INTRODUCTION

CHARACTERIZATION OF EFFECTS OF FORMULATED PLANT EXTRACTS (CLOVE AND CARDAMOM) ON HYPHAL MORPHOGENESIS IN *Candida albicans*

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1. INTRODUCTION

There is a recent estimation of about 8.7 million eukaryotic species on earth, out of which 7% (611,000 species) are fungi. Among them only around 600 species are pathogenic to humans. This relatively small group circumscribe fungi that causes relatively mild infections of the skin (e.g., dermatophytes and *Malassezia* species), fungi that causes severe cutaneous infections (eg., *Sporotrix schenkii*) and fungi that have the possibility to cause life threatening systemic infections (eg., *Aspergillus fumigates*, *Cryptococcus neoformans*, *Hisyoplasma capsulatum* and *Candida albicans*).Actually, *Candida* species are the fourth most widespread systemic infections in US with crude death rates of upto 50%. In humans, *C. albicans* causes two major types of infections: 1) Superficial infections (eg. oral or vaginal Candidiasis) and 2) life threatening systemic infections (**Mayer F *et al*., 2013**).*Candida* show two modes of proliferations: 1) hyphal mode, in which elongated tubes are formed by the continuous growth at the tips where the separate cells are marked by septa. 2) Yeast growth mode, where distinct cells are elongated or bud-off daughter cells that typically dissociate from mother cells**.** In nature, yeast form is less commonly found but it is generally seen in economically and scientifically important organisms such as *Saccharomyces cerevisiae.* Some fungi are not restricted to specific growth mode i.e. they can grow either in yeast form or in hyphal form, depending on certain environmental conditions. Such fungi are termed as dimorphic fungi **(Whiteway M *et al*., 2007).**

1.1. *Candida albicans*

1.1.1. Taxonomy

Fig 1*: <u>Candida albicans</u>*

Domain = Eukaryota

Phylum = Ascomycota

Class = Saccharomycetes

Order = Saccharomycetales

Family = Saccharomycetaceae

Genus = *Candida*

species = *albicans*

1.1.2. <u>Description</u>

Candida species belong to the normal microflora of a person's mucosal oral cavity, alimentary canal and vagina **(Shao *et al.*, 2007)**, and are responsible for various clinical indications from mucocutaneous overgrowth to bloodstream infections **(Eggimann *et al.*, 2003)**. Due to their great versatility, these yeast causes systemic infections in humans during immunocompromised conditions and these are commensal in nature. The genus is composed of a varied group of organisms and more than 17 different *Candida* species are known to be the disease causing agents of human infection; however, more than 90% of interfering infections are caused by

Candida albicans, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* **(Pfaller *et al*., 2007)**.

In immunocompromised patients such as those infected by HIV, transplant recipients, chemotherapy patients and etc, excessive growth of these organisms will cause certain disease. **(Kabir MA *et al*., 2012).** *Candida albicans* are the causative agents of Candidiasis in human hosts as they are opportunistic fungal pathogen. Candidiasis is further divided into four major groups: oropharyngeal candidiasis, vulvovaginal candidiasis, cutaneous candidiasis and invasive candidiasis. A whitish film formed on the palate, tongue and inside of the cheeks are the results of the infection, Thrush which is caused by Candidiasis. Esophagitis is caused when thrush extends into the throat and this is known as oropharyngeal candidiasis. Vulvovaginal candidiasis are found in approximate 75% women which are the results of extensive growth of *C. albicans* in the vaginal region identified by rashes, itchiness and secretion of fluid from the genitals. Cutaneous Candidiasis is generally known as diaper rash and can create problems anywhere in the skin such as armpits, hands and under breasts **(Pfaller MA *et al*., 2007)**. The best way to recognize invasive candidiasis is incurable fever when patient is given the antibiotic.

The increasing population of immunocompromised patients that use intravenous catheters, total parenteral nutrition, invasive procedures and the continuous use of wide range-spectrum antibiotics, cytotoxic chemotherapies and transplantation are factors that devote to the increase of these infections **(Ortega *et al*., 2011)**.

