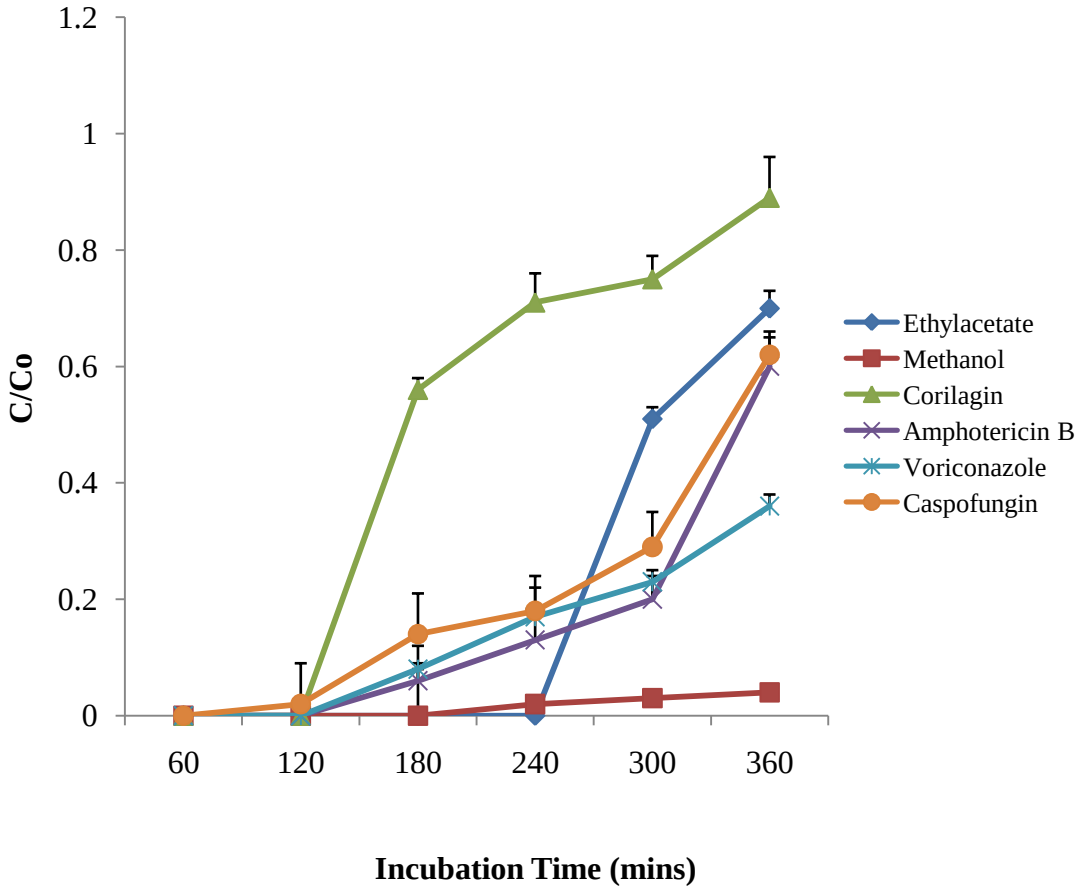


Figure 4.3: Penetrability Concentration of Ethylacetate, Methanol Extracts of *Acalypha wilkesiana*, Corilagin, Voriconazole, Amphotericin B and Caspofungin on Adhered Cells of *Candida albicans* II

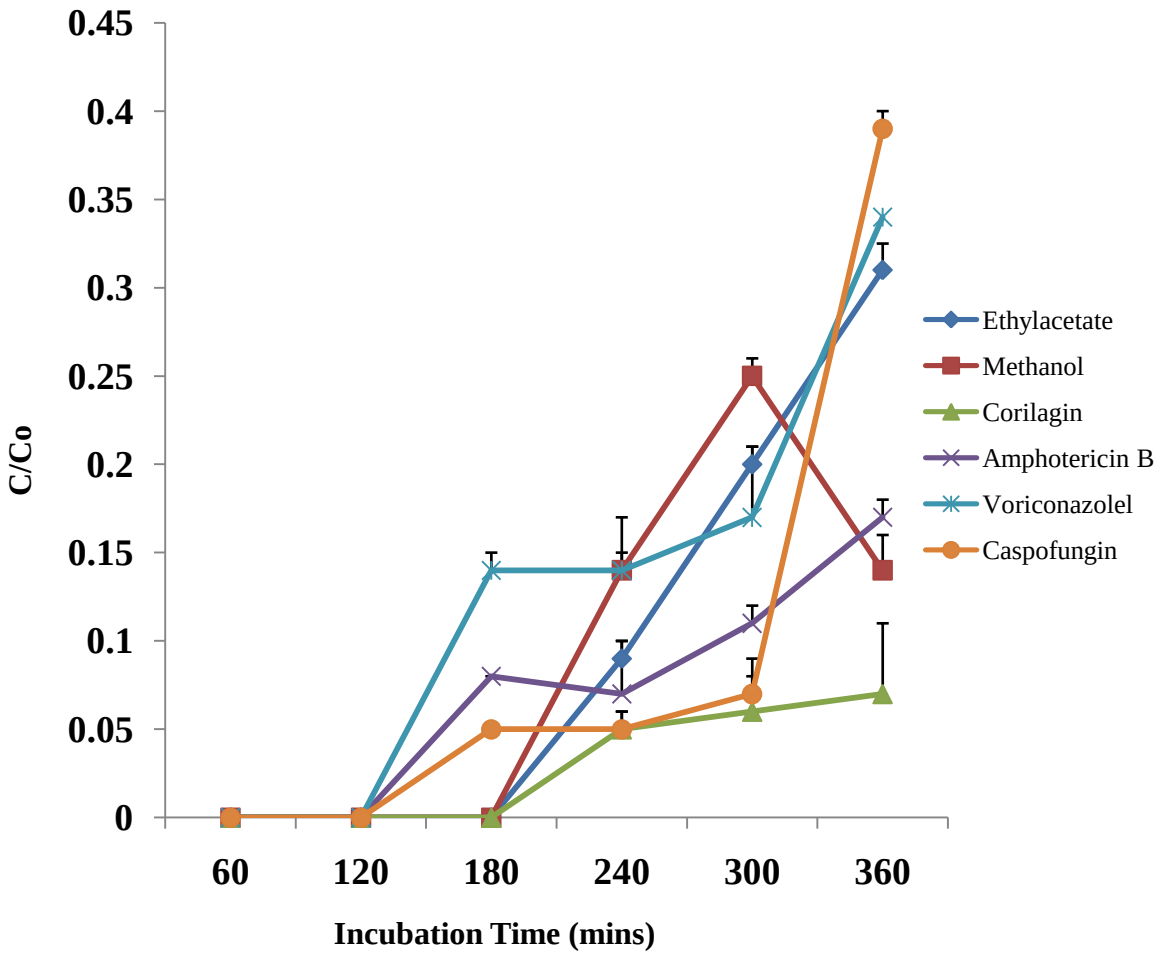


Figure 4.4:Penetrability Concentration of Ethylacetate, Methanol Extracts of *Acalypha wilkesiana*, Corilagin, Voriconazole, Amphotericin B and Caspofungin on Adhered Cells of *Candida parapsilosis* .

4.3 Median Adhered Cells Eradication concentration ($AdEC_{50}$)of Ethylacetate and Methanol extracts of*Acalypha wilkesiana*leaves,Corilagin Voriconazole, Amphotericin B and Caspofunjin on *Candida albicans* I, II*and C. parapsilosis*

Table 4.6shows the median adhered cells eradication concentration of Ethylacetate, Methanol Extracts of *Acalypha wilkesiana,* Corilagin, Voriconazole, Amphotericin B and Caspofungin on *Candida albicans (I and II) and C. parapsilosis.*The two extracts were selected on the basis of their fungicidal activity. The ethylacetate extract had lower $AdEC_{50}$of 124.1, 130.0 and 151.5 mg/ml on *C. albicans* I, II and *C. parapsilosis* while the methanol extract had 192.5, 202.5 and 230.2 mg/ml on *C. albicans* I, II and *C. parapsilosis.*Corilagin at 0.04mg/ml, Voriconazole (0.01mg/ml), Amphotericin B. (0.01mg/ml) and Caspofungin (0.01mg/ml) showed significantly ($p \leq 0.01$) higher percentage of biofilm eradication compared to the extracts.$AdEC_{50}$of corilagin, voriconazole, Amphotericin B and caspofungin on *Candida albicans* I were 0.09, 0.007, 0.008 and 0.007 mg/ml while on *C. albicans* II, the $AdEC_{50}$ were 0.102,0.008,0.009 and 0.007 and 0.14, 0.015, 0.015 and 0.012 mg/ml on *C. parapsilosis.*

Table 4.6: Median Adhered Cells Eradication Concentration ($AdEC_{50}$) of Ethylacetate, Methanol Extracts and Standard Drugs on *Candida albicans* I, II and *C. parapsilosis.*

Extracts and Drugs	***C. albicans I*** **(mg/ml)**	***C. albicans II*** **(mg/ml)**	***C. parapsilosis*** **(mg/ml)**
Ethylacetate	124.1	130.0	151.5
Methanol	192.5	202.5	230.2
Corilagin	0.09	0.102	0.14
Voriconazole	0.007	0.008	0.015
Amphotericin B	0.008	0.009	0.015
Caspofungin	0.007	0.007	0.012

4.4 Isolation and PartialPurification of most Potent Fraction of Ethylacetate Extract of *Acalypha wilkesiana* Leaves

Ethylacetate extract hadsignificant antibiofilm activity and was therefore chosen for bioassay guided fractionation. Table 4.6 shows the weight and retention factor of the fractions of ethylacetate extract. Column chromatographic separation using two solvent systems starting from absolute n-hexane (100%), n-hexane: ethylacetate (4:1, 3:2, 2:3, 1:4), and absolute ethylacetate (100%) gave 62 fractions where fractions with the same retention factors were pooled together and to obtain seven fractions (A,B,C,D, E, F and G). The seven pooled fractions were then developed in 100% absolute ethylacetate in a TLC tank and allowed to dry and then sprayed with vanillin sodium sulphate to give the chromatogram shown in (A*ppendix vii).* The MIC, MFC, % Biofilm Eradication (BE) and Biofilm Penetrabillity (BP) were determined in all the seven pooled fractions (A, B, C, D,E, F and G).

Table 4.7 presentsFractions from the separation of ethylacetate extract, their percentage recovery and retention factor.

