# CHARACTERIZATION OF EFFECTS OF PLANT EXTRACT (NUTMEG AND PEPPER) ON HYPHAL MORPHOGENESIS IN *Candida albicans*

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## 1. INTRODUCTION

*Candida* species are normally harmless commensal organisms of the gastrointestinal and genitourinary tract but sometimes in immunodeficiency conditions of host this fungus becomes pathogen which causes a range of disease from superficial to life threatening systemic superficial infections in human immunodeficiency virus (HIV) patients and immunocompromised individual. Life-threatening blood branched infections along compromising or threatened intensive care patients (mainly those undergoing cancer chemotherapy or immunosuppressive medication because of organ or bone marrow transplant procedures). They spread out or enlarge in many different or distinguishable morphological forms which purview or reach from unicellular budding yeast to true Hyphal with collateral or symmetric -side wall **(Sudbery P et al., 2004).** In a typical manner regularly, *C. albicans* capture about or nearly 70% of the population or group of individuals and *C. albicans* are mainly occurring as harmless commensal i.e. it profits other without any negative effect while some *C. albicans* are also found in the urinary tract and GIT in human body**. (Kabir M AA et al., 2012).**

It is found that *Candida* causes some kind of diseases like oral, pharyngeal, vaginal, and invasive or incursive Candidiasis. It is also found that approx. 70% of the females will face or experience these kinds of diseases which are influenced by *C.albicans*, generally vaginal Candidiasis, in their lifespan. More often, the infections occur by *C.albicans* is inheritable by patients in hospitals with the death rate of approx. 45% **(Pfaller MA *et al.*, 2006).** Oral and pharyngeal fungal infection is a white spot or patch of C.albicans on mucous membranes and this fungal disease come about or arise in the buccal chamber or throat. Vulvovaginal fungal infection is known by the unreasonable growth of Yeast in the vulvovaginal area causing some haptic sensation, and extraction of fluid from the vaginal region. Infection comes about when the *Candida* move into the inpatients blood

stream and can conveniently spread through a higher level to almost many distinct organs of end to end body. Incursive candidiasis is best acknowledged when body (of host or individual) fever becomes incurable with antibiotics. Mainly, azoles are meant for curing Yeast or fungal diseases while it is also found that fluconazole is meant for curing some intense disease **(Ortega *et al.*, 2011).**

The Pathogenecity of *Candida* species is characterized by definite hostility parameters, likewise the acquirement to make a sudden movement of human body immunity, acceptance, formation of *C.albicans* surface biofilm (on human body or organelle surfaces and on instruments) and bring out of organ-detrimental enzymes likewise nucleases, lipases and peptidases. Unwanted or unenviable responses, noxious and impression of azoles blocking are the drawbacks for application of these antifungal medicine. Also major cause or parameter liable for human or mammalian illness is the colonization of azoles impedance of surface aggregation of *C.albicans* by the *C.albicans*. Also the large or big quantity of *Candida* biofilms **(Rajendran *et al.*, 2010),** planktonic cells inside fungal tissue surface aggregation are mainly dominated to antifungal parameters than are permanently attached to substratum or stalk less cells **(Kuhn & Ghannoum, 2004).** The development or progress of drug resistance inside *Candida* biofilms has been related with an integrated shoot up in the full development process. In addition, many researchers have uncovered that in the occurrence of a simple or alleged surface, cellular matrix of yeast have embroiled consistently and same grade of prevention to azoles i.e. (Terconazole and Nistatin) is exhibit or show as cells grown in a shaker and disclosing ample quantity of aggregated biofilms. The gain in defiance *Candida* species restrains a need for fresh novel targets for new antifungal or anti *Candida* agents.

## 1.1 ***Candida***

*Candida***,** genus of yeast and is usually give rise to fungal disease worldwide. Many species are benign or innocuous commensal or endosymbionts of mammalian hosts including humans; despite anything to contrary, when mucosal barriers are disrupted or breaks or the immune system is compromised they can attack or infest and cause disease **(Ortega *et al.*, 2011).***Candida albicans* is very commonly discriminated or secluded species, and can cause infectious disease (Candidiasis or thrush) in humans and other animals. Many species of *Candida* are found in intestinal microbial flora, including *C. albicans* in mammalian or human hosts, whereas others

live as endosymbionts in insect hosts. Systemic infections of the bloodstream and major organs (Candidemia or invasive Candidiasis), particularly in immunochallenged patients, affect over more than 90,000 people per year in the U.S (**Eggimann *et al*., 2003**). The DNA of several *Candida* species has been sequenced. Antibiotics contribute to the progress of *Candida* infections or disease, including gastrointestinal *Candida* profusion of growth and penetration of the gastrointestinal mucosa. While females are more unprotected or predisposed to genital yeast infections, men can also be infected. Some agents or factors, such as relatively long use of antibiotic, increase the risk of infection for both men and women. People with diabetes or impaired immune systems such as those with HIV, are more susceptible to yeast infections. *Candida Antarctica* is a source of industrially important lipases.

## 1.2*C.albicans* Pathogenesis

The proficiency or fitness of fungal strain or yeast to pathogenesis to varied hosts is because of ample of Pathogenecity inducing features or components and many distinctive attribute of *C.albicans*. Also characters like Hyphal transformation, impression or persona of some organic compounds on the superficies of *Candida*, synthesis of *C.albicans* through sensing, biosynthesis of *C.albicans* on superficies of medical or surgical tubes and hyper formation of proteolysis enzymes. *C. albicans* is polymorphic or polymorphous yeast which can mature or divides also as circular shaped and longitudinal simple unsubdivided cells having narrowing or bottleneck at the septa (pseudohyphal) or as parallel- walled true Hyphal. To a wide level or point outer or physical appearances consists light colored and opaque cells, formed while shifting, and dictiospores, which are thick-walled spore-like lookout. While Candida or yeast are regularly appear or feel during Pathogenecity and have pellucid role (as shown in next section), the function of false hyphal and movement inside body is also not clear and spores have not been found inside patient body. The movement in the interval of non hyphal and Hyphal is known as dimrphism and also awaited or anticipated that yeast and hyphal forms are disease inducing. The hyphal form has been found to be to a great extent offensive in comparison with non hyphal forms. On the contrary the littler yeast is thought anticipated or thought to display the form primarily involved in incident or circulation.

**Biofilms expression**: At a greater extent important disease causing cause or parameter of *C. albicans* is its capability or adaptability to imply fungal mass depositions on living or dead substratum. Surgical tubes used (dead) and intestinal cell substratum (living) is the higher degree most common substrates. Biofilms form in a sequential or ordered way including adherence of Yeast cells to the substrate, fractionation of these fungal or *Candida***,** synthesis or aggregation of pathogenic yeast in the more higher part of the yeast aggregation on membranes, development of Matrix material outside cell or tissue and, finally**,** circulation or transmission of yeast cells from the biofilms complex. Developed or grown up biofilms are highly resistant to antifungal agents and host immune causative intrinsic factor in analogy or confrontation to individual's tissue. The intrinsic causative factors liable for elevated impedance include the non simple structure of biofilms, the cell aggregate surfaces, higher synthesis of azoles effluence pumps and metabolite rigidity. Self indulgence of fungal cells from the well mature biofilms has been shown to inducing Pathogenecity, as degenerated cells were higher disease causing in a mouse model of dispersed or distributed infection.

**Thigmotropism**: A significant environmental mark or signal that activates or takes place fungal/hyphal and biofilms biogenesis in *Candida* (figure.1) is getting through sensing of fungal cells by an abiotic surface. On sensing with an abiotic surface, yeast cells switch or change accordingly to hyphal growth. On definite but not specified substrates, like as agar or mucosal surfaces, this Hyphal can then encroach or intrudes into whatsoever surface. Contact to any solid substratum also influencing or leading to the formation or biosynthesis of biofilms. On surfaces with certain or definite topology (like presence of natural depression) hyphae growth (contact sensing) occurs.