1.2. Epidemiology

Mostly *Candida* species are commensal in nature and may form colonies on the skin and on the mucosal surfaces of humans. Critically ill or diversely immunocompromised patients are more prone to establish both superficial and life-threatening Candida infections **(Hasan *et al*., 2009)**. *Candida* contagion also embodies the most common fungal infections in AIDS patients **(Fidel, 2006; Hasan *et al*., 2009)**. These patients potentially develop oropharyngeal Candidiasis, which can lead to malnutrition and obstruct the absorption of medication. *C. albicans* is the predominant cause of invasive fungal infections **(Horn *et al*., 2009)** and shows a serious public health challenge with increasing medical and economic importance due to the high death rates

and increased costs of care and duration of hospitalization **(Almirante *et al*., 2005; Lai *et al*., 2012)**.

1.3. Pathogenesis

1.3.1. Transmission

Transmission of *Candida albicans* generally takes place from mother's microflora to infant through childbirth. When there is certain imbalance in the body like variation in the normal acidity level of the vagina, the excessive growth of *C. albicans* leads to symptoms of diseases. The spread of *C. albicans* infections through sexual intercourse is very seldom known. The typical pool for *C. albicans* is in the normal human microflora, and is not at all found in animal vectors. *C. albicans* are the 4^{th} most leading cause of nosocomial infection where most of these infections cause about 40% mortality. **(Fanelloa S *et al.*, 2001)**.

1.3.2. Distinct morphological forms of *Candida albicans*

- **Yeast-form growth:** Asymmetric budding takes place when yeast cells grow resulting into diploid cells which forms smooth and round colonies. Formation of Septin rings takes place before budding, and division of nuclei takes place across the mother-daughter junction. Bud site selection in *C. albicans* yeast cells is dependent on temperature. At lower temperature, a mixture of cells containing large number of cells with axial pattern is generally present in the culture.
- **Pseudohyphal growth**: Budding in *C. albicans* pseudohyphal cells occurs in a unipolar pattern. After cytokinesis the cells remain attached which forms branched chains of elongated buds and fibrous or rough colonies. Just below the colony, agar is invaded by the filaments and these filaments extend from the colony edge below the agar. As in yeast cells, formation of Septin rings takes place before budding, and division of nuclei takes place across the mother-daughter junction. Like *S. cerevisiae* pseudohyphae, *C. albicans* pseudohyphal cells consume more time growing in a polarized manner and tend to remain in G_2 phase longer than yeast cells.
- **Hyphal growth:** As compared to the pseudohyphal cells, hyphae are narrower (~2µm). They have parallel side walls with no constriction at the septation points. There is continuous evagination and elongation of the germ tube that contains the septum where

the nucleus starts dividing. Checkpoints that are said to coordinate bud growth do not function in *C. albicans* hyphae

- **Mating projection:** Mating projection or shmoo is the structures which are formed when *C. albicans* responds to the surrounding pheromone. The nucleus present in the cell migrates into the shmoo and it undergoes Kar3p-mediated fusion with other nucleus when this shmoo fuses with the cell of different mating type.
- **Formation of chlamydospore:** Chlamydospore which are formed at the tip of suspensor cells have thicker cell wall and are comparatively larger than blastospores. Under adverse condition, chlamydospores are formed through the process of cell division that is different from that of yeast, pseudohyphal or hyphal growth. The function of chlamydospore is ambiguous; it may show resting state but still there is no confirmation about this.