Table 4.7: Fractions, Percentage Recovery and Retention Factor of Ethylacetate Extract of *A. wilkesiana* Leaves

Fractions	Weight (g)	% Recovery	RF
A	0.33	6.49	0.87
B	0.18	3.54	0.56
C	0.14	2.76	0.93
D	1.65	32.48	0.87 0.83
E	0.25	4.92	0.83 0.93
F	1.24	24.41	0.13 0.40 0.47 0.53 0.67 0.73
G	1.29	25.39	0.20 0.35 0.39 0.47 0.56

4.4.1 Minimum Inhibitory Concentration (MIC) Minimum FungicidalConcentration (MFC), IC50, AdEC$_{50}$ of Subfractions G, F and Standard Drugs

The MIC ofsubfraction G was found to be 5mg/ml on *C. albicans* I, IIand *parapsilosis*with MFC of 5mg/ml *on C. albicans*IandIIbut 10mg/ml on *C. parapsilosis* (Table 4.8).Fractions F and G showed inhibitory activities as presented in Table 4.9. The IC_{50} of fraction G on *Candida albicans* I, II and *Candida Parapsilosis* was 27.0, 25.0 and 23.0 mg/ml which were lower than the IC_{50} of fraction F for the three strains of *Candida* used.

Table 4.10 shows the median adhered cells eradication concentration ($AdEC_{50}$) of fractions G, F and the reference drugs. It was observed that the subfraction G had $AdEC_{50}$of2.9,2.7and 3.0 against *C. albicans I, II* and *C. parapsilosis*which were less than the $AdEC_{50}$ of subfraction F.

Table 4.8: Minimum Inhibitoryand Minimum Fungicidal Concentrations (MIC / MFC) of Fractions F and G of Ethylacetate Extract of *A. wilkesiana*Leaves and Reference Drugs on Planktonic Cells of *C. albicans*(I and II)and *C. parapsilosis*.

actions	***C. albicans I*** **(mg/ml)**	***C. albicans II*** **(mg/ml)**	***C. Parapsilosis*** **(mg/ml)**
	5 / NTCD	10/ NTCD	10 / 10
	5/ 5	5/ 5	5/ 10
rilagin	4×10^{-2}/ 4×10^{-2}	4×10^{-2}/4×10^{-2}	4×10^{-2}/ NTCD
riconazole	12.5×10^{-6}/ NTCD	12.5×10^{-6}/ NTCD	12.5×10^{-6}/ NTCD
mphotericin B	12.5×10^{-6} / 25×10^{-4}	12.5×10^{-6} / 25×10^{-4}	12.5×10^{-6} / 25×10^{-4}
spofungin	12.5×10^{-6} / 25×10^{-4}	12.5×10^{-6} / 25×10^{-4}	12.5×10^{-6} / 25×10^{-4}

NTCD = No Total Cell Death

Table 4.9: Median Inhibitory Concentration (IC_{50}) of Fractions F and G of Ethylacetate Extract of *A. wilkesiana* Leaves and Reference Drugs on Planktonic cells of *C. albicans* (I and II)*and C. parapsilosis*

Fractions / Drugs	***C. albicans I*** **(mg/ml)**	***C. albicans II*** **(mg/ml)**	***C. parapsilosis*** **(mg/ml)**
A- E	NA	NA	NA
F	50.0	80.0	35.0
G	27.0	25.0	23.0
Corilagin	0.8×10^{-1}	1.3×10^{-1}	1.0×10^{-1}
Voriconazole	66.0×10^{-5}	69.0×10^{-5}	74.0×10^{-5}
Amphotericin B.	71.0×10^{-5}	70.0×10^{-5}	70.0×10^{-5}
Caspofungin	71.0×10^{-5}	71.0×10^{-5}	69.0×10^{-5}

NA= No Inhibitory Activity

Table 4.10: Median Adhered Cells Eradication Concentration ($AdEC_{50}$) of Subfractions F, G and Standard Drugs against *Candida albicans* I, II and *C. parapsilosis*

Fractions and Standard Drugs	***C. albicans* I** (mg/ml)	***C. albicans* II** (mg/ml)	***C. parapsilosis*** (mg/ml)
F	5.9	6.8	6.5
G	2.9	2.7	3.0
Corilagin	0.09	0.102	0.14
Voriconazole	0.007	0.009	0.009
Amphotericin B	0.0075	0.008	0.008
Caspofunjin	0.008	0.004	0.006

4.4.2 Minimum Inhibitory Concentration (MIC) Minimum Fungicidal Concentration (MFC), IC50, $AdEC_{50}$ of Subfractions G7, G17-19 and Standard Drugs

Table 4.12 presents the minimum Inhibitory concentrations (MIC) and minimum Fungicidal concentrations (MFC) of G7, G17-19 and the reference drugs. G7 had MIC of 1.25mg/ml each on C.*albicans* I, II and *C.parapsilosis*. MFC of 2.5mg/ml on *C. albicans* IandIIbut there was no total death observed for *C. parapsilosis at* 2.5mg/ml. Subfraction G17-19 showed MIC of 2.5mg/ml each for all the three strains but there was no total cell death at 2.5mg/ml on *C.albicans* I, II and *C. parapsilosis.*

The reference drugs hadlow concentrations of MIC and MFC except voriconazole which had no fungicidal activity on all the *Candida* strains.Corilagin showed MIC of 0.04mg/ml each on *C.albicans* I, II and*C. parapsilosis*but, there was no total cell death observed on *C. parapsilosis.*

Subfraction G7 had IC_{50}(5.8, 6.0 and 10.0 mg/ml) on *C. albicans* I, IIand *C. parapsilosis,* while pooled subfraction G9-16 had $IC_{50}$of 50, 90 and 100 mg/ml on *C.albicans* I, II and *parapsilosis.* Pooled subfractions G17-19had IC_{50} of 41, 35 and 40 mg/ml) on *C.albicans* I, II and *C. parapsilosis*,respectively(Table 13). Subfractions G7 and G17-19 were selected for further bioassays based on their potency.

Table 4.14 showed median Adhered cells eradication concentration ($AdEC_{50}$) of subfractions G17-19,G7 and the reference drugs on *C.albicans* I, II and *C. parapsilosis*. Subfraction G7 had BEC_{50} of 2.31, 1.405 and 1.523 mg/ml on the three strains of *Candida* used while G17-19 had $AdEC_{50}$ of 3.801, 4.12 and 4.503 mg/ml.

However, identication of the purified compound was attempted using proton NMR but perhaps we got an artifact (Appendice IX)

Table 4.11: Weight, Percentage Recovery and Retention Factor of subfractions G of Ethylacetate Extract of *A. wilkesiana* Leaves

Subfractions Fractions	Weight (g)	% Recovery	RF

G1-G6	-	-	-
G7	0.25	25.77	0.83
G8	0.08	8.25	0.76
G9 - G16	0.02	2.06	0.25
G17- G19	0.19	19.59	0.65
G20 –G21	0.27	27.84	0.27
G22	0.16	16.49	0.40

Table 4.12: **Minimum Inhibitory and Fungicidal Concentrations (MIC/ MFC) ofSubfractions G7, G17-19and Reference Drugs against *Candida albicans* I, II and *C. parapsilosis***

Fractions	*C. albicans* I mg/ml	*C. albicans* II mg/ml	*C. p* mg/m
G7	1.25 / 2.5	1.25 / 2.5	
G17-19	2.5/ NTCD	2.5 /NTCD	
Corilagin	4×10^{-2}/ 4×10^{-2}	4×10^{-2}/ 4×10^{-2}	
Voriconazole	12.5×10^{-6} / NTCD	12.5×10^{-6}/ NTCD	
Amphotericin B	12.5×10^{-6}/ 25×10^{-4}	12.5×10^{-6}/ 25×10^{-4}	
Caspofungin	12.5×10^{-6}/ 25×10^{-4}	12.5×10^{-6}/ 25×10^{-4}	

NTCD = No Total Cell Death

Table 4.13 : Median Inhibitory Concentration (IC_{50}) of Subfractions G and the Reference Drugs on Planktonic Cells of *C. albicans* I, II and *C. parapsilosis*

Subfractions and Drugs	***C. albicans* I mg/ml**	***C.albicans* II mg/ml**	***C. parapsilosis* mg/ml**
G7	5.8	6.0	10.0
G8	87.0	100.0	100.0
G9-16	50.0	90.0	100.0
G17-19	41.0	35.0	40.0
G20-21	100	NA	NA
G22	NA	NA	NA
Corilagin	0.9×10^{-1}	1.2×10^{-1}	0.9×10^{-1}
Voriconazole	70.0×10^{-5}	75.0×10^{-5}	77.0×10^{-5}
Amphotericin B	69.0×10^{-5}	71×10^{-5}	72.0×10^{-2}
Caspofungin	72.0×10^{-5}	72.0×10^{-6}	72.0×10^{-5}

NA = No Inhibitory Activity.

Table 4.14: Median Adhered Cells Eradication Concentration ($AdEC_{50}$) of Subfraction G7, G17-19 and Reference Drugs on *C.albicans* I, II and *C. parapsilosis*

Subfractions / Standard Drugs	***C.albicans* I** (mg/ml)	***C.albicans* II** (mg/ml)	***C.parapsilosis*** (mg/ml)
G7	2.31	1.405	1.523
G17-19	3.801	4.12	4.503
Corilagin	0.089	0.073	0.09
Voriconazole	0.006	0.007	0.007
Amphotericin B.	0.007	0.007	0.008
Caspofungin	0.006	0.006	0.006

4.5 Median Biofilm Eradication (BEC_{50}) of Fraction G7 and Scanning Electron Micrograph of Bīōfilms Formed from 48 hours Cultured*Candida albicans* I, II

and *C. parapsilosis* then Treated with Fraction G7, Corilagin, Voriconazole, Amphotericin B and Caspofungin

Table 4.15 showed the BEC_{50} of partially purified G7 and reference drugs on *C. albicans* I, II and *C. parapsilosis*. The $BEC_{50}$of G7 were 2.42, 1.56 and 1.543 mg/ml on the three strains of *Candida* used.