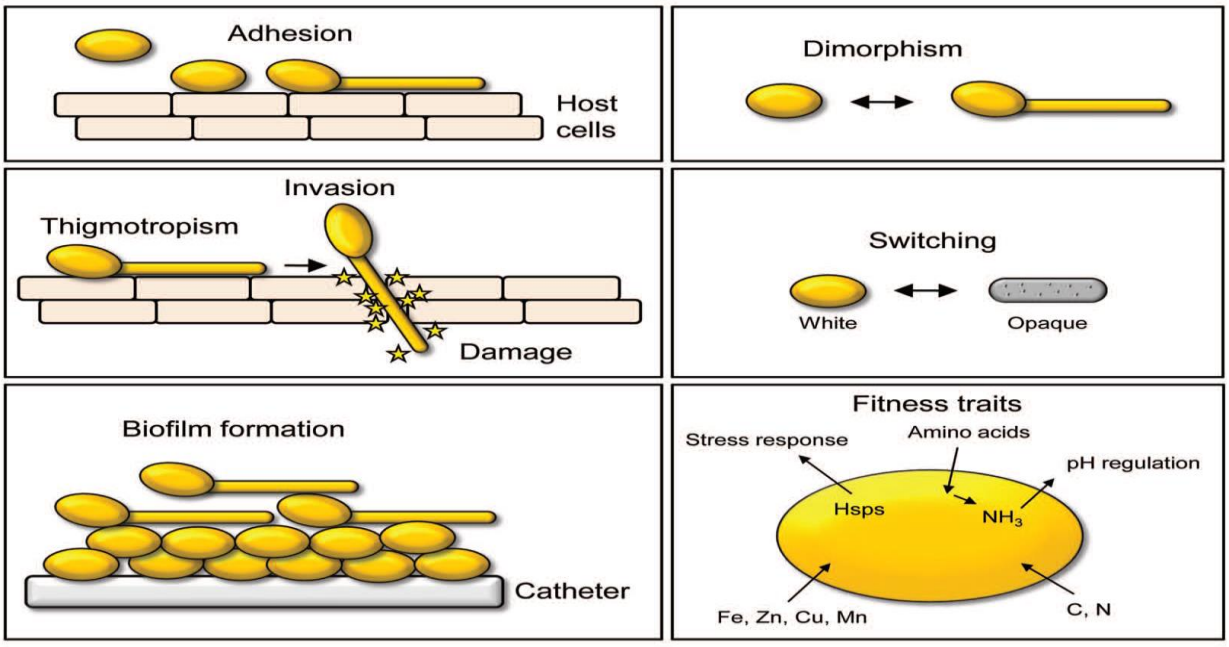


**Fig1: *C.albicans* Pathogencity methods (Mayer F *et al*., 2013)**

## 1.3. Microbial Resistance

It can be primary resistance (intrinsic) or secondary resistance (acquired):

- **Primary resistance**: It is establishing of course among specified fungi without previous vulnerability to the drug and underscore or bring out the importance of identification of fungal species from clinical specimens. Examples have or cover resistance of *Cryptococcus neoformans* to echinocandins and *Candida krusei* to fluconazole.

- **Secondary resistance:** It evolved among previously susceptible strains after vulnerability to the antifungal or antimicrobial agent and is generally relying on altered gene expression. The development or evolution of fluconazole resistance microbes among *Candida* albicans and also C. neoformans strains illustrates this type of resistance.

## 1.4. Antifungal Drugs

There are many classes of antifungal drugs present but some important ones are azoles drugs and polyenes drugs with different actions on fungus or *Candida* like polyenes dugs works on nucleic acid biosynthesis of fungus while azoles drugs act differently on fungus i.e. it repress ergosterol synthesis**.**

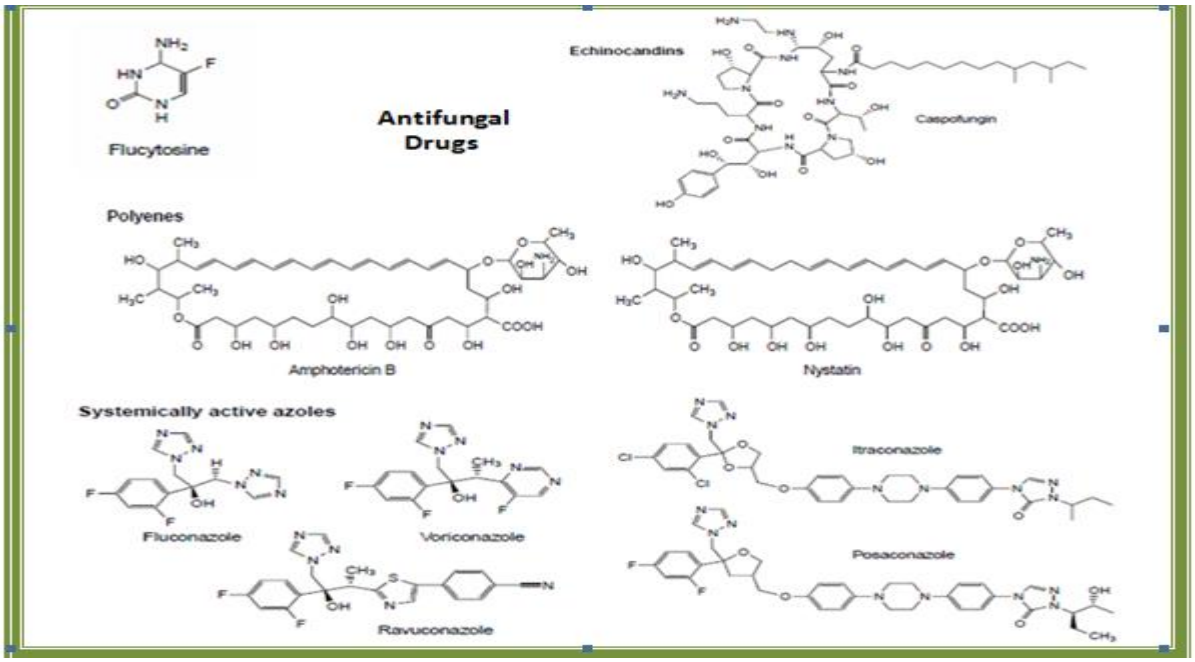


**Fig.2 some antifungal drugs**

## 1.5 Antifungal or Anticandida Drug Targets

There are 4 major antifungal drug or place targets in *C. albicans*:

The fluorinated pyrimidine similar 5-fluorocytosine causes abnormal or deviated RNA synthesis and hinder or get involved with DNA replication (**Akins, 2005; Sanglard and Bille, 2004).**

1) The latest developed class of antifungals is the cyclic lipopeptides i.e. echinocandins. These antifungals are thought to impede with fungal plasma membranes synthesis by resisting an enzyme Glucan synthase and are antifungal or arrest fungal growth *C. albicans*

2) There is an impression that Polyenes are also influence oxidative damage. The azoles antifungals, such as the triazole and fluconazole, are hinder or impede with sterol biosynthesis
3) Some antifungal drugs like polyenes (amphotericin B and nystatin) are heterocyclic chemical group that imbed or plug into sterol bilayers, attached or get bonded to sterols, and clustered mass in circular to forms mall opening in membrane. Fungal membrane wholeness or rawness is disrupted or discontinues through these epithelial channels and outflow of positively charged atoms likewise potassium ion, which is antifungal.