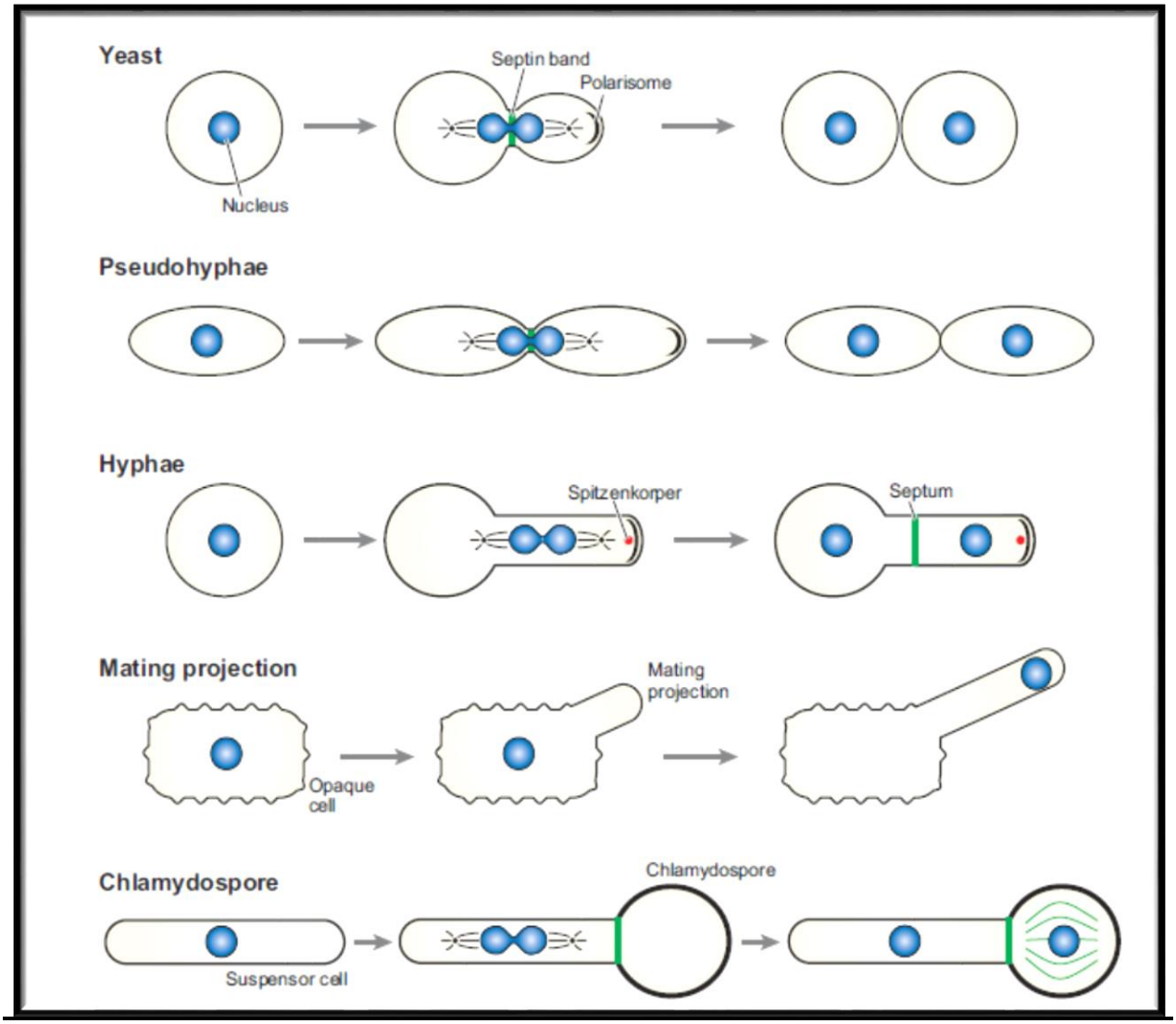


Fig 2: Different morphological forms of *C. albicans* (Whiteway M *et al.*, 2007)

1.4. Pathogenecity mechanism in *C. albicans*

Certain factors and characteristics features of *Candida albicans* are responsible for causing disease to different host. And those characteristic features includes polymorphism, adhesion of cells on the host cell surface, invasion on the cell surface, thigmotropism, biofilm formation and production of certain secreted Hydrolases.