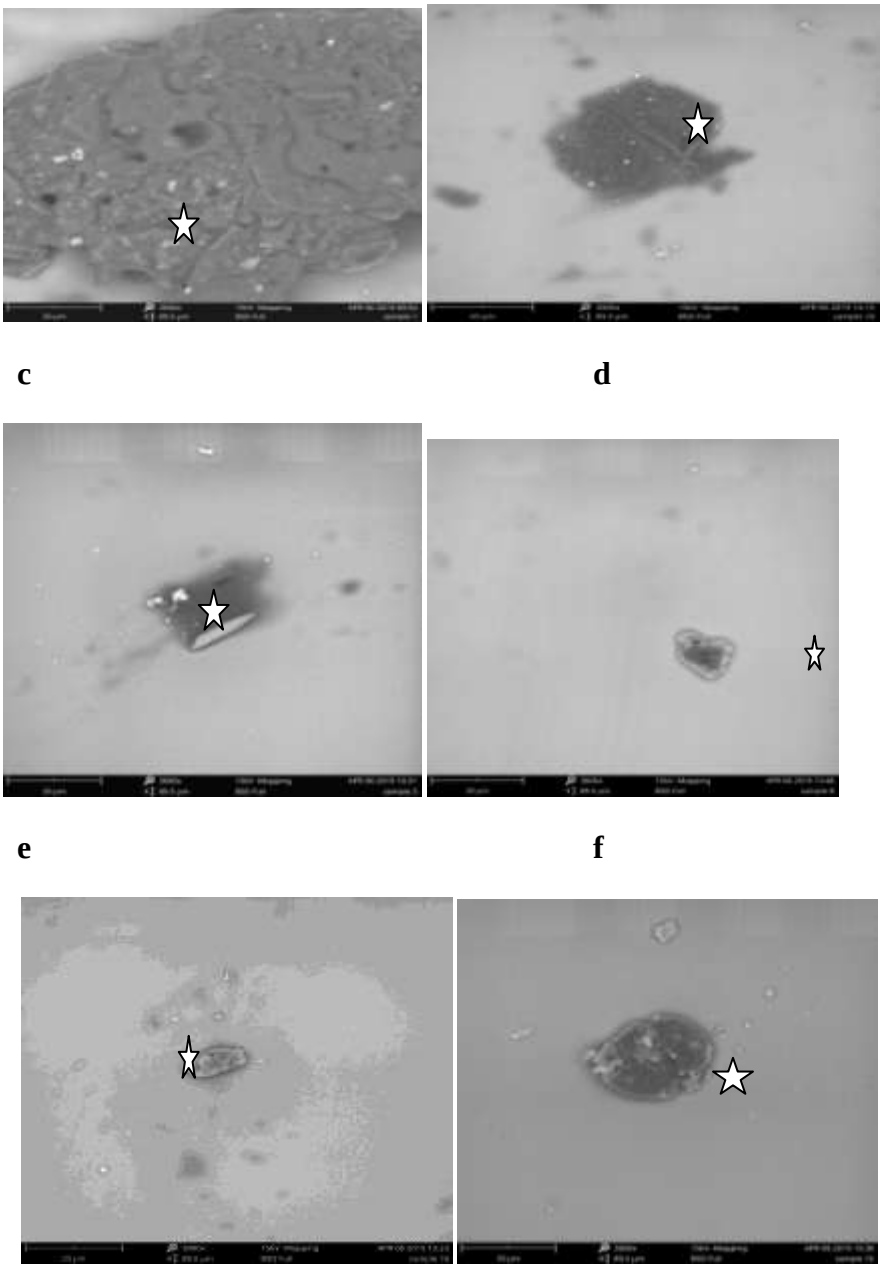
Plates V,Plates VII and Plates VIII showedthe scanning electron micrograph of cultured *C.albicans* I , *C. albicans*II and *Candida parapsilosis*, respectively,

The observationof the negative control groups (a) in plates VI, VII and VIII for *C. albicans* I,*C. albicans* II and *C.parapsilosis* respectively revealed agglomerates ofyeasts and filamentous forms whereas the groups (b-f) which were exposed to Corilagin (0.04 mg/ml), Voriconazole (0.01mg/ml), Amphotericin B (0.01mg/ml), Caspofungin (0.01mg/ml) and the purified fraction of ethylacetate extract (fraction G7) of *Acalypha wilkesiana* leaves comprised sparsed yeasts.

Table 4.15: Median Biofilm Eradication Concentration (BEC_{50}) of Subfraction Purified Fraction G7and Reference Drugs on *C.albicans* I, II and *C. parapsilosis*

Subfractions / Standard Drugs	***C.albicans* I** (mg/ml)	***C.albicans* II** (mg/ml)	***C.parapsilosis*** (mg/ml)
G7	2.42	1.56	1.543
Corilagin	0.089	0.073	0.09
Voriconazole	0.006	0.007	0.007
Amphotericin B.	0.007	0.007	0.008
Caspofungin	0.006	0.006	0.006

a **b**



c **d**

e **f**

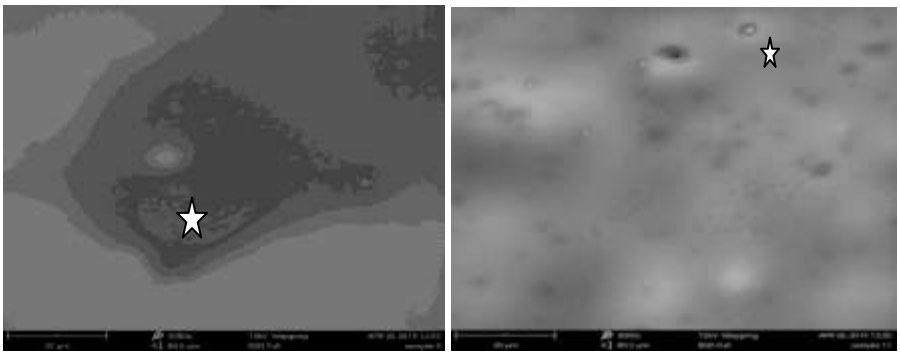
Plate X: Scanning Electron Micrograph of 48Hours Cultured *C. albicans I*

Key : control plate (a) Loss of Biofilm Biomass in treated cells with Corilagin plate (b), Voriconazole plate (c), Amp B. plate (d), Caspofungin plate (e) and Purified Fraction G of Ethylacetate Extract of A.wilkisiana Leaves plates (f).

☆ = Biofilm Biomass

a **b**

c **d**



e **f**

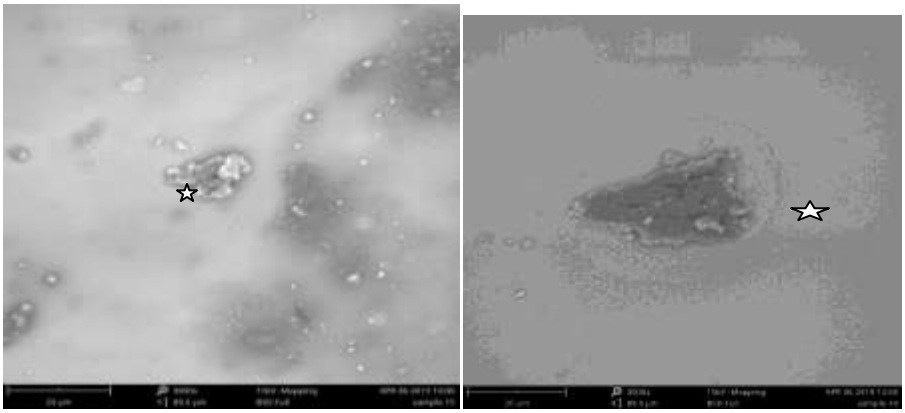


Plate XI: Scanning Electron Micrograph of 48Hours Cultured *C. albicans* II

Key: control plate (a) Loss of Biofilm Biomass in treated cells with Corilagin plate (b), Voriconazole plate (c), Amp B. plate (d), Caspofungin plate (e) and Purified Fraction G of Ethylacetate Extract of*A. wilkesiana* Leaves plates .

☆ =Biofilm Biomass

a **b**

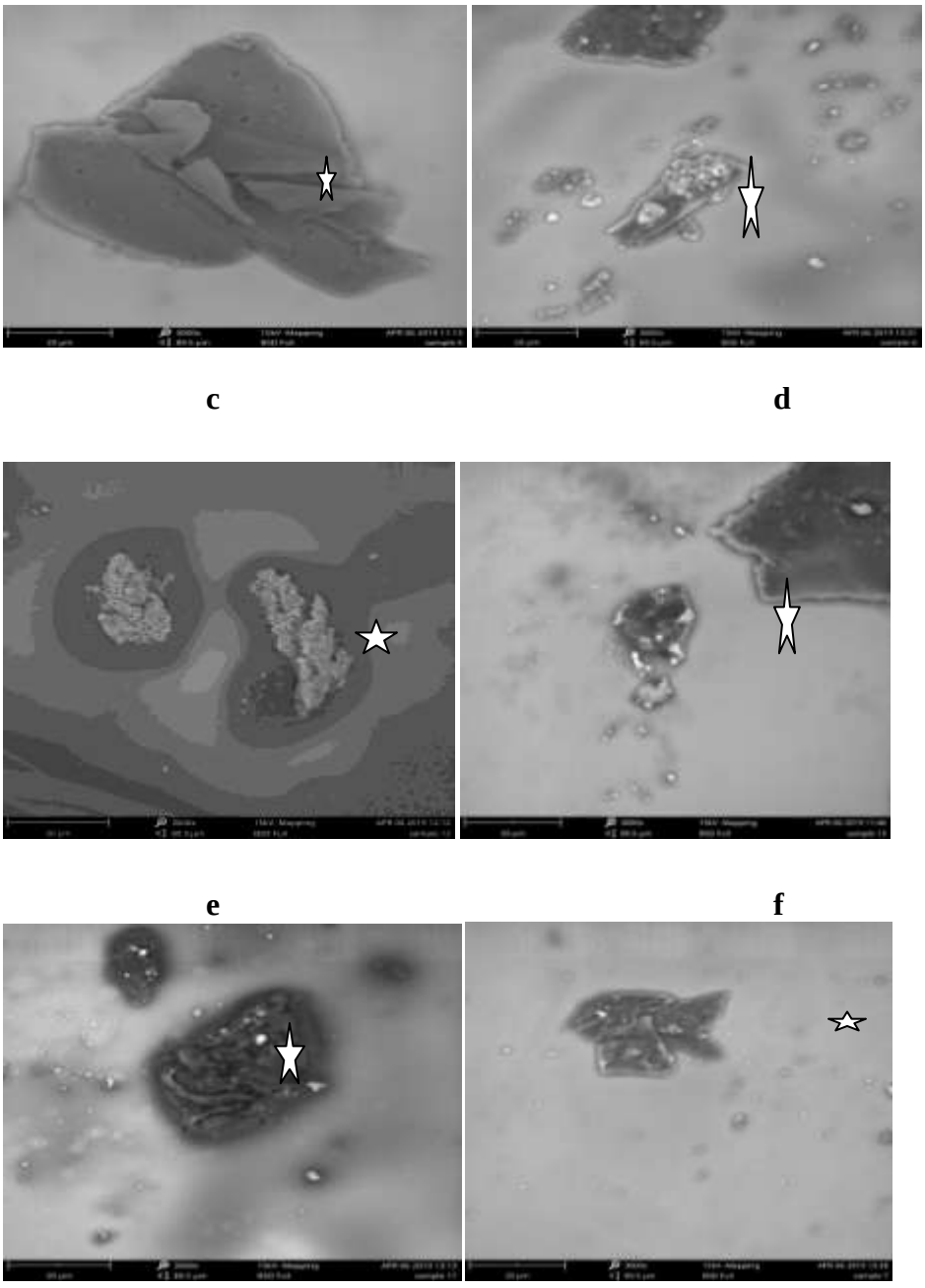


Plate XII: Scanning Electron Micrograph of 48Hours Cultured *C. parapsilosis*

Key: control plate (a) Loss of Biofilm Biomass in treated cells with Corilagin plate (b), Voriconazole plate (c), Amp B. plate (d), Caspofungin plate (e) and Purified Fraction G of Ethylacetate Extract of *A. wilkesiana* Leaves plates (f).

☆ = Biofilm Biomass.