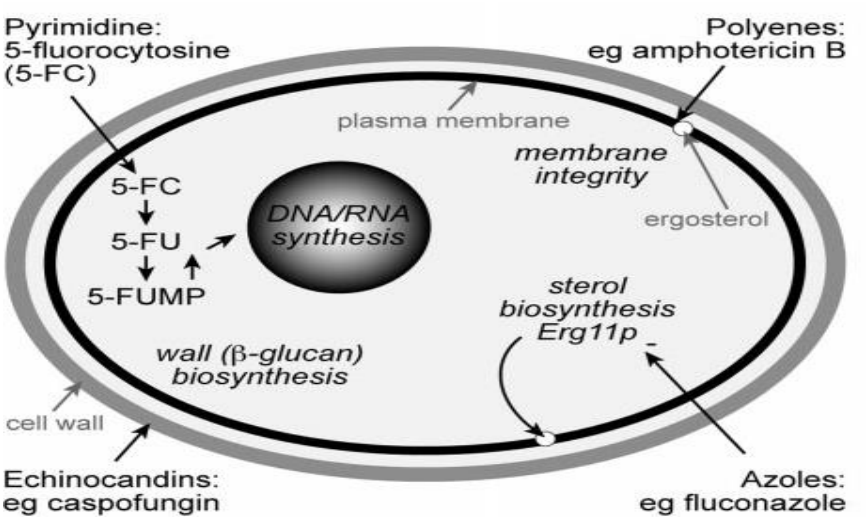


**Fig3: *C.albicans* diverse drug targets (Cannon R.D *et al.*, 2007)**

## 1.6. Mechanisms of Azole Resistance

- **Up-regulation of target enzyme:** Found that some of the *Candida albicans* sporadic with weakened or shriveled sensitivity to anti-fungal drugs have more intra-cellular concentrations amount of *ERG11p* than do fungal drugs-vulnerable or perusable variety. It is found that the azoles or drugs used against *Candida* is, known now, induced and grooves therapeutic levels or amount can no longer efficaciously obstruct or forbids ergosterol biosynthesis. *ERG11p* encoded enzyme up-regulation or increase can be attained through gene amplification or gene caption or figure, elevated or high transcription rate, or decreased debasement or degradation of the gene product. Moreover, this methodology or process is seen to contribute diminutive to

the overall resistance encumbrance or load in *Candida* species, this is obviously because of only humble or unassuming increases in enzyme levels have been described.

- **Reduced antifungal drug concentration:** The advancement of efflux pumps impel reduction in antifungal drug concentration or amount at/near the target site. Efflux pumps are coded or encrypted in *Candida albicans* by mainly 2 genetic cassettes of instrument of transmission: the *CDR* super family gene, and *MDR* super family genes of the leading supporter or assistant group. Up-regulation of these two gene super family has been incontestable in azoles drugs or fungal-resistant *C. albicans*. There are some other gene families that are used for transportation in *Candida*, like *MEP* and *eft 2* and in *C.grayi*. Also found that this *CDR* gene upward-regulation focuses on or revolved around defense to all the antifungal drugs, Multi Drug resistance encrypted outflow channels have a very narrow ambit window and are very specialized for antifungal drugs. (**Kanafani Z.A *et al.*, 2009)**

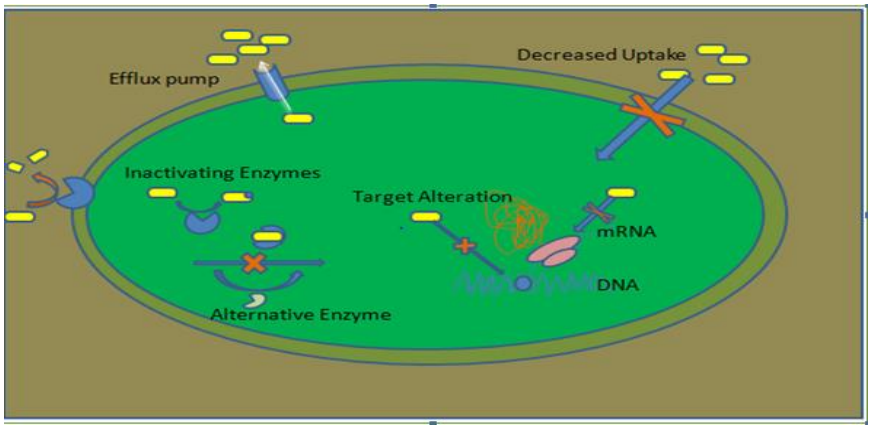


**Fig.4 Different Drug Resistance Mechanisms**

## 1.7. Importance of Lipid in *Candida*

Lipids are critical or indispensable structural components of biomembranes or plasma membranes and play a valuable or principal role in cellular signaling, plasma membrane micro domain organization or structure and dynamics, membrane aggregation or assemblage and energy storage exploiting cellular lipid droplets and also plasma lipids and proteins. There are various sections or types of yeast lipids, among which plasma membrane sterol is one of the valuable and critical constituents. It is liable or prudent mainly for rigidity and stability of

membrane which resistance from physical stresses (Mukhopdhyay K *et al.,* 2004). It is found that azoles have adverse effects on fungal plasma membranes. Therefore modification or alteration of it is more often caused by sterol forfeit, giving rise to much more increase in membrane permeability and fluctuation or drop-off of azoles sensitivity in fungal cells. Falsifications suggest that dysfunctional import of azoles or drugs involve membrane dysfunctions via**.** Decrease in the ergosterol profile is key factor in antifungal drug resistance. The engagement of membrane lipid phase in drug resistance is now deciphered in the dysfunction and it is also seen that (Pap/MDR1) of human is homologues to ABC drug transporter and it affects the structural integrity and arrangement of lipids (Lave Y *et al.,* 2001). The rise or beginning of drug resistance in serial isolates of *C. albicans* from patient's implicit antifungals or azoles cure is in most cases from antecedently more vulnerable strain **(Prasad T *et al.,* 2013).**

## 1.8. Distinct Morphological Forms Of *Candida albicans*

- **Mating protrusion:** It is pioneer that a shmoo, opaque cell respond to the mating pheromone present in the encompassing by elongating a mating projection. It is seen that the nucleus moves in vicinity to projection and then undergo fusion.
- **Yeast-form growth:** A blastospore buds off a novel cell, concomitant or accompanying into 2n cells. Spindles are instantly present in the detached or isolated spindle bodies that compound the chromosomes to the facing or other side of mother-daughter crossway**.**
- **Pseudohyphae growth:** Also the band i.e. septin band is also traversed by from nuclear fragmentation. **(Whiteway M *et al.,* 2007)**
- **Hyphal growth:** It is seen that due to division of nucleus toward the direction of germ tube one of the nucleus moves far away while other come back to mother cell.

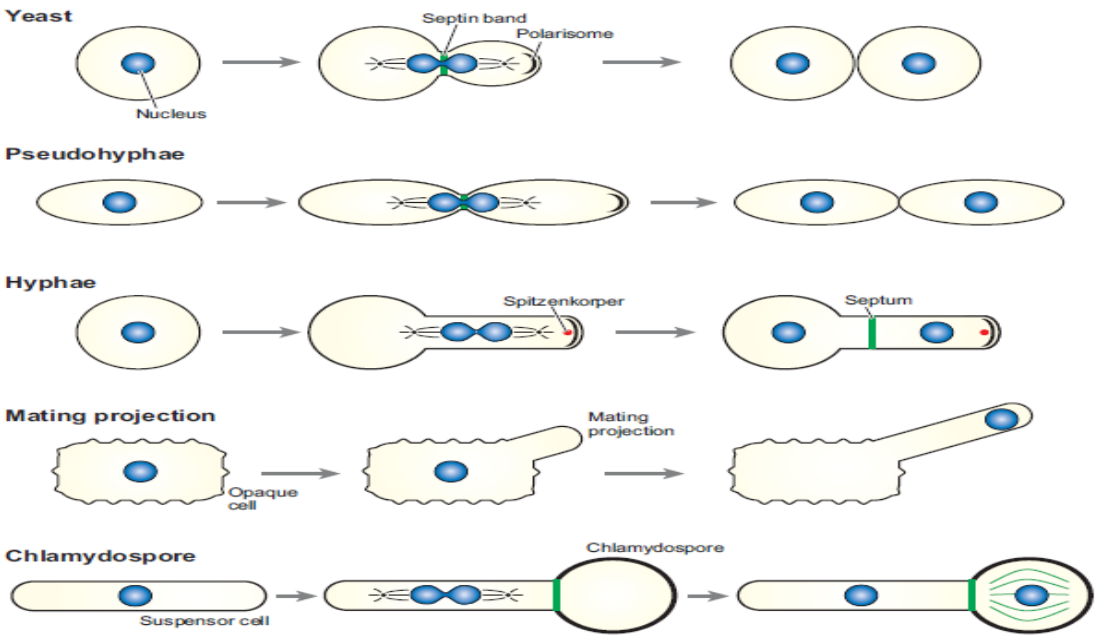


**Fig 5: Different structural forms of *C. albicans***

## 1.9. Why Plant Extracts Used As Antifungal Drugs

Recently it is found that due to the multiple advancement of drug resistance in human microbes and also the impression of drug resistance strains leads to the continuous search for new antifungal agents or antimicrobial agents without toxicity and side effects. Although a huge number of antimicrobial or antifungal agents have been discovered to treat pathogenic microorganisms but developing resistance to these agents is main involve. Thence it is requisite to seeking for more impressing or efficacious and less toxic novel antifungal or antimicrobial agents that would overcome or pull through above limitations. It is also found that *C. albicans* is famous for inducing Candidiasis. In dictation to tackle the difficulty of diminished accessibility of azoles requisite to cure Candidiasis, customary preventive drugs extracts are synthesized from plants are without change being used in many distinguishable or varied parts of the world.