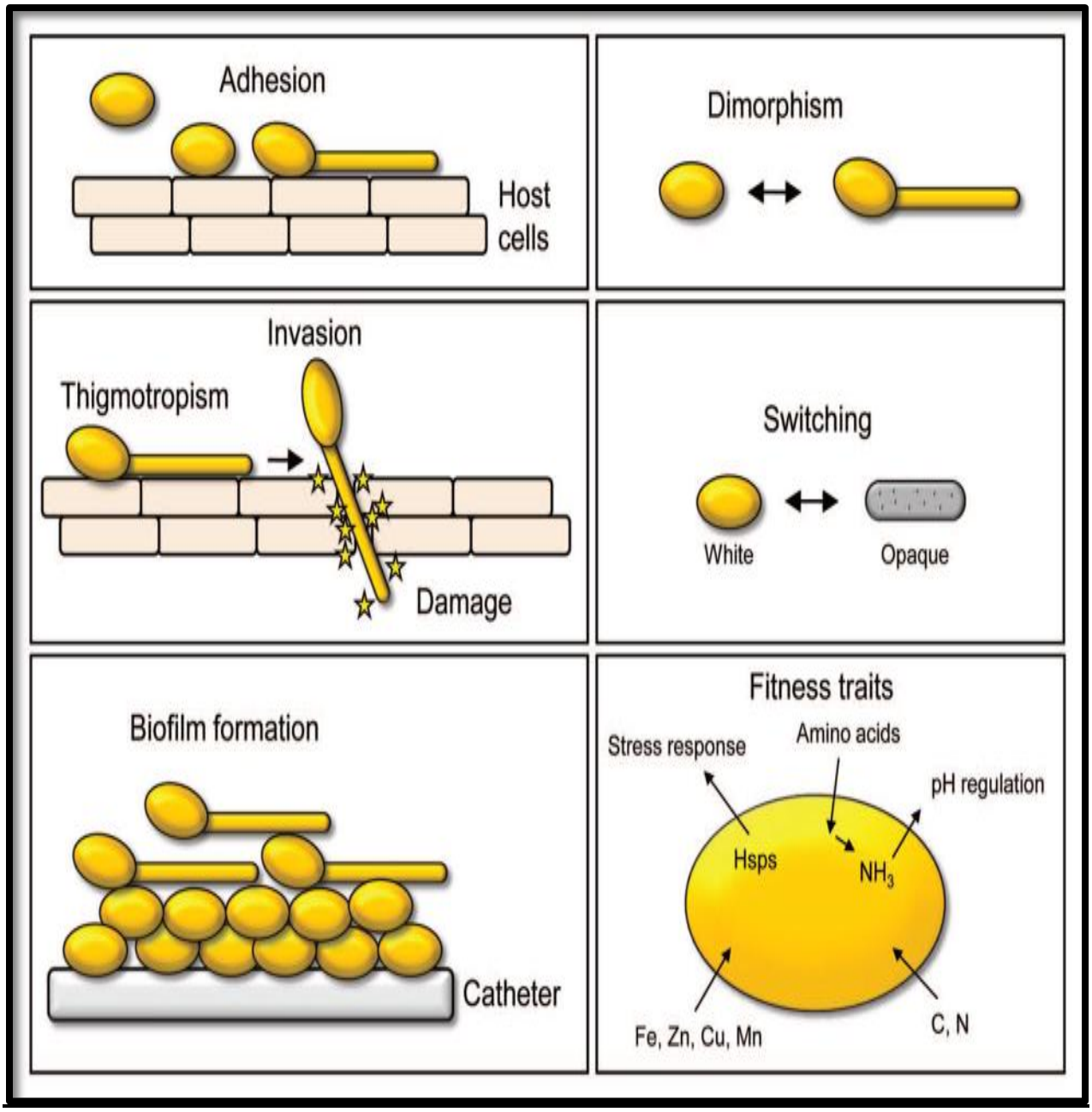


Fig 3: An overview of selected *C. albicans* pathogenicity mechanisms (Mayer F *et al*.,2013)

Polymorphism: *C. albicans* is a polymorphic fungus that undergoes certain morphological changes and grows either as yeast, as pseudohyphal or as true hyphal forms. The switching of yeast form to true hyphal form, called as dimorphism, is the major disease causing determinant of this organism. During switching many opaque cells are formed. The function of pseudohyphae

and chlamydospore is still unclear. The true hyphal form is known to be more virulent than any other morphological forms.

Adhesins and invasions: Adhesins are the specialized set of proteins which mediate attachment to other *C. albicans* cells to other microorganisms, to abiotic surfaces and to host cells. *C. albicans* has two different mechanisms for invasion into host cells: 1) Induced endocytosis and 2) Active penetration. In induced endocytosis, engulfment of the fungal cell into the host cell takes place by host ligand binding to the specialized set of proteins known as invasins which are expressed on the fungal cell surface. Infact, it is a passive process, indicating that even killed hyphae are taken up along with the viable cells. In contrast, active penetration requires only viable *C. albicans* hyphae; therefore it is a fungal driven process.

Biofilm formation: Expression of biofilm on the biotic or abiotic surface is one of the major virulent determinants of *C. albicans.* Transplantation procedures, Immunosuppression, use of indwelling medical devices like catheters, dental implants heart valves, vascular grafts and artificial joints and prolonged ICU stays have increased the chance of biofilm infection. Biofilms form in a continuous process which includes initial attachment of cells on the surface followed by proliferation and finally a mature biofilm is formed. Enhanced antifungal resistance and protection from host immune factors are the two consequences of biofilm formation with deep clinical indications.