4.6 Mode of Action of Purified Antibiofilm Principle (G7) on Cell Wall and Cell Membraneof *Candida albicans*I *and* II *and C. parapsilosis.*

Table 4.16 and Table 4.17 show the mode of action of the purified fraction of G7 on cell wall and cell membranes of *Candida albicans* I, II and *C. parapsilosis*using exogenous sorbitol and ergosterole, respectively.

Table 4.16 shows the effect of exogenous sorbitol used as osmotic protectant on the $IC_{50}$of purified G7 of ethylacetate extract of *Acalypha wilkesiana* leaves, corilagin and reference drugs. It was found that there was increase in IC_{50} of the purified G7 of ethylacetate extract of *Acalypha wilkesiana* leaves and caspofungin on the three *Candida* strains of *Candida* in the presence of exogenous sorbitol compared to their IC_{50} without sorbitol but there was no significant increase in IC_{50} of Corilagin in the presence of sorbitol compared to the IC_{50} of Corilagin without sorbitol in all the three *Candida* strains used.

Table 4.17 shows that there was no significant difference between the IC_{50} of purified G7 in the presence of ergosterol compared to the IC_{50} without ergosterol. There was increase in the IC_{50} of voriconazole and corilagin in the presence of ergosterol compared to that without ergosterol but there was difference between the IC_{50} of purified G7 in the presence of ergosterol and without ergosterol on the three strains of *Candida* used in this research work.

Table 4.16: Mode of action of Purified Subfraction G7 on Cell Wall of *Planktonic Cells of Candida albicans* I *and* II *and C. parapsilosis* using IC_{50}

	IC_{50} Without Sorbitol			IC_{50} With Sorbitol		
gents	***C. albicans I***	***C.albicans II***	***C. parapsilosis***	***C. albicans I***	***C.albicans II***	***C. par***
	6.8	**5.0**	**9.5**	**8.0**	**14.0**	**13.5**
/ml)	**1.2×10^{-2}**	**1.0×10^{-2}**	**1.2×10^{-2}**	**4.0×10^{-1}**	**4.4×10^{-1}**	**5.0 ×**
	72.0×10^{-5}	**72.0×10^{-5}**	**72.0×10^{-5}**	**12.6×10^{-4}**	**10.1×10^{-4}**	**10.0×**

Values are mean values from triplicate tests

Table 4.17: Mode of Action of Purified Subfraction G7 on Cell Membrane of *Planktonic Cells of Candida albicans* IandIIand *C. parapsilosis* using IC_{50}

	IC_{50} Without Ergosterole			IC_{50} With Ergosterole		
Antifungal agents	***C. albicans I***	***C. albicans II***	***C. parapsilosis***	***C. albicans I***	***C. albicans II***	***C. parapsilosis***
G7	**6.0**	**5.5**	**9.4**	**6.2**	**6.5**	**10.9**
Corilagin (0.04mg/ml)	**1.2×10^{-2}**	**1.0×10^{-2}**	**1.2×10^{-2}**	**3.5×10^{-2}**	**2.9×10^{-2}**	**1.4×10^{-2}**
Amphotericin B (0.01mg/ml)	**69.0×10^{-5}**	**71.0×10^{-5}**	**70.4×10^{-5}**	**3.1×10^{-3}**	**2.9×10^{-3}**	**3.4×10^{-3}**

Values are mean values from triplicate tests

CHAPTER FIVE

5.0 DISCUSSION

Fungal cells in a human host adhere, arrange, and protect themselves according to the environments that allow cells to survive in hostile environments and disperse planktonic cells to colonize new niches by biofilm formation therefore, contributing to their resistance strategy. Plants have been recognized as useful medicinal herbs that serve as leads in the discovery of new antimicrobials with new possible mode of action (Ibukun *et al*., 2019). To counter resistance caused by biofilms, new drugs must be multitargeted such that it must be able to penetrate the fungal biofilms to get to its targets. This study focused on the biofilm penetrability and eradication abilities of *A. wilkesiana* leaves.

Preliminary assay on the phytochemical sreening of *A. wilkesiana* leaves revealed the presence of alkaloids, phlobotannins, flavonoids (catechins and flavones) and saponins, all of which have potential health promoting effects (Basu *et al*., 2007).This study revealed that extracts of *Acalypha wilkesiana* leaves had significant amount of phenolics and alkaloids which are known for their antimicrobial activities this agrees with the findings of Awe and Eme (2014).

Ethylacetate extract of *Acalypha wilkesiana* leaves recordedsignificant activity against the planktonic cells of *C.albicans*I, II and *C. parapsilosis*compared to n-hexane, methanol and aqueous extractswhich was in line with the report ofMuyideen *et al***.**(2013).

Biofilm penetrability capability of the potent fractions and reference drugs increased with time which agreeswith the findings of Al-Fattani (2007) withincrease in antifungal penetration with time due to increase in the concentration of the drugs absorbed by the membrane barriers.

A significant ($p \leq 0.05$) reduction in biomass of sessile cells treated with the purified fraction of *Acalypha wilkesiana* leaves compared to the negative control as shown by the scanning electron micrograph was revealed. This conforms with the findings of Silva *et al*. (2011) who reported that antifungal agents reduced germ tube formation, fungal metabolism and proliferation.The reduction in biomass by the purified fraction of ethylacetate extract of *A.wilkesiana* leaves may be as a result of itsinterferance with biofilm proliferation which eventually reduces the formation of multilayer cell clusters. Janiel (2014) reported that proliferation is the most common mode of growth of *Candida* spp biofilms.

The possible mode of action of the purified fraction of *Acalypha wilkesiana* leaves was predicted by culturing the *Candida* isolates in the presence and absence of sorbitol and exogenous ergosterol, respectively. Cell wall and cell membrane are the two most common targets of antifungal drugs. Specific fungal cell wall inhibitors share a distinctive characteristic where their antifungal effects are reversed in media containing sorbitol.This effect is detected by increase in IC_{50} of the purified fraction of ethylacetate extract of *Acalypha wilkesiana* leaves (G7) in the presence of sorbitol (osmotic protectant) as compared to IC_{50} without sorbitol.This is possibly because cells protected with sorbitol can grow in the presence of fungal cell wall synthesis inhibitors, whereas growth would be inhibited in the absence of sorbitol. There was

also increase in the IC_{50} of caspofungin used as a standard with known mode of action which is the fungal cell wall as the target. However, the cell wall is most likely target of subfraction G7 as indicated by the increase in IC_{50} in the presence of sorbitol.This finding conforms with that of Maria *et al*. (2014) which reported that increase in inhibitory concentration of antifungal in the presence of sorbitol implicates cell wall as the possible target. Purified G7 did not form complex with the ergosterol as there was no increasein $IC_{50}$of the G7containing exogenous ergosterol.Amphotericin B was used as a control due to its known mode of action to complex with the membrane ergosterol and createspores that interfere with the membrane integrity leading to leakage of cellular contents and death (Ostrosky *et al*., 2010).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

Four solvents (n-hexane, ethylacetate, methanol and aqueous) were used to extract the phytochemicals from air dried and pulverized samples of *Acalypha wilkesiana* leaves (copper leaves).The results of phytochemical screening showed that methanol extract hadhigher total phenolic and flavonoids content which were significantly different ($p \leq 0.05$) from other extracts.Three clinical isolates of *Candida* strains were characterized as *Candida albicans* I, II and *parapsilosis*. Amphotericin B, voriconazole and caspofungin were standard drugs used in this experiment. Corilagin is a compound purified from *Acalypha wilkesiana* leaves and found to be potent against planktonic cells of *Candida* species as reported in literature. Antifungal effects ofn-hexane, ethylacetate, methanol and aqueous extracts of*Acalypha wilkesiana* leaveswere tested on the planktonic and sessile cells of *Candida albicans* I, II and *C.parapsilosis.* The ethylacetate extractwas the most potent as it was shown to have the lowest MIC and MFC.The ethylacetate extract penetrated the adhered cells of *Candida albicans* I, II and *parapsilosis* at a higher percentage than n-hexane, methanol and aqueous extracts. The median adhered cells eradication concentration of ethylacetate extract was lower than that of n-hexane, methanol and aqueous extract. It was in viewof these that ethylacetate extract was selected for fractionation in a bioassay guided manner using column chromatography-TLC. The most potent fraction was purified using Preparative tin-layer chromatography(P-TLC). IC_{50} of the purified fraction increased significantly in the presence of sorbitol but no significant increase was observed in the presence of exogenous ergosterol which showed that the mode of action of the purified fraction is possibly by inhibiting 1,3-

glucan synthase which is an enzyme that is involved in the synthesis of glucan, an important cell wall component of *Candida* species.Scanning electron micrograph indicated that the purified fraction reduced the biomass of the biofilm to a significant degree.

6.2 Conclusion

Based on the findings of this study, the following conclusions were made:

Ethylacetate extract of *Acalpha wilkesiana* leaves had the potential to significantly reduce thebiomass of *C. albicans* I, II and *C. parapsilosis* sessile cells in a dose dependent manner**.**

Ethylacetate extract of *Acalpha wilkesiana* leaves penetrated the biofilms formed by *C. albicans* I, II and *C. parapsilosis.*

The mode of action of the partially purified fraction of *A. wilkesiana* leaves implicated the fungal cell wall as the most likely target due to theincrease in IC_{50} of the purified fractionin the presence of sorbitol (osmotic protectant) compared to in the absence of sorbitol.

6.3 Recommendations

The purified fraction of ethylacetate extract of *Acalypha wilkesiana* leaves should be further studied to identify the most potentcomponent.

There should be focus on combinatorial therapy for fungal infection in view of reducing resistance of fungal cells to existing drugs.

More studies should be carried out on coatings of medical equipments such as catheters with antifungals particularly those of natural originswith a view of reducing the incidence of nosocomial infections.