The frequency of life-forbidding infections caused by disease causing microorganisms has raised or exaggerated worldwide, becoming an most important cause of morbidity or cognitive state of mind and mortality in immune compromised patients in developing countries **(Ara N *et al.*, 2009).** Although a huge number of antimicrobial or antifungal agents have been discovered but

rise of resistance caused by pathogenic organism cause a big concern **(Al-Bari M.A *et al.*, 2006).**Therefore it is required to search for more efficacious and less toxic novel antifungal agents that would expel or pull round these disadvantages. An important group of the skin or dermal pathogens are the fungi, among which dermatophytes and *Candida* spp. are striking **(Fan S.R *et al.*, 2008; D Toledo CEM *et al.*, 2011)**. Under certain situations, usually correlated with a compromised host immune system, *C. albicans* and related species can become disease causing, causing oral, vaginal and/or systemic Candidiasis **(Odds F.C., 1994).**

Medicative plants or plant extracts are inexhaustible in nature different or unlike to the imitative drugs that are taken out from exhaustible sources of crude or unrefined materials likewise fossil or forgey sources and petrochemicals **(Samantha M.K *et al.*, 2002)**. Because of all these expediency, plants go along to be a prima or leading source of new major heterocyclic compounds. Nowadays, the unperceptive application of mercantile antimicrobial or azoles drugs has been also mainly caused manifold azoles resistance in human illness inducing microbes or fungus. Therefore for substituting conventional antifungal drugs scientists are induced for researching new drugs. **(Sharapa R *et al.*, 2013).**

## 2. MATERIALS USED

Media cultures and chemical compounds that were used in experiments were procured or get in from Thermo Fisher Scientific (Mumbai, India) and Hi-Media (Mumbai, India) and the solvents utilized for research project were procured from Thermo Fisher Scientific.

### 2.1. Strains and fungal growth media:

The *Candida* strain was preserved or kept up on liquid broth and Agar plates for solid media. Wild or intractable type *Candida albicans* strain (Fonzi, W. R. 1983) has been utilized for this research experiments. Strains were preserved in 15 % (v/v) glycerol at -80ºC. Fungal cells were revitalized on YEPD (Yeast extract, peptone and dextrose) plates from frozen glycerol stocks and preserved at low temperature (4°C). Liquid media cell cultures were mature or grown up at a temperature of 30°C with regular agitation or shaking of 140-150 rpm (revolution/min) for a time period of 14-16 hrs and these growing or dividing cells were further utilized for all the research experiments.

### 2.2. Yeast peptone Dextrose (YEPD) Media

**Table 1: composition of YEPD Media**

| **COMPOSITION** | Yeast extract | Peptone(Soluble Protein) | Dextrose(Sugar) |
|---|---|---|---|
| **PERCENTAGE (%)** | 1% (w/w) | 2% (w/w) | 2% (w/w) |

## 2.3. Spider Broth Media

**Table 2: Spider Broth Media for 1 liter**

| COMPOSITION | Liq. nutrient media (broth) | Osmitrol | Potassium phosphate dibasic |
|---|---|---|---|
| PERCENTAGE (%) | 10% (w/w) | 10% (w/w) | 2% (w/w) |

Using 1Normal Hydrogen chloride set pH 7.2

## 2.4. NAG MEDIA

**Table 3: N-Acetyl Glucosamine Media**

| Composition | YNB | Sodium Chloride |
|---|---|---|
| Percentage (%) | 0.335% (w/w) | 0.45% (w/w) |

**Add 25mM (milli Molar) NAG for 10 milli litter media solution**

## 2.5 Chemicals Used For Experiments

**TABLE: 4**

| Chemicals used | Composition (%) |
|---|---|
| Saline(NaCl in water) | 0.9% (w/w) |
| Methanol ($CH_3OH$) | (0.5% [w/v]) |
| Potassium hydroxide (KOH) | 1.5ml (60% [wt/vol]) |
| Pyrogallol ($C_6H_6O_3$) | 1ml |
| *n*-heptane ( $C_7H_{16}$) | 3-5ml |
| Hydrogen Chloride (HCl) | 1N |

## 3. METHODS

### 3.1. Extract Preparation:

Naturally occurring plant extracts (Pepper and Nutmeg) were grinded into very fine powder and subsequently taken away with various HPLC grade organic solvents. The active components or elements of nutmeg and pepper were then extracted or taken away with the help of isopropyl alcohol and finally distillation process was used to concentrate it. Finally nutmeg and pepper extract were modified by exploiting ethyl acetate.

### 3.2. Drug Susceptibility Assay:

### 3.2.1. Spot Assay:

- In this assay, first of all *Candida* cells were grown overnight in YEPD agar media plate and then were suspended or supported in 0.9% saline (i.e. sodium chloride solution in water) and then OD (at 600 nm) of fungal cell (*Candida*) suspension was determined and set to 0.1 using spectrophotometer. **(Mukhopadhyay *et al.*, 2006)**
- Then after, 5 micro Liter of 5 times serial dilutions of *Candida* cell culture was spotted onto growth culture i.e. YEPD plates in the state of being absence (Growth control) and Presence (of the nutmeg and pepper). Finally after incubating the plates (48 hr) the differences occurred in growth were recorded

### 3.3. Growth Curve of *Candida Albicans*

- First of all take 50 ml of yeast extract peptone dextrose (YEPD) media.
- After that pour 60 µl yeast cell suspensions (in 0.9% saline) into it.
- Now switch on the spectrophotometer and make it auto zero by placing cuvette inside it , having 3 ml of YEPD media (blank). Then add 3 ml of cultured media in cuvette.
- Analyze or check the O.D or optical density with the help of spectrophotometer
- Now, finally repeat this process after every 4 hrs.

## 3.4. STEROL ESTIMATION

- Inoculate the primary culture with Candida cells and grow it whole night at 30°C
- Then inoculate the secondary YEPD cell culture and again grow the cells whole night at same temperature.
- Before centrifugation weigh and label the empty tubes
- Pellet down the cells at 6000rpm with the help of centrifugation for 15 min and at a very low temperature i.e. 4°C.
- Wash the yeast or Candida cells with mille Q water two times by centrifuging at 6000rpm for 15 min and temp. At 4°C
- Weigh the dried cells i.e. Without moisture content in it
- Alkaline saponification is done with 3ml of methyl alcohol, 2ml of 60% KOH and 2ml of 0.5% pyrogallol i.e. $C_6H_6O_3$
- Swirl well to blend in the solution.
- The mixture was preserved for refluxing at a temperature 80°C for the time 2 hrs and give in to at lower temperature.
- The ergosterol was taken out with the help of normal heptane i.e. $C_7H_{16}$ and was recurrent by mixing notable volume of *n*-heptane i.e. $C_7H_{16}$ two to three times.
- Collect or stash away the supernatant because the weight density of n-heptane i.e. $C_7H_{16}$ is lower than water.
- To calculate content of Sterol or ergosterol

  % Ergosterol + % 24(28)-DHE = $[(A_{281.5nm}/290) \times F]$ / pellet weight;

  % Ergosterol = [% ergosterol + % 24(28) DHE] - % 24(28) DHE,

Where, F is also known as factor for dilution in hexane i.e. With the chemical formula $C_6H_{14}$

## 3.5. Hyphal Morphogenic Study of *C.albicans* on Spider Media

- In this study, spider media is used unlike YEPD growth media to study hyphal morphogenesis of fungal cells (*Candida*).
- It contains mixture of 1% nutrient broth, 1% Mannitol with chemical formula $C_6H_{14}O_6$ and 0.2% $K_2HPO_4$ i.e. Potassium hydroxide dibasic and set the pH of spider media to 7.2 using 1N hydrogen chloride i.e. HCl.