Thigmotropism: An important environmental sign or signal that triggers fungal or hyphal and biofilm synthesis in *C. albicans* (fig.) is contact sensing or thigmotropism. On contact with the abiotic surface, yeast cells transformed to hyphal growth. On certain substrates, like as agar or mucosal surfaces, these hyphals can then encroach into any surface. Contact to any solid substratum also persuading to the formation of biofilms. On surfaces with particular topologies (like presence of ridges) hyphal growth (contact sensing) occurs.

Secreted hydrolases: After attachment to the cell surface of the host and hyphal growth, to the Hydrolases are secreted by the hyphae of *C.albican* cells, which help in the penetration of these cells In addition, secreted hydrolases are thought to enhance the efficiency of extracellular

nutrient acquisition. Three different classes of secreted hydrolases are expressed by *C. albicans*: proteases, phospholipases and lipases.

1.5. Conventional Antifungal Drugs

Table 1: Conventional Antifungal Drugs

Chemical Class	Drug	Target
Azoles	Miconazole Ketoconazole Fluconazole Itraconazole Terconazole Voriconazole Posaconazole	Ergosterol synthesis
Polyenes	Amphotericin B Nistatin	Ergosterol (membrane function)
Pyrimidine	Flucytosine	DNA and RNA Synthesis
Echinocandins	Caspofungin Micafungin Anidulafungin	Glucan synthesis

1.6. Treatment

For treating candidiasis in healthy adults, fluconazole (a triazole) with 800mg loading dose along with 400mg daily dose is given as a primary treatment. Fluconazole is administered intravenously in Candidemia patients, but echinocandin and lipid formulation amphotericin B are again preferred for critically ill patients. Also, when low- dose of amphotericin B is given it resulted in 40% less side-effects as compared to the high dose treatment and on removing the infection both have the same effect. Treatment with fluconazole resulted in negligible side effects **(Nguyen MH *et al.*, 1995).**

1.7. Antifungal drug targets in *C. albicans*

There are four main antifungal drug targets in C. albicans:

1) 5-fluorocytosine (5-FC) (fluorinated pyrimidine analogue) is known to cause unusual synthesis of RNA and hinders DNA replication **(Akins, 2005; Sanglard & Bille, 2002)**.
2) When polyenes, such as amphotericin B and nystatin, get inserted into lipid bilayers, they bind to sterols and form pores. Integrity of plasma membrane integrity is lost by these pores and causes the efflux of cations such as K+, which is fungicidal for *C. albicans*.
3) These polyenes are also known to cause oxidative damage **(Sanglard & Bille, 2002)** whereas azole antifungal drugs, such as triazole fluconazole, interfere with the synthesis of sterol.
4) Echinocandins, the cyclic lipopeptides are known to be the recent developments in the class of antifungal drugs. These drugs inhibit the enzyme (1,3)-D-b-glucan synthase which is responsible for the cell wall synthesis and acts as fungicide for *C. albicans* cells **(Cannon R.D *et al.*,2007).**