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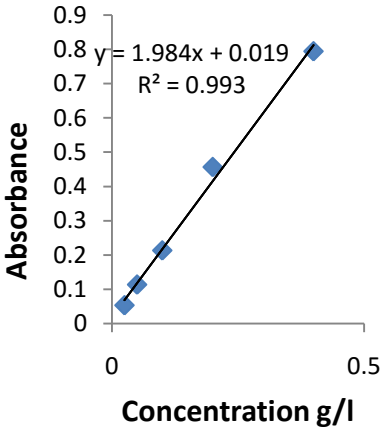
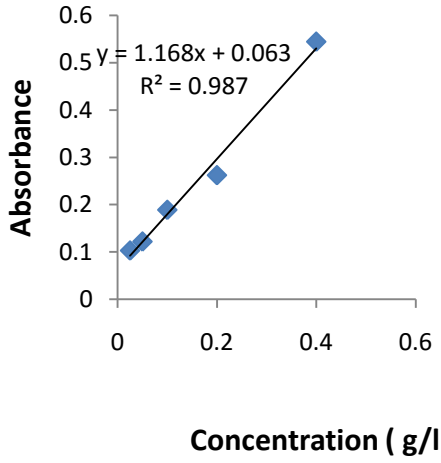
APPENDICES

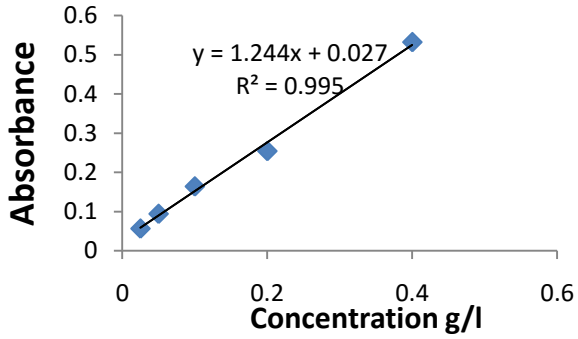
Appendice I: Standard Curves for Determination of Total Phenolics, Flavonoids and Alkaloids



Garlic acid Standard Curve



Quarcetine Standard Curve

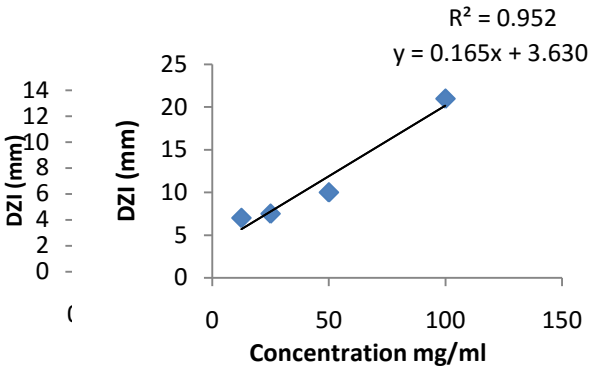


Atropine Standared curve for Total Alkaloids

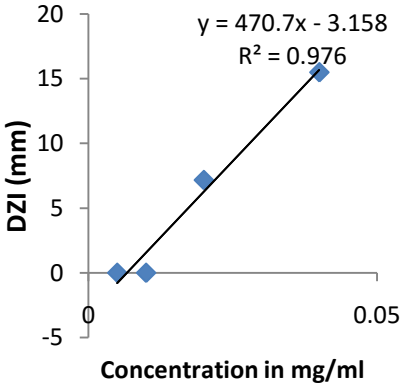
APPENDICE II: Biofilm Formation of *Candida albicans* I, II and *Candida parapsilosis* over 72 Hours Incubation.

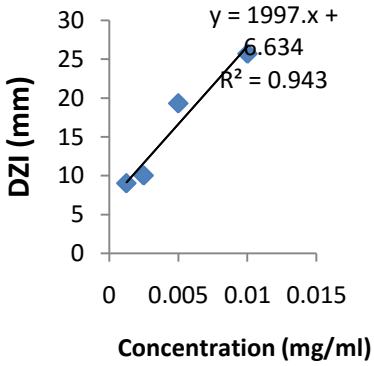
	Log_{10} Biofilm (cfu/ml)		
Incubation Time(Hours)	***Candida albicans I***	***Candida albicans II***	***Candida parapsilosis***
24	8.85±0.67	6.61±0.4	6.36±0.76
48	9.35±0.98	7.45±0.86	7.34±1.1
72	9.89±0.3	7.87±0.7	7.88±0.9

APPENDICE III :Standard Curve of Ethylacetate, Methanol, C orilagin, Voriconazole, Ampotericin B and Caspofungin on Biofilms of *C.albicans I*, *C. albicans II* and *C.parapsilosis*, respectively : Diameter Zone of Inhibition (DZI) plotted against concentration of the Antifungals.

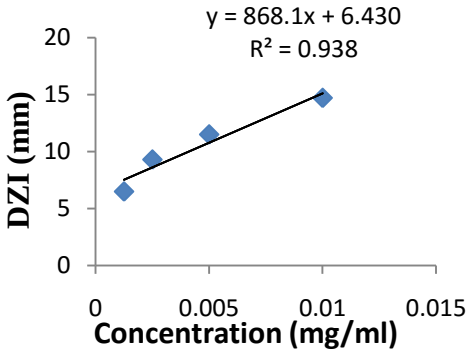


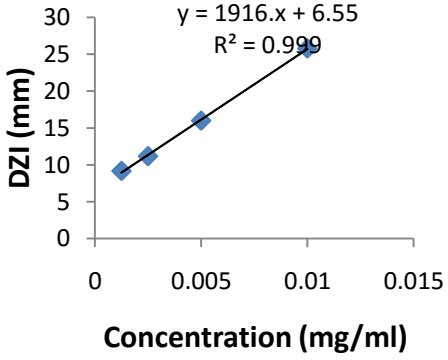
Standard Curve for Ethylacetate and Methanol Extract of *A. wilkesiana*on Biofilms of *C. albicans I.*





Standard Curves for Corilagin and Amphotericin B on *C.albicans I*





Standard Curves of Voriconazole and Caspafungin on Biofilms of *C. albicans I.*

APPENDICEIV: Mean Values of Penetrability Concentration (C / Co) of Ethyl acetate, Methanol Extracts of *Acalypha wilkesiana,* Corilagin, Voriconazole, Amphotericin B and Caspofunjin on *Candida albicans (I and II) and C. parapsilosis*

Candida albicans I

Time (minutes)	**60**	**120**	**180**	**240**	**300**	**360**
Ethylacetate	0	0	0	0.18	0.35	0.47
Methanol	0	0	0	0	0.3	0.4
Corilagin	0	0	0	0.54	0.64	0.78
Amp. B.	0	0	0.04	0.06	0.18	0.52
Voriconazolel	0	0	0.07	0.14	0.18	0.38
Caspofungin	0	0	0.095	0.12	0.19	0.49

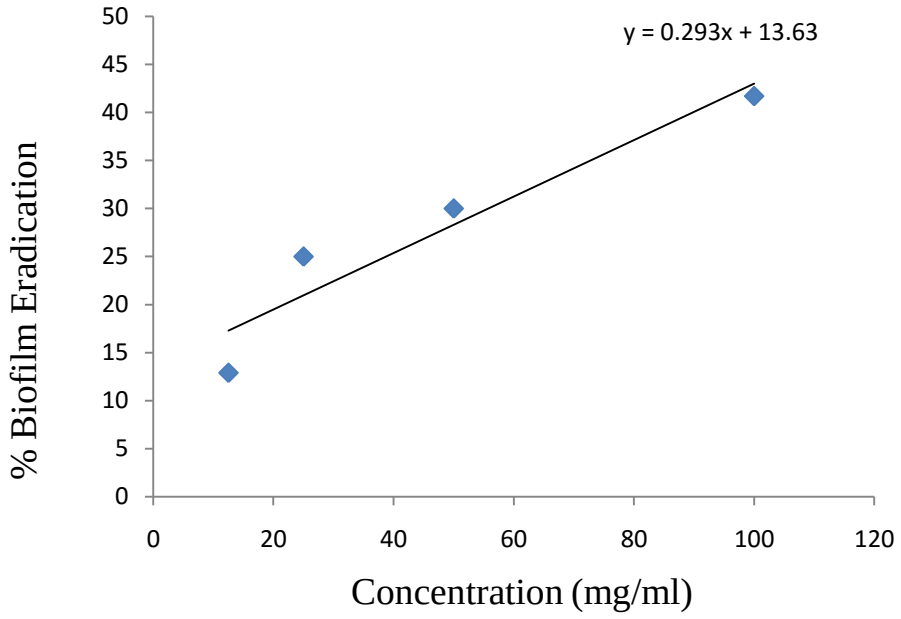
C. albicans II

Time (minutes)	**60**	**120**	**180**	**240**	**300**	**360**
Ethylacetate	0	0	0	0	0.51	0.7
Methanol	0	0	0	0.02	0.03	0.04
Corilagin	0	0	0.56	0.71	0.75	0.89
Amp. B.	0	0	0.06	0.13	0.2	0.6
Voriconazole	0	0	0.08	0.17	0.23	0.36
Caspofungin	0	0.02	0.14	0.18	0.29	0.62

C. parapsilosis

	60	**120**	**180**	**240**	**300**	**360**
Ethylacetate	0	0	0	0.09	0.2	0.31
Methanol	0	0	0	0.14	0.25	0.14
Corilagin	0	0	0	0.05	0.06	0.07
Amp. B.	0	0	0.08	0.07	0.11	0.17
Voriconazolel	0	0	0.14	0.14	0.17	0.34
Caspofungin	0	0	0.05	0.05	0.07	0.39

APPENDICE V: Extrapolation of $BEC_{50}$of Ethylacetate Extract from Plot of %Biofilm Eradication of Ethylacetate Extract on *C. albicans I*against Varied Concentration



BEC_{50}

From the linear equation above,

Y=0.2936x + 13.639

When Y= 50

50= 0.2936x + 13.639

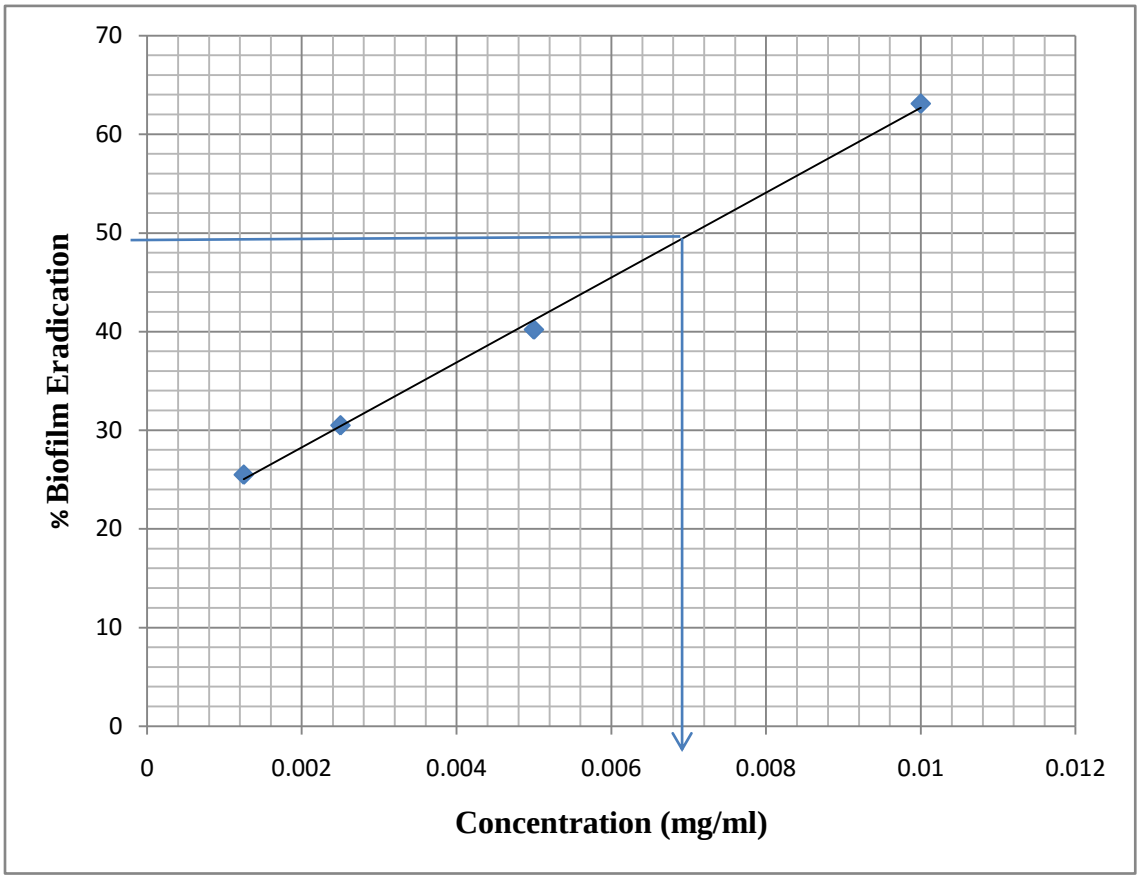
50-13.639= 0.293x

X= 36.36/0.293

X= 124.1

Therefore, BEC_{50} of G7 on *C.albicans I* = 124.1mg/ml

APPENDICE VI: Extrapolation of BEC_{50} of Voriconazole against *C.albicans I* from Plot of %Biofilm Eradication of Voriconazole against Varied Concentration