- The treated yeast cells were inoculated or impregnated in media and are allowed to grow or mature at normal room temperature i.e. 37ºC. Yeast Cells were extracted at regular time intervals and patterned the hyphal growth.
- Nutmeg and pepper were inoculated in spider to check or patterned the reduction or inhibition of hyphal morphogenesis.
- Then finally allow the *Candida* cells to grow at normal room temp of 37ºC in incubator and capture the pictures or images at confocal microscope *FluoView™ FV1000*.

## 3.6. Hyphal Morphogenic Study of *C.albicans* on NAG Media

- Here N-acetyl glucosamine media is used unlike YEPD growth media to study hyphal morphogenesis of fungal cells (*Candida*). It contains mixture of 0.335% Yeast nitrogenous extract (without nitrogenous base) and 0.45% Sodium chloride i.e. NaCl
- The treated yeast cells were inoculated or impregnated in media and are allowed to grow or mature at normal room temperature i.e. 37ºC. Yeast Cells were extracted at regular time intervals and patterned the hyphal growth.
- Nutmeg and pepper were inoculated in spider to check or patterned the reduction or inhibition of hyphal morphogenesis.
- Then finally allow the *Candida* cells to grow at normal room temp of 37ºC in incubator and capture the pictures or images at confocal microscope *FluoView™ FV1000*.

## 4. RESULT AND DISCUSSION

### 4.1. Growth curve of *C.albicans* cells showed sigmoid growth pattern

- Yeast or *Candida* Cells were inoculated and grown on YEPD (i.e. yeast extract peptone and dextrose) growth media.
- Growth and maturation of *Candida* cells in YEPD media were examined by measuring OD i.e. optical density of cells at a wavelength of 600nm.
- The obtained values were plotted against time (Fig: 4.).
- Yeast cells exhibit the characteristic sigmoidal or S-shaped pattern of growth. The middle i.e. exponential time or log time was noted and saved for future experiments.

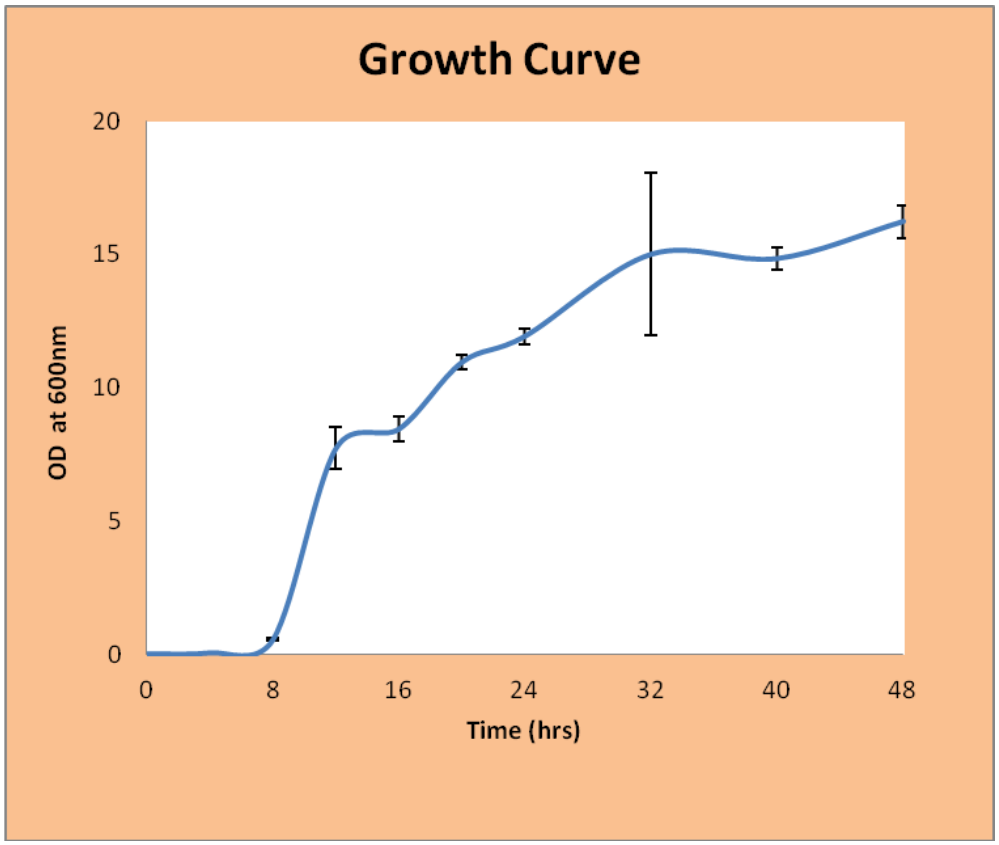


**Fig6.Sigmoidal Growth Curve of *C. albicans***

## 4.2. Spot Assay Used In Determination of MIC of Plant Extracts

In this assay, Candida cell culture (5 µL), at an optical density ($OD_{600}$=0.1), were spotted on growth media plates. Candida cells were spotted in fivefold serial dilution in presence of plant extracts i.e. nutmeg (20µl/ml) and Pepper (20µl/ml) and in absence of extracts (control)**.** Then the plates having *Candida* along with extracts were kept for incubation (48 hours) at a temperature of 30°C to check the differences in growth of Candida in comparison of control. Therefore this assay was used to find minimum conc. Of plant extracts required to inhibit the growth of Candida cells in solid agar media.

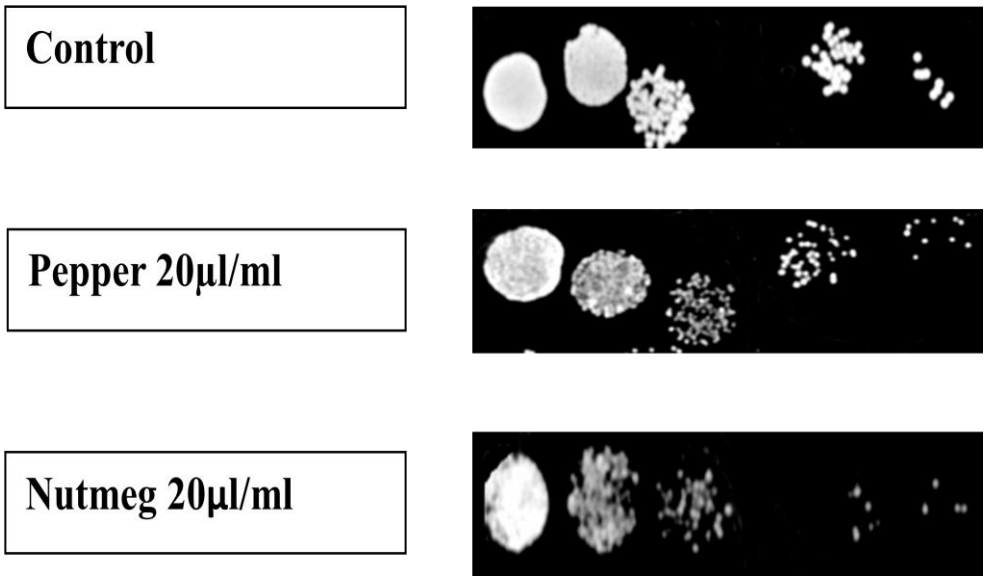


**Fig7. Spot assay showing growth of cells in presence and absence of plant extracts**

## 4.3 Nutmeg and Pepper exhibits inhibition on hyphal Morphogenesis on spider media

Before this experiment MIC of nutmeg and pepper was determined with the help of spot assay. In this study effect of extracts on hyphal morphogenesis of *Candida* in spider media was determined. In this cells which were already induced in NAG media were kept with nutmeg and pepper for incubation at normal room temperature. Then Candida cells were observed regularly after 3-4 hours under confocal microscope. It was found that there was complete inhibition of hyphal growth in Candida when treated with nutmeg and pepper or 100% hyphal inhibition.