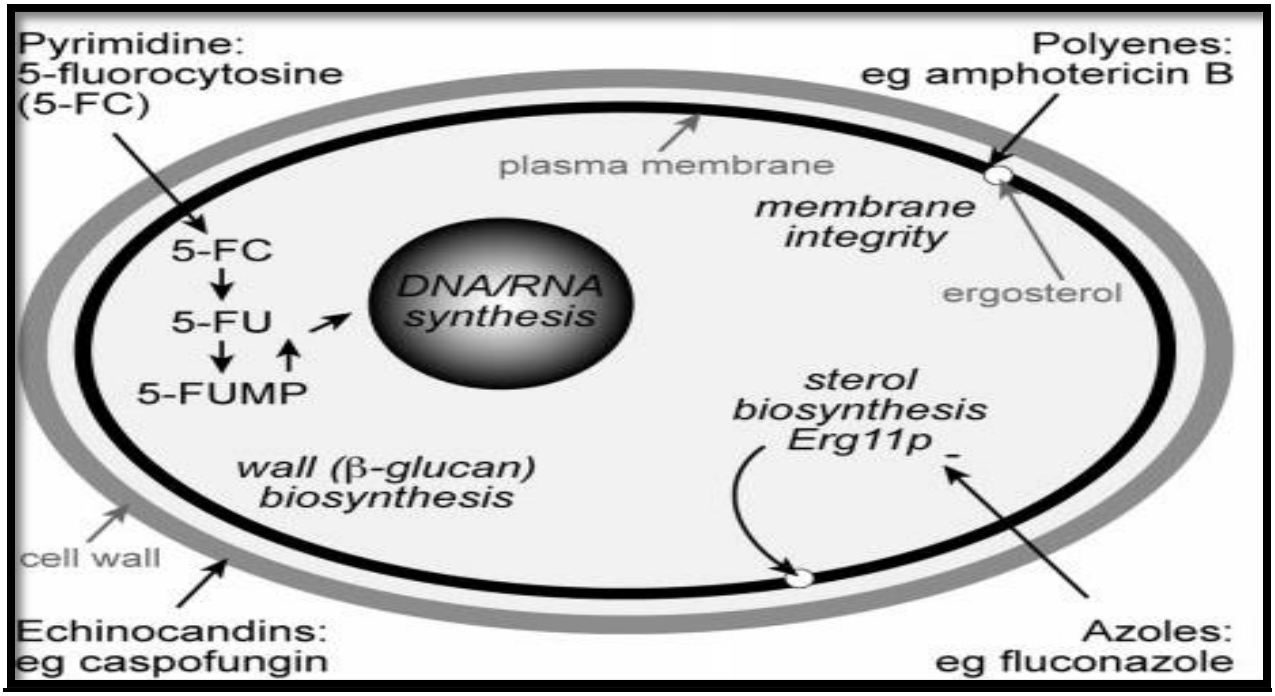


Fig 4: Targets of current antifungal drugs in C. albicans (Cannon R.D *et al.*, 2007)

1.8. Mechanisms of azoles resistance

- **Decreased drug concentration:** The development of efflux pumps causes decrease in drug concentration at the site of action. Efflux pumps are encrypted in Candida species by two gene super families of transporters: the *CDR* genes of the ATP-binding containing super family, and the multi drug resistance genes of the major facilitators class .Upward regulation of *CDR1*, *CDR2*, and *MDR1* has been demonstrated in azoles or fungal-resistant *C. albicans*. Other transporter genes have been found in many other *Candida* species, such as CgCDR1 and *PDH1* in *Candida glabrata* and *CdCDR1* and *CdMDR1* in *Candida dubliniensis*. Whereas *CDR* gene upward-regulation confers resistance to almost all azoles, *MDR*-encoded efflux pumps have a low range spectrum and very specific for fluconazole.

- **Target site alteration:** It has been found that point mutations in *ERG11*, the gene coding for the reference enzyme lanosterol C14α-demethylase, inhibits binding of azoles to the enzyme binding site. Then after, integral resistance to fluconazole in *Candida krusei* isolates has been assigned to lower affinity of Erg11p to the drug. In the excess of 80 amino acids substitutions or replacement in Erg11p have been detected. Different mutations can also coexist in the same gene with additive effects.

- **Up-regulation of target enzyme:** Some of the *Candida* isolates with decreased sensitivity to fungal drugs have higher intracellular concentrations of Erg11p than do fungal drugs-susceptible strains types. The antifungal drugs is, therefore, elicited or provoked and routine therapeutic concentrations can no longer efficaciously prevent or inhibits ergosterol synthesis. Target enzyme up-regulation can be gained through gene amplification or gene illustration, increased transcription rate, or decreased subversion of the gene product. Moreover, this methodology is thought to contribute little to the overall resistance burden in *Candida* species, because only modest increases in enzyme levels have been described.

- **Development of bypass pathways:** Exposure to azoles compounds results in depletion of ergosterol from the fungal membrane and accumulation of the toxic product 14α-methyl-3,6-diol, leading to growth arrest. Mutation of the ERG3 gene prevents the formation of 14α-methyl-3,6-diol from 14α-methylfecosterol .Replacement of ergosterol with the latter product

leads to functional membranes and negates the action of azoles on the ergosterol biosynthetic pathway. *Candida* strains with *ERG3* mutation are also resistant to polyenes, because their cell membranes are devoid of ergosterol **(Kanafani Z.A *et al.*, 2009).**