Linear equation from the graph Y = 4305.4 x + 19.643

When y= 50,

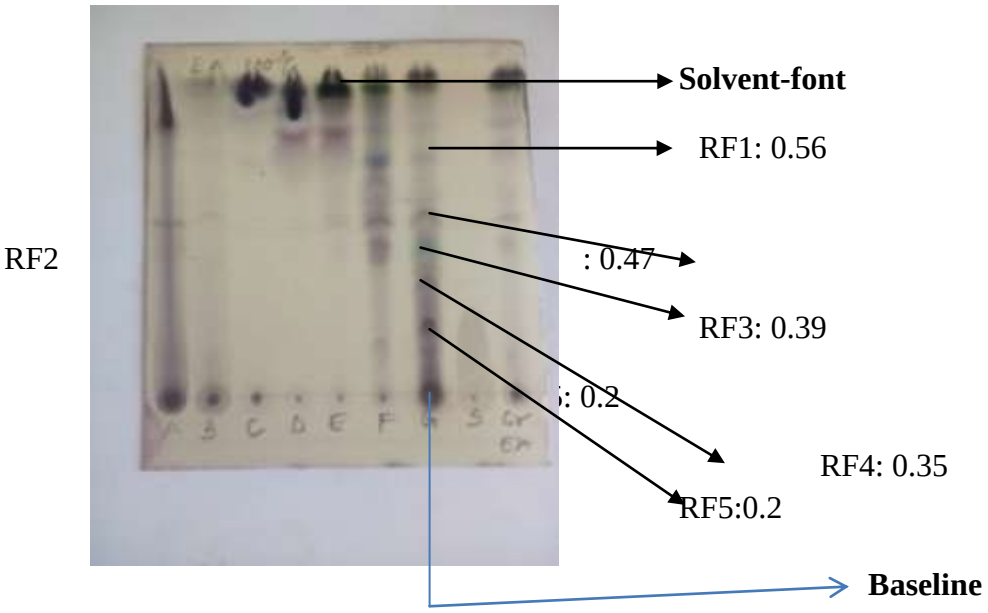
50= 4305.4x + 19.643

50-19.643= 4305.4x

X= 30.36/4305.4= 0.0071

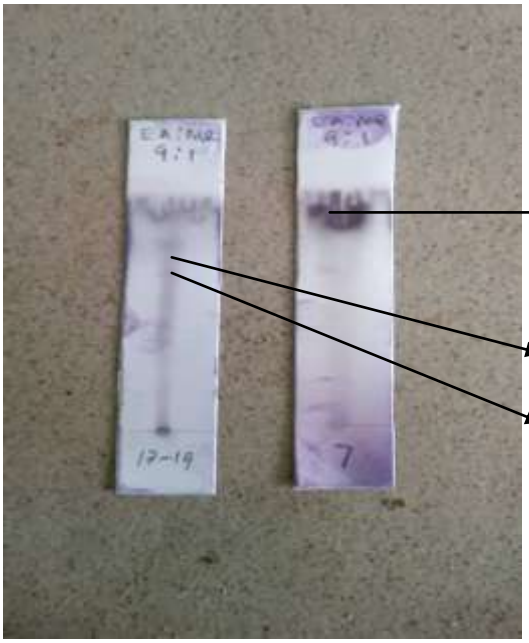
BEC_{50} of voriconazole on *C.albicans I* = 0.0071 mg/ml

APPENDICE VII: Chromatogram and Retention Factor of Fractions of Ethylacetate Extract Developed in Absolute Ethylacetate



APPENDICE VIII: Chromatogram and Retention Factors of Subfractions G7 and G17-19 sprayed with Vanillin Sulfuric Acid (i) and Viewed with UV Lamp (ii).

(i)



Rf 0.83

Rf 0.7

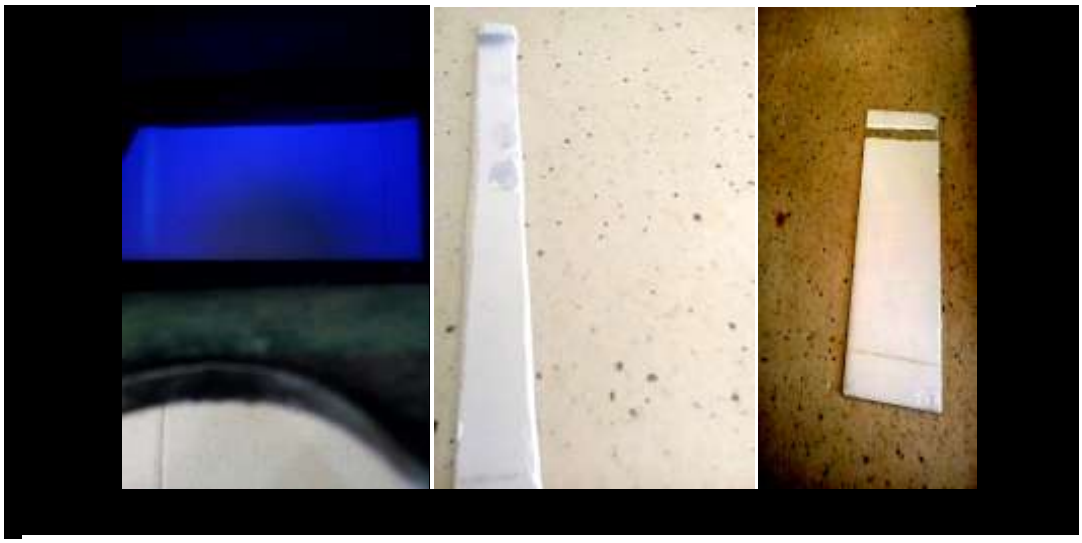
Rf 0.65

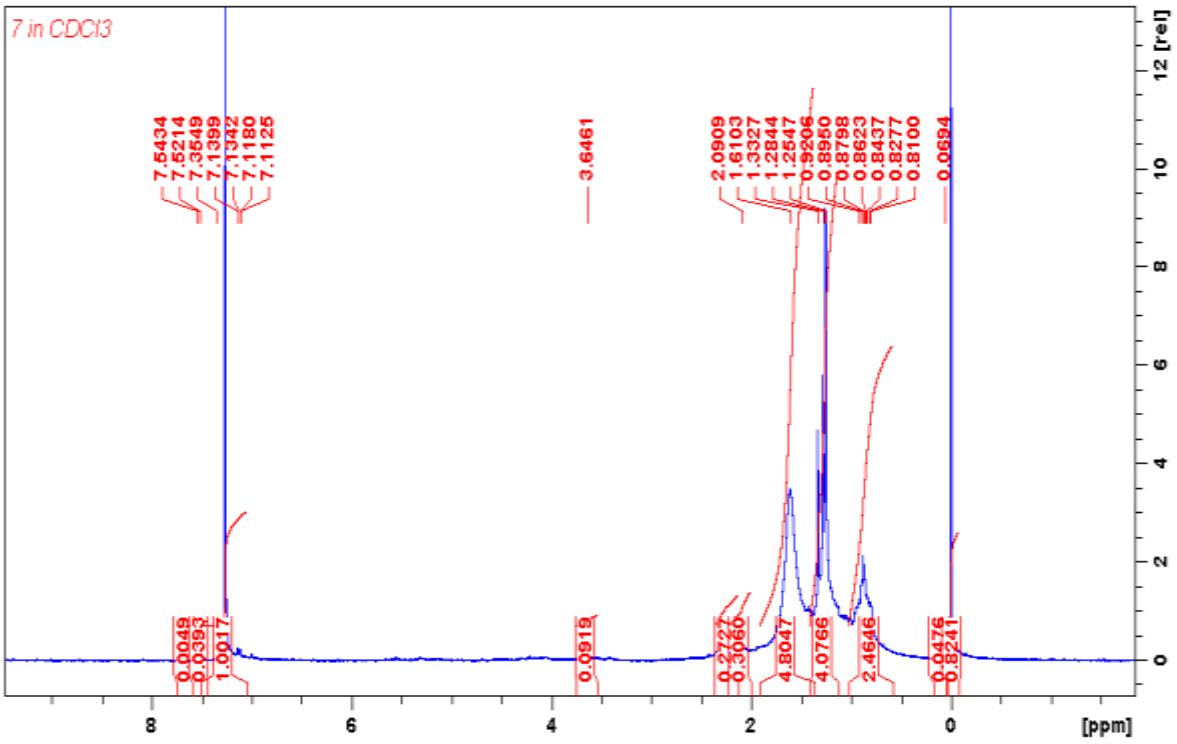
(ii)