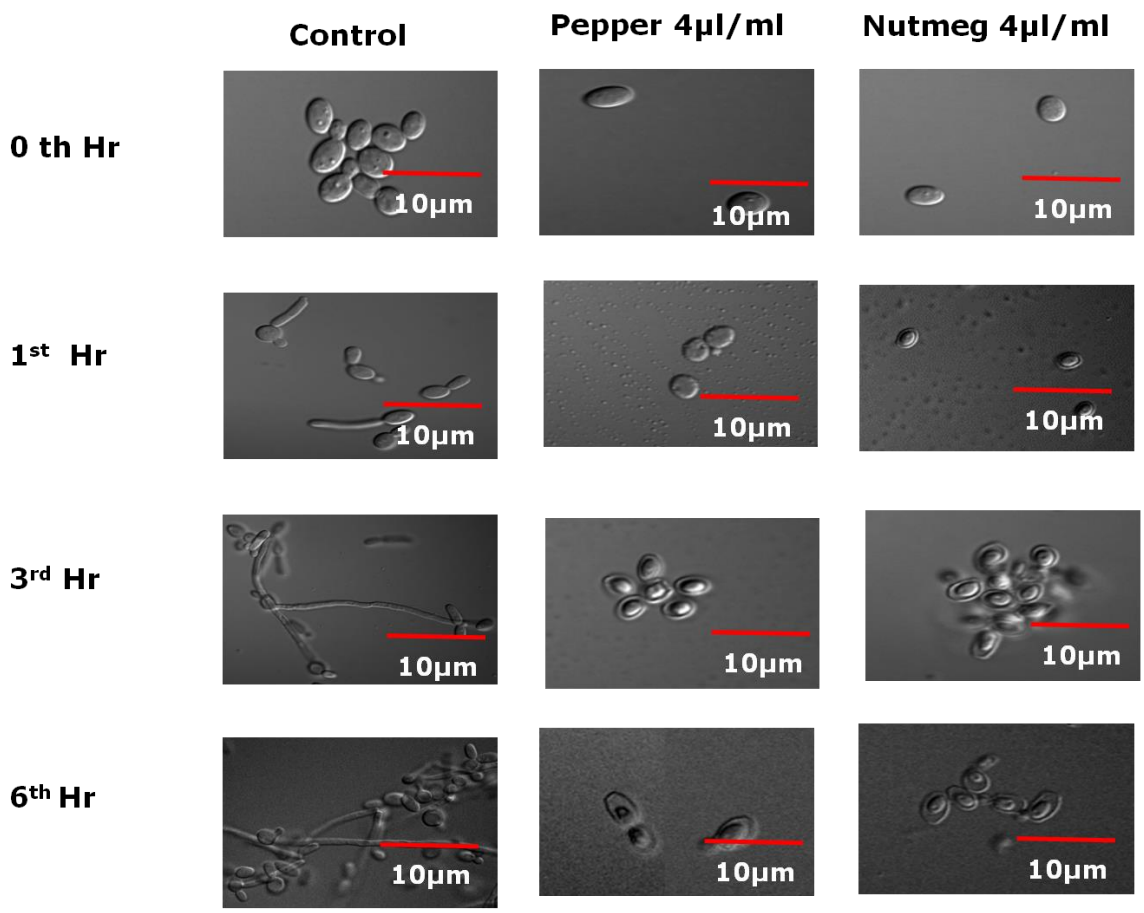


**Fig.8. Hyphal morphogenesis in spider media**

## 4.4 Nutmeg and Pepper Exhibits Inhibition on hyphal morphogenesis on NAG furnished media

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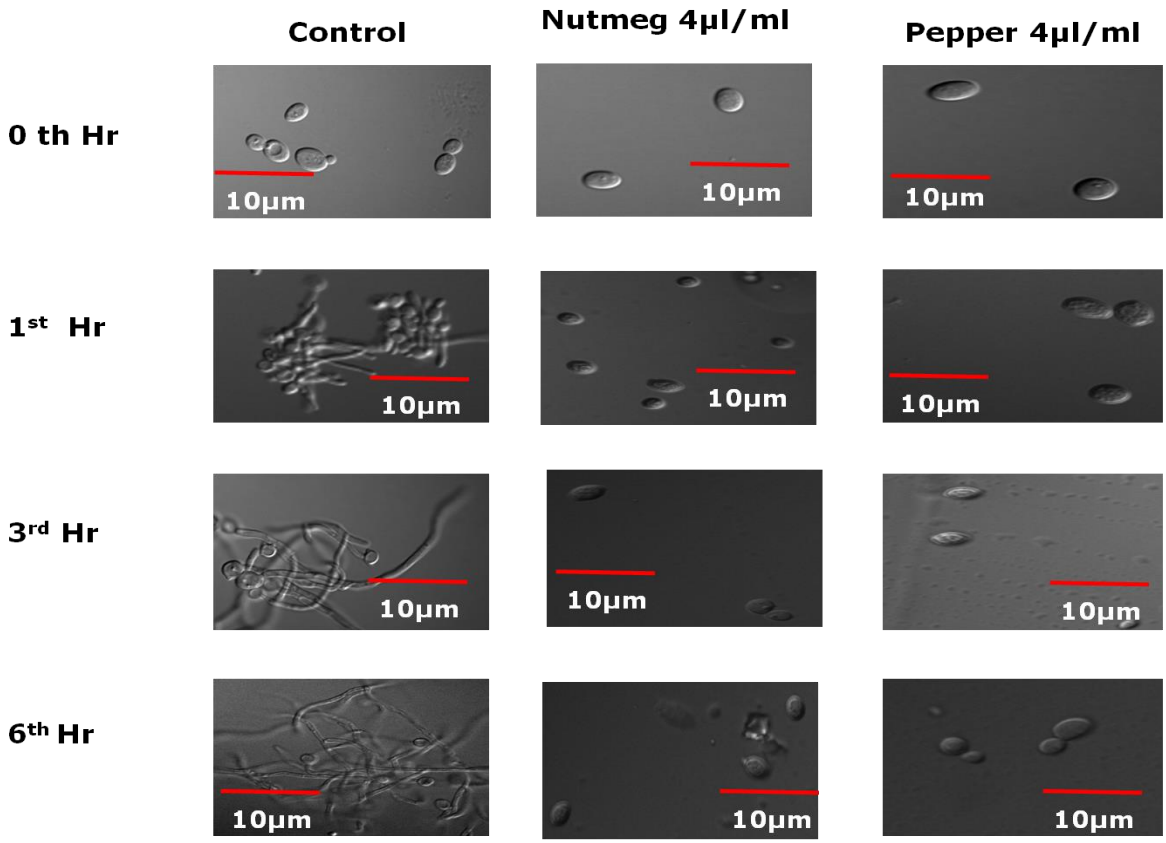


**Fig.9. Hyphal morphogenesis in spider media**

## 4.5. Observed significant decrease in sterol count of *Candida Albicans* after treatment with plant extracts i.e. Nutmeg and Pepper in spider media

The ability of plant extracts in the inhibition of hyphal morphogenesis was well illustrated in the susceptibility assay. But the molecular mechanism behind the both plant extracts inhibition on hyphal morphogenesis was not well known. This study was an effort to elucidate the role of sterol on hyphal morphogenesis. Ergosterol is the main component of cell membrane which is involved in membrane homeostasis. Induced cells were grown on spider media and incubated at 37ºC to induce hyphal cells. Cells were harvested and extracted the sterols. The unique four peak absorption pattern between 200nm to 400nm indicates the presence of both Ergosterol and Dihydro ergosterol (DHE) at 281.5 nm and only DHE at 230nm. Therefore, the amount of ergosterol can be determined by subtracting the absorption of only DHE from total ergosterol and DHE content **[Arthington-Skaggs].** Cells treated with pepper and nutmeg showed significant

reduction in sterol level (Fig. 8). When compared to control, pepper and nutmeg showed 23% and 2% reduction in spider media.