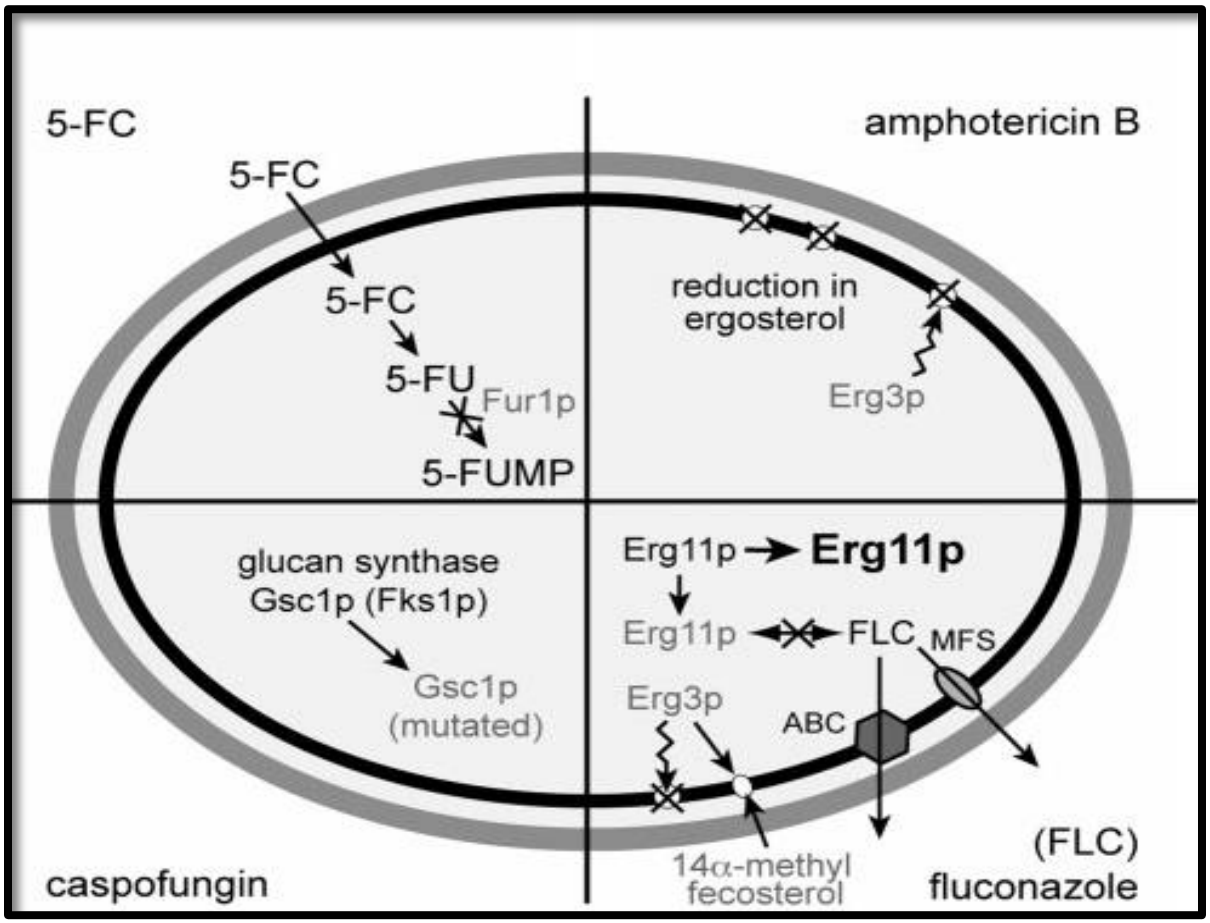


Fig 5: Mechanisms of resistance to antifungal drugs in *C. albicans* (Cannon R.D *et al.*, 2007)

1.9. Prevention

Candidiasis is mainly caused by excessive growth of the *Candida albicans*. An individual can be prevented from the overburdening of microbes by keeping a healthy lifestyle. *C. albicans* are not able to outcompete other commensal microbes by using proper hygienic conditions, good nutrition and careful use of antibiotics. Recurrent infections or Candidemia can be seen in immunocompromised patients, but anti-fungal drugs, such as clotrimazole (Lotrimin, Mycelex), can prevent such recurrence.