APPENDICE IX:Chromatogram of G7 developed in two solvent system (n-hexane : Ethyl acetate 2:8) which glows when viewed with UV Lamp (A1) the TLC glass sprayed with Vanillin Sulphuric Acid which showed purple

colored spot (A2) and Scraped (A3): Proton- NMR Spectra of Purified Fraction G7

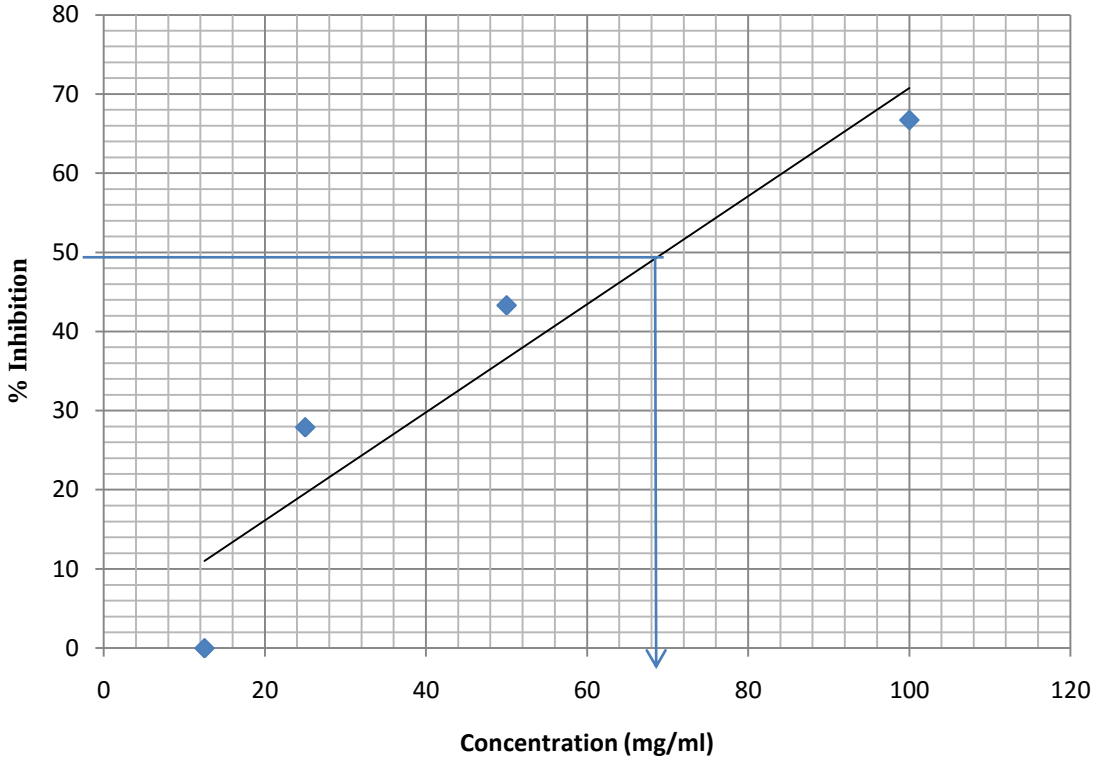
A1 **A2** **A3**



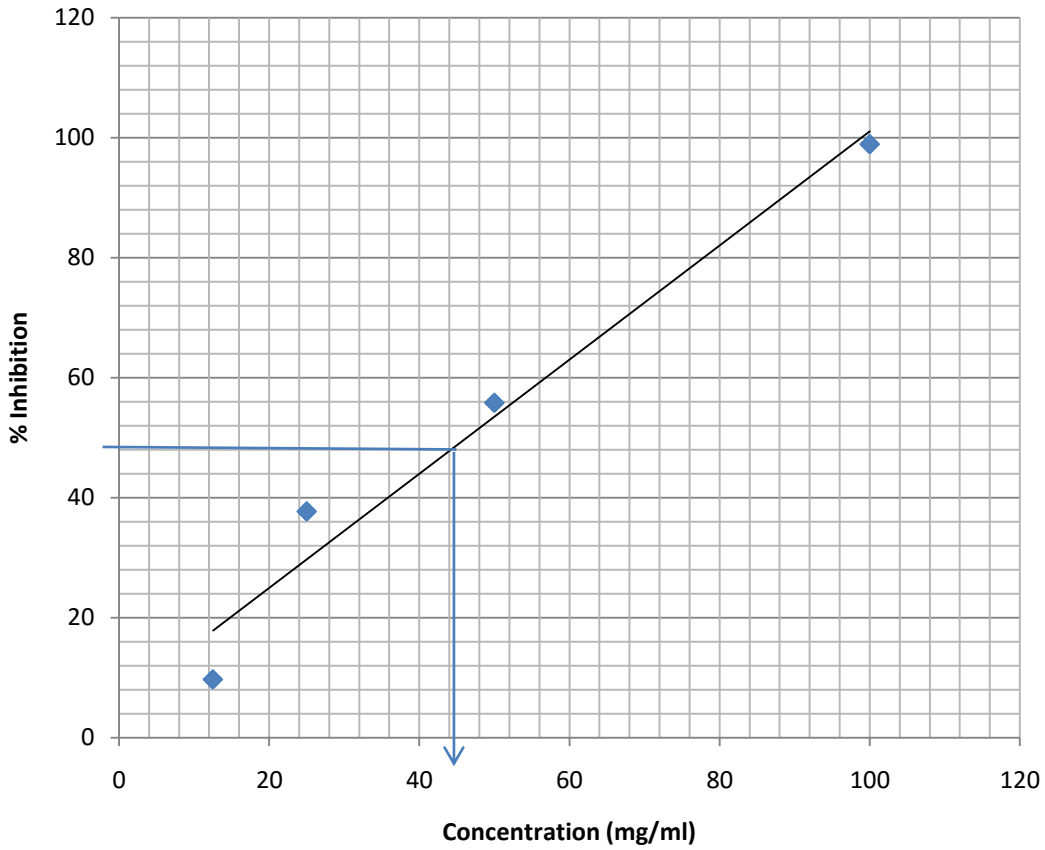


Proton- NMR Spectra of Purified Fraction G7

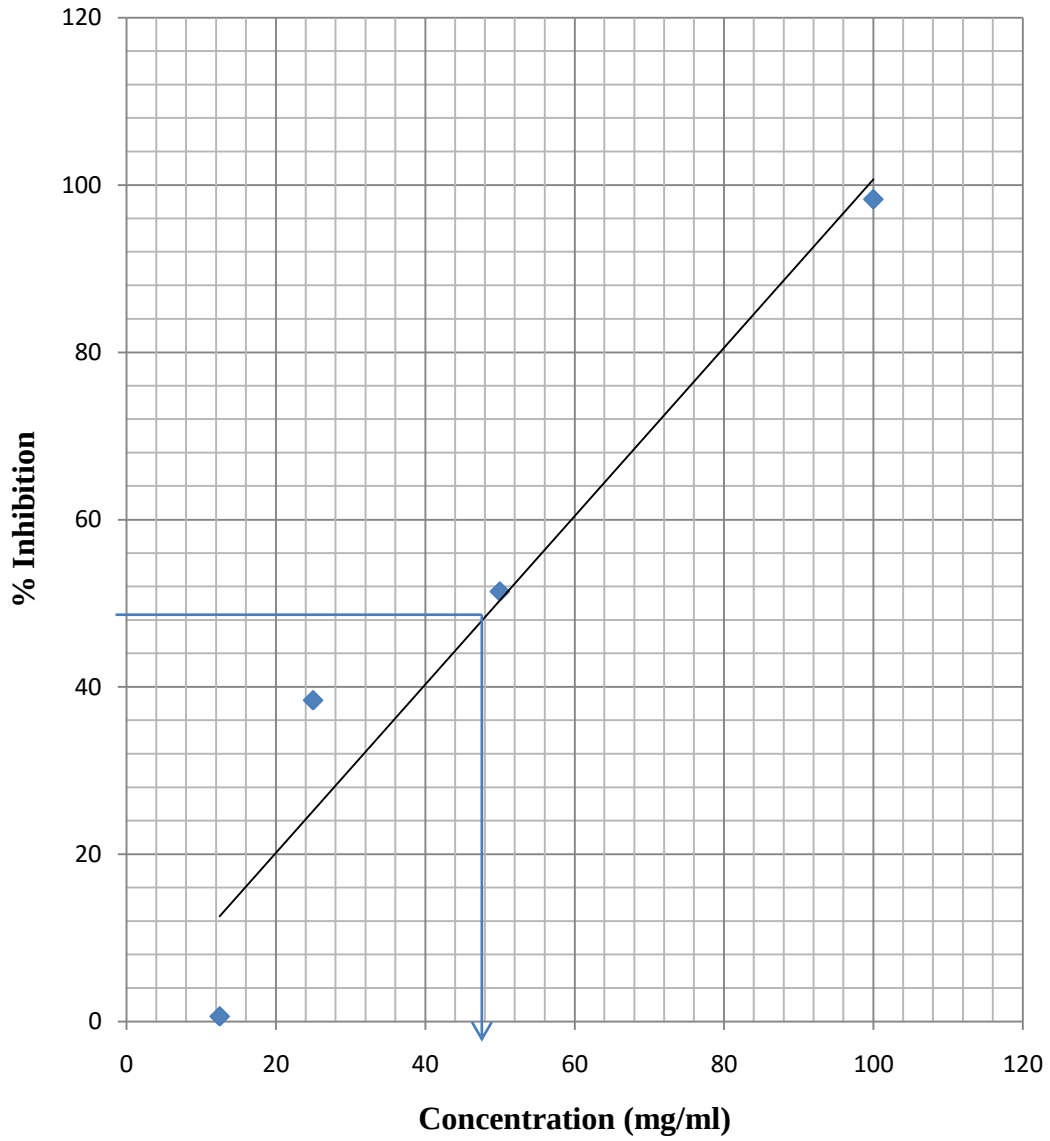
APPENDICE X: Extrapolation of IC_{50} from Plots of % Inhibition against Concentration of the Antifungals.



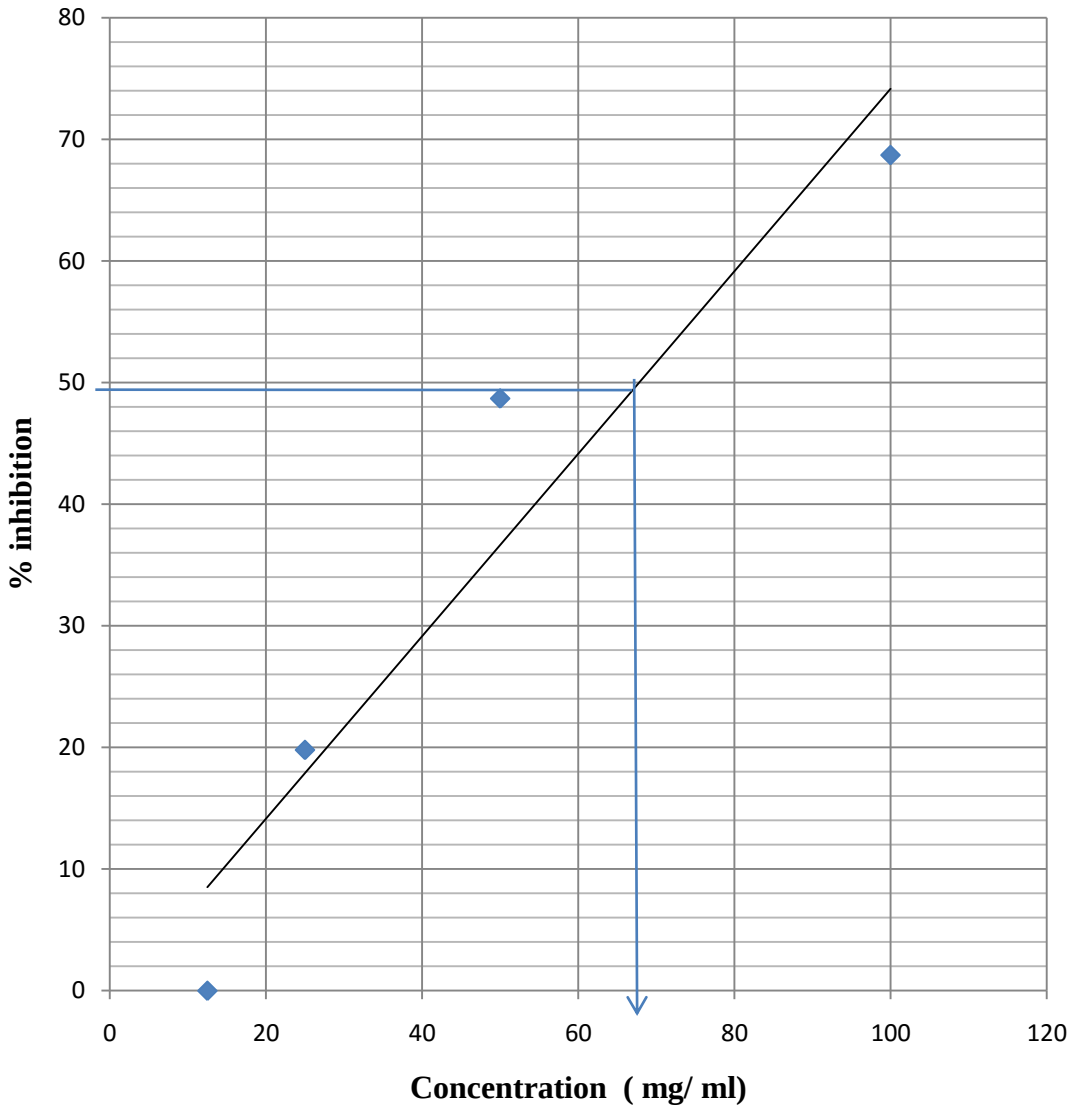
IC_{50} ofn-Hexane Extract against Planktonic Cells of *C.albicans* I