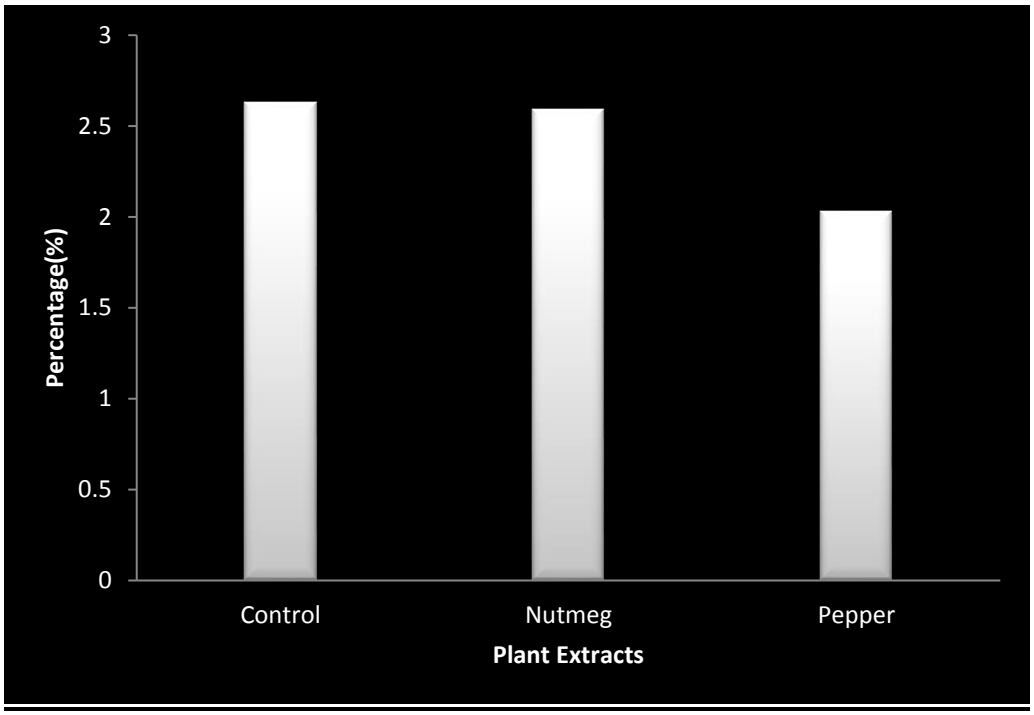


**Fig10. Estimation of sterol in spider media**

## 4.6. Observed significant decrease in sterol count of *Candida Albicans* after treatment with plant extracts i.e. Nutmeg and Pepper in NAG media

Ergosterol is the critical part of cell's plasma membrane which is involved in membrane homeostasis. *Candida* cells were grown on NAG furnished media and incubated at 37 ºC to induced hyphal cells. Yeast Cells were harvested and extracted the sterols. The distinct 4 peak absorption pattern forms between 200- 400nm shows the occurrence of Ergosterol and Dihydro3, 4ergosterole at 281.5 nm and only Dihydro ergosterol (DHE) at 230nm. Thus, the quantity of ergosterol can be examined carefully by reducing the absorption of only Dihydro ergosterol (DHE) from total ergosterol and Dihydro ergosterol (DHE) content **[Artheengton-Skags,**]. Yeast Cells activated with plant extracts used in experiment exhibits remarkable decrease in sterol level

(Fig. 9). When analyzed with control and plant extracts (i.e. pepper and nutmeg) exhibit 19% and 90% decrease in NAG furnished media.

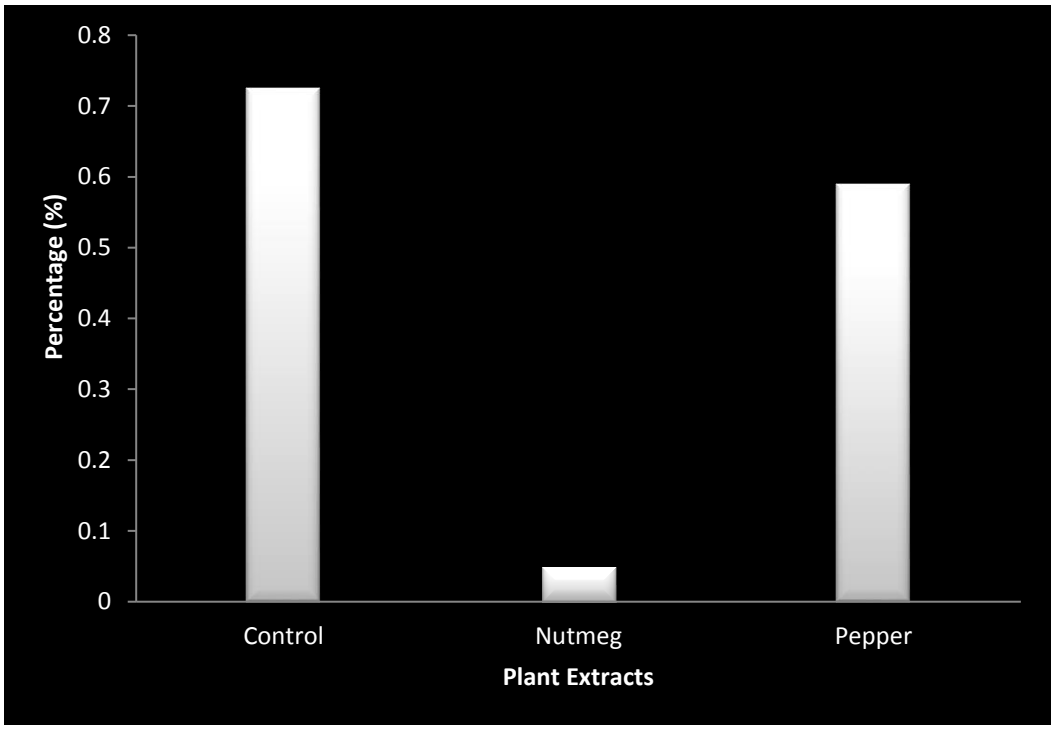


**Fig.11. Estimation of sterol in NAG media**

## 5. CONCLUSION

Plant extracts i.e. nutmeg and pepper were essentially required before performing experiments and these extracts were first ground into fine powder and subsequently extracted with different HPLC grade organic solvents. Then the active components of Nutmeg and Pepper were first extracted by isopropanol and then concentrated by distillation method and finally the concentrated extract was emulsified using an organic compound i.e. ($C_4H_8O_2$) ethyl acetate. Then Spot assay was performed which was aimed only to find the minimum inhibitory concentration (MIC) of pepper and nutmeg i.e. minimum concentration of these extracts to inhibit *Candida* cells completely**.** It was also found that nutmeg and pepper causes complete hyphal inhibition i.e. when *Candida* cells were treated with one of the plant extracts i.e. nutmeg or pepper (4µl/ml) in spider or NAG supplemented media there were 100% inhibition in hyphal morphogenesis and untreated cells i.e. control shows individual cells . Moreover, the sterol (ergo-sterol) profile was examined carefully to track the actual molecular mechanism behind the inhibition on hyphal morphogenesis by Nutmeg and Pepper. Remarkable decrease in ergosterol content was found in pepper and nutmeg treated cells grown in spider and NAG furnished media. These results acquired were compared with control (non-treated) cells. Ergosterol is the major sterol found in plasma membrane and preserves the stability of plasma membrane; therefore by changing their sterol profile it may restrain the hyphal morphogenesis of *C. albicans* towards plant extract used in this experiment. Many more advanced studies are currently in progress to decipher the condition or state of Nutmeg and Pepper and their other mechanism.