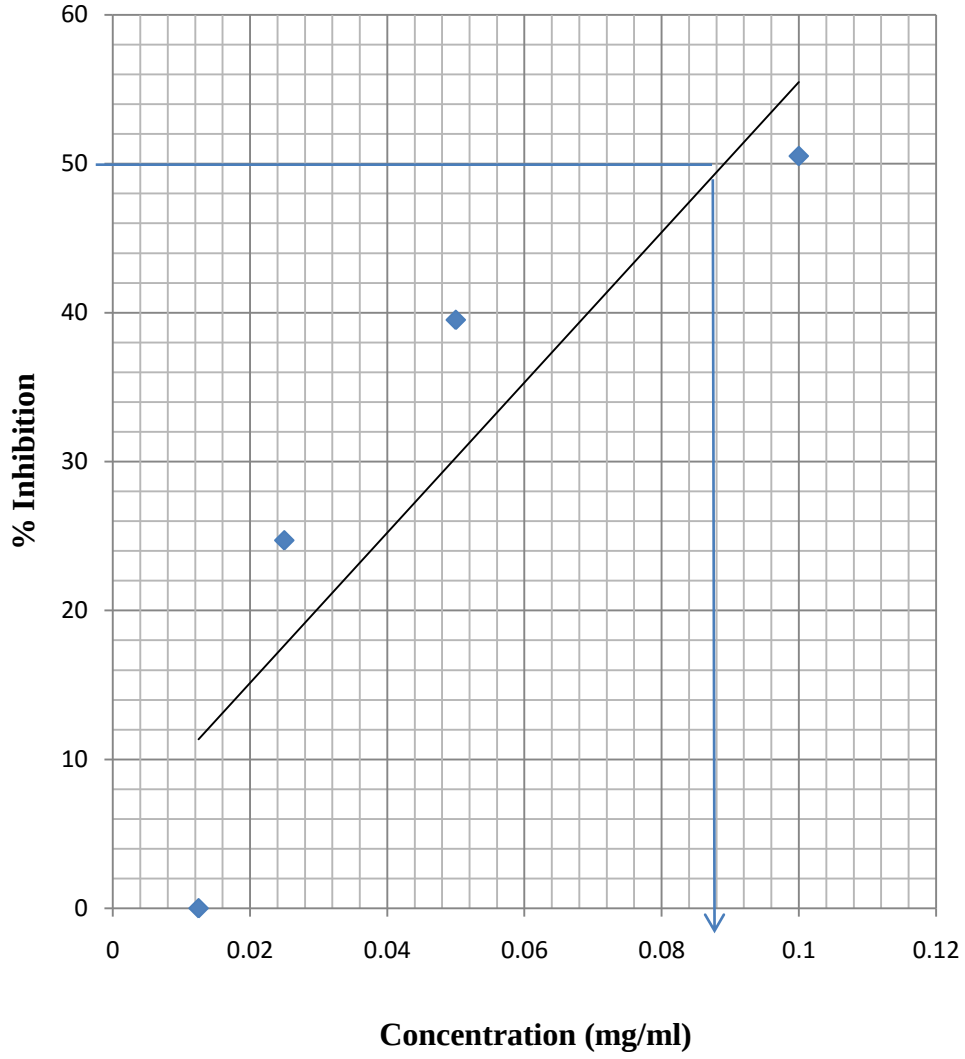
IC_{50} ofEthylacetate Extract against Planktonic Cells of *C.albicans* I



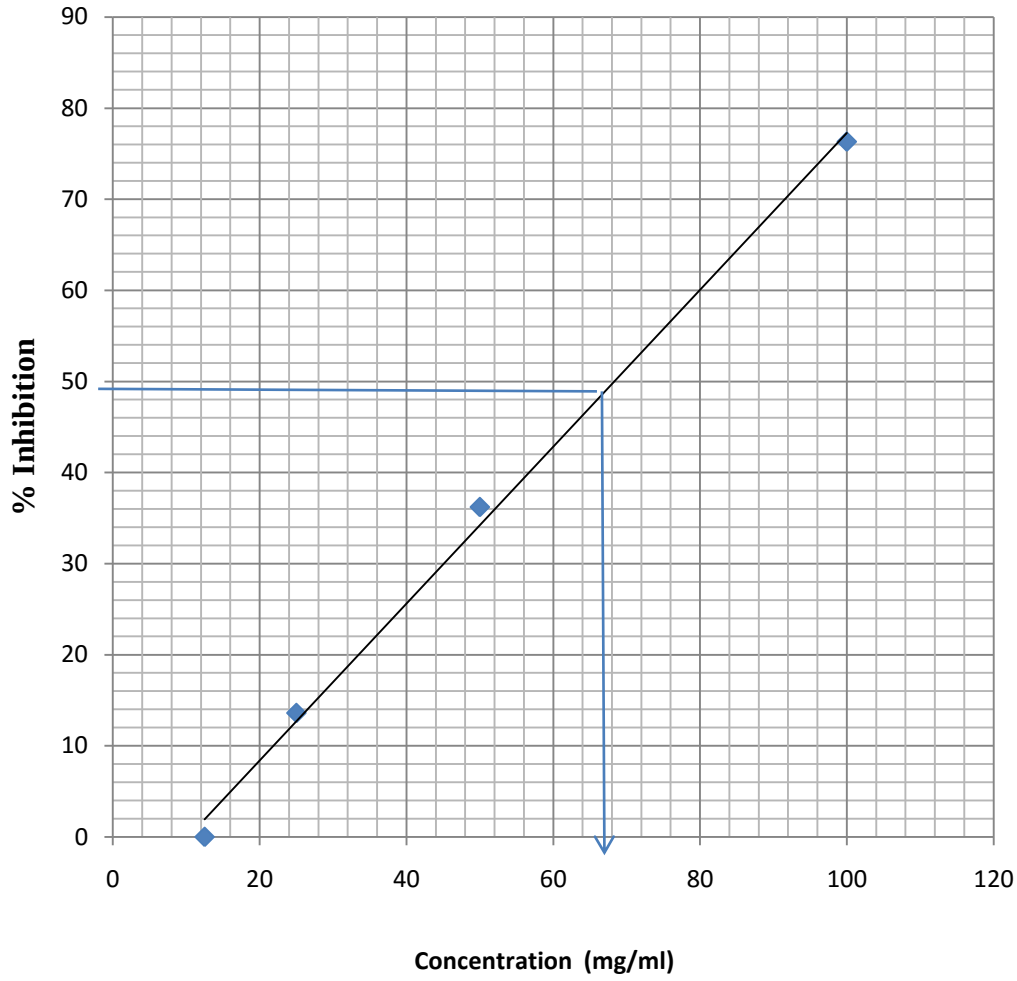
IC_{50} of Ethylacetate Extract against Planktonic Cells of *C.albicans*II



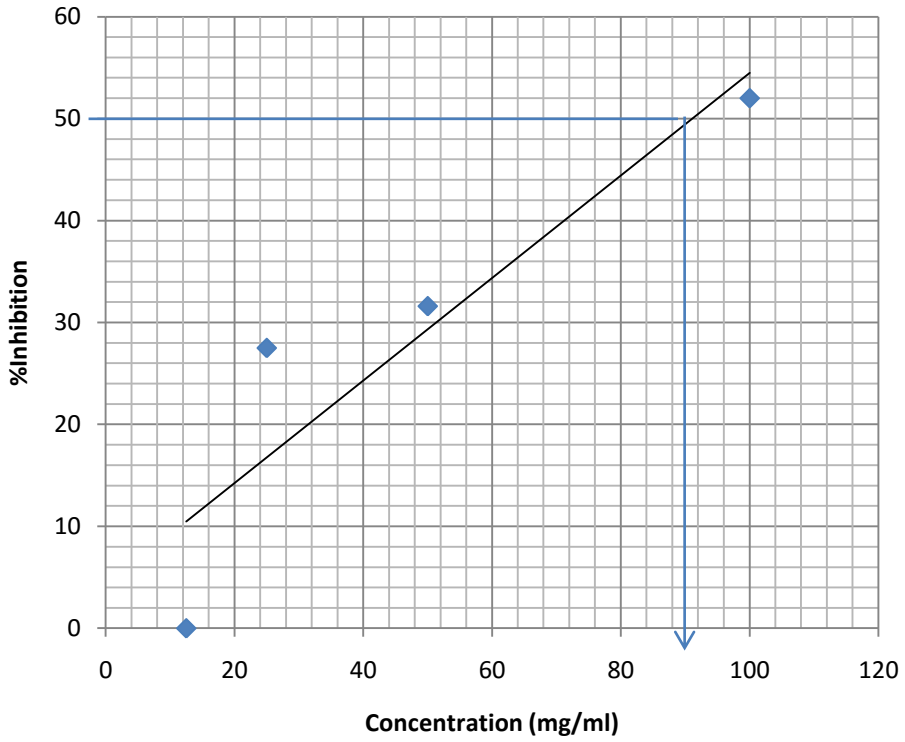
IC_{50} of Aqueous Extract against Planktonic Cells of *C.albicans* I



IC_{50} of Corilagin against Planktonic Cells of *C.albicans* I



IC_{50} of Methanol Extract against Planktonic Cells of *C.parapsilosis*



IC_{50} of n-Hexane Extract against Planktonic Cells of *Candida albicans II*