Therefore, it seems from the above experimental results that the plant extracts used i.e. nutmeg and Pepper have some clinical significance because they are efficacious against hyphal morphogenesis of *Candida* cells. It is apparent from the above experimental results that the Nutmeg and Pepper used for this study are also efficient in intrusive the bio-synthetic pathway of ergosterol. It is also significant to find that other cellular targets of plant extracts i.e. pepper and nutmeg have potential to contribute to advancement of new therapeutic plan of action for clinical uses. More advanced stages investigations are currently in progress.

## 6. REFERENCE


1. Sudbery P, Gow N and Berman J. (2004). The distinct morphogenic states of *Candida albicans*. Trends microbial; 12(7):317-24.

2. Kabir M.A, Hussain M.A and Ahmad Z. (2012*). Candida albicans*: A Model Organism for Studying Fungal Pathogens. ISRN Microbiology: 1-15.

3. Pfaller M.A and Diekema D.J. (2007). Epidemiology of Invasive Candidiasis: a Persistent Public Health Problem. Clin Microbiol Rev; 20(1):133-63.

4. Ortega M, Marco F, Soriano A, Almela M., Martınez, J. A, Lo´ pez, J., Pitart C. and Mensa J. (2011). *Candida* species bloodstream infection: epidemiology and outcome in a single institution from 1991 to 2008. J Hosp Infect; (77):157–161.

5. Rajendran R, Robertson D. P, Hodge P. J, Lappin D. F. and Ramage G. (2010). Hydrolytic enzyme production is associated with Candida albicans biofilm formation from patients with type 1 diabetes. Mycopathologia; (170):229–235.

6. Kuhn DM and Ghannoum MA (2004). *Candida* biofilms: antifungal resistance and emerging therapeutic options. Curr Opin Investig Drugs; 5(2):186-97.

7. Sardi J.C.O, Almeida A.M.F and Giannini M.JS.M. (2011). New antimicrobial therapies used against fungi present in subgingival sites—A brief review. Arch Oral Biol.; 62(1):951-959.

8. Eggimann P, Garbino J and Pittet D. (2003). Epidemiology of *Candida* species infections in critically ill non immune suppressed patients. Lancet Infect Dis; (3): 685–702.

9. Mayer F, Wilson D and Hube B. (2013). Candida albicans pathogenicity mechanisms. Virulence.; 4(2): 119–128

10. Wayne P.A. (2008). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Approved Standard M27-A3, third ed. Clinical and Laboratory Standards Institute. CLSI; 15(14):1-13.

11. Akins R. A. (2005). An update on antifungal targets and mechanisms of resistance in Candida albicans. Med Mycol; (43):285–318.

12. Sanglard, D. and Bille, J. (2002). Current understanding of the modes of action of and resistance mechanisms to conventional and emerging antifungal agents for treatment of Candida infections. In Candida and Candidiasis. Edited by R. A. Calderone. Washington, DC: American Society for Microbiology; 49(2): 668–679.

13. Cannon R.D, Lamping E, Holmes A.R, Niimi K, Tanabe K, Niimi M and Monk B.C. (2007). *Candida albicans* drug resistance – another way to cope with stress. Microbiology; (153):3211-3217**.**

14. Kanafani Z.A and Perfect J.R. (2008). Resistance to antifungal agents: Mechanisms and clinical impact. Clinical infectious diseases; (46):120-8.

15. Mukhopadhyay K, Prasad T, Saini P, Pucadyil TJ, Chattopadhyay A and Prasad R. (2004). Membrane sphingolipid-ergosterol interactions are important determinants of multidrug resistance in *Candida albicans*. Antimicrob Agents Chemother; 48(5):1778-87.

16. Mukhopadhyay K, Kohli A.K and Prasad R. (2002). Drug susceptibilities of yeast cells are affected by membrane lipid composition. Antimicrob. Agents Chemother; 46:3695–3705.

17. Lavie Y and Liscovitch M. (2001). Changes in lipid and protein constituents of rafts and caveolae in multidrug resistance cancer cells and their functional consequences. Glycoconjugate J; (17):253–259.

18. Prasad T, Sethumadhavan S and Fatima Z. (2011). Altered Ergosterol biosynthetic pathway - an alternate multidrugresistance mechanism independent of drug efflux pump in humanpathogenic fungi *C. albicans*. A. Méndez-Vilas (Ed.): 1-12.

19. Ara N, Nur MH, Amran MS, Wahid MII and Ahmed M. (2009). In vitro antimicrobial and cytotoxic activities of leaves and flower extracts from *Lippiaalba*. Pakistan Journal of Biological Sciences; 12(1):87-90.

20. Al-Bari MA, Sayeed MA, Rahman MS and Mossadik MA. (2006). Characterization and antimicrobial activities of a phenolic acid derivative produced by *Streptomyces bangladeshiensis*a novel species collected in Bangladesh. Res J Med Sci; (1):77-81.

21. Muschietti L, Derita M, Sülsen V, Muñoz J, Ferraro G, Zacchino S and Martino V. (2005). In vitro antifungal assay of traditional Argentine medicinal plants. J Ethnopharmacol; (102):233-238.

22. Fan SR, Liu XP and Li JW. (2008). Clinical characteristics of vulvovaginal candidiasis and antifungal susceptibilities of *Candida* species isolates among patients in Southern China from 2003 to 2006. J ObstetGynaecol Res; 34(4):561-566.

23. De Toledo CEM, Britta EA, Ceole LF, Silva ER, De Mello JCP and FilhO BPD. (2011). Antimicrobial and cytotoxic activities of medicinal plants of the Brazilian cerrado, using Brazilian cachac, as an extractor liquid. J Ethnopharmacol; (133):420-425.

24. Odds FC. (1994). *Candida* species and virulence. ASM News; (60):313–318.

25. Chong PP, Lee YL, Ian BC and Ng KP. (2003). Genetic relatedness of *Candida* strains isolated from women with vaginal candidiasis inMalaysia. J Med Microbiol; (52):657–666.

26. Sharanappa R and Vidyasagar G.M. (2013). Anti-*candida* activity of medicinal plants. A review. International Journal of Pharmacy and Pharmaceutical Sciences; 5(4): 1-8.

27. Sukdeb Pal, Yu Kyung Tak and JoonMyong Son. (2007). Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A Study of the Gram-Negative Bacterium *Escherichia coli*. *Appl. Environ. Microbiol*; 73(6): 1712-1720.

28. Fonzi, W. A., and M. Y. Irwin. (1993). Isogenic strain construction and gene mapping in *Candida albicans*. Genetics; (134):717–728.

29. Prasad T, Chandra A, Mukhopadhyay CK, and Prasad R. (2006). Unexpected Link between Iron and Drug Resistance of Candida spp.: Iron Depletion Enhances Membrane Fluidity and Drug Diffusion, Leading to Drug-Susceptible Cells. Antimicrob. Agents Chemother; 50(11):3597–3606.

30. Wayne, PA. (2008). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Approved Standard M27-A3, third ed. Clinical and Laboratory Standards Institute. CLSI: 1-13.

31. Mukhopadhyay K, Prasad T and Prasad R. (2004). Membrane sphingolipid and ergosterol interactions are important determinants of multi drug resistance in *Candida albicans*. Antimicrob. Agents Chemother; 48(5):3695–3705.

32. Prasad T, Hameed S, Manoharlal R, Biswas S, Mukhopadhyay CK, Goswami SK and Prasad R. (2010). Morphogenic regulator *EFG1* affects the drug susceptibilities of pathogenic *Candida albicans*. FEMS Yeast Res; (10):587–596.

33. Prasad T, Siani P, Gaur NA, Viswakarma RA, Khan LA, Haq QMR and Prasad R. (2005). Functional Analysis of Ca*IPT1*, a Sphingolipid Biosynthetic Gene Involved in Multidrug Resistance and Morphogenesis of *Candida albicans*. Antimicrobial Agents and Chemotherapy. 2005; 49(8): 3442 – 3452.

34. Arthington-Skaggs BA, Jradi H., Desai T and Morrison C. J. (1999). Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of Candida albicans. Journal of Clinical Microbiology; 37(10), 3332–3337.