1.10. Functional relevance of lipids in *Candida*

Biomembranes of *Candida* are mainly made up of lipid components which play a very vital role in signaling process, organization of membrane microdomain, membrane trafficking and storage of energy in the form of lipid droplets and plasma lipoproteins. Fungal lipids are divided into many classes, among which sterol is the major constituent which is responsible for stability of membrane, rigidity, provide resistance to physical stress and prevent from external environmental factors **(Mukhopadhyay K *et al.*, 2004).** Antifungal drugs, mainly azole drugs targets membrane sterols which causes destabilization of the membrane leading to increase in membrane permeability and drug susceptibilities in yeast cells also gets altered **(Mukhopadhyay K *et al.*, 2002).** Evidences suggest that impaired import of an antifungal agent involve membrane alterations through changes in the sterol and/ or lipid content and are an important attribute of azole resistance. The involvement of membrane lipid phase in drug resistance is now well documented and ABC drug transporter Cdr1p of *C. albicans* and its homologue, human P glycoprotein/ multidrug resistance protein (Pgp/MDR1) have been found to be sensitive to the nature and the physical state of the lipid matrix. Farnesol (FA), a 15-carbon oxygenated lipid made up of isoprene moieties, is one of the key players in lipid signaling pathways of pathogenic fungi. Hyphal formation in *Candida* is inhibited by FA by altering the gene expression using Ras1-adenylate cyclase and MAP kinase pathways **(Lavie Y *et al.*, 2001; Pasrija R *et al.*, 2008)**. The emergence of drug resistance in serial isolates of *C. albicans* from patients undergoing azole treatment is in most cases from a previously more susceptible strain.

1.11. Why plant extracts used as antifungal drugs

- The increased development of drug resistance in human microorganisms as well as the appearance of unwanted side effect of certain antifungal agents leads to constant requirement of new antifungal agents without host toxicity. Although a huge number of antifungal agents have been discovered against disease causing microorganisms but constant development of drug resistance is big concern. Therefore it is required to search for more effective with less toxic novel antifungal agents that would overcome these disadvantages**.** *C. albicans* is notorious for causing Candidiasis. In order to relieve or ease the problem of

decreased availability of drugs required to treat Candidiasis, conventional medicine derived from plants are still being used in many different parts of the world.

- Medicinal plants or plant extracts are renewable in nature dissimilar to the artificial or synthetic drugs or antifungal agents that are extracted or derived from non-renewable sources of primary raw materials such as fossil sources and petrochemicals **(Samanta M.K *et al.*, 2000).** Due to all these advantages, plants continue to be a major source of new major compounds. Today, the indiscriminating use of commercial antimicrobial or antifungal drugs has caused multiple drug resistance in human disease causing microorganisms **(Aliero A *et al.*, 2008).** This condition compelled scientists to search for new and effective antimicrobial or antifungal agents to substitute the present regimens **(Sharanappa R *et al.*, 2013).**

MATERIALS

2. MATERIALS

2.1. Strains and Growth media:

2.3. Spider Media

The composition of spider media include nutrient broth 1% (w/v), Mannitol 1% (w/v), K_2HPO_4 0.2% (w/v) and finally the pH of the media is maintained at 7.2 using 1N HCl

2.4.NAG (n-Acetyl Glucosamine) Media

The composition of NAG media includes yeast nitrogenous base (w/o nitrogenous base) 0.335% (w/v), NaCl 0.45% (w/v). After autoclaving the mixture add 25mM NAG for 10 ml media

METHODS

3. . METHODS

3.1 Extract preparation:

Plant extracts of Clove and Cardamom were first crushed into fine powder and then extracted with various HPLC grade organic solvents. Isopropanol is used for the extraction of the active components of Clove and Cardamom which is then concentrated by distillation method. Lastly, the emulsification of the concentrated extract is done by ethyl acetate.

3.2 Growth Curve of *Candida albicans*

- 50 ml of YEPD media is taken
- Add 60 µl of cell suspensions into that media.
- Pour 3 ml of media into the cuvette and set the spectrophotometer to autozero
- After making autozero add 3 ml of cultured media into the cuvette
- Check the Optical density in spectrophotometer
- Repeat the process after every 4 hrs

3.3. Drug susceptibility assay:

3.3.1. Spot assay:

In the process of spot assay, cells were allowed to grow for overnight on YEPD agar plate and then these cells were suspended in 0.9% saline and then setting the OD_{600} of the suspension to 0.1. Then, 5 µL spot of yeast culture was spotted onto YEPD plates in the absence (Growth control) and presence of the plant extract by fivefold serial dilution. Growth difference was checked after incubating the spotted plates for 48 hrs at 30°C (**Mukhopadhyay K *et al.*, 2004)**.

3.3.2. Sterol estimation by using Spider and n-acetyl glucosamine media

- Inoculate the primary culture and grow it overnight at 30°C.
- From the primary culture inoculate the secondary culture and allow it to grow for overnight at 30°C.
- Sterols were extracted **(Prasad T, 2010)** from cells grown in the absence (control) and presence of Clove and Cardamom at 30°C with slight modification in it.

- The cells with hypha which were treated with the plant extracts (Clove and Cardamom) are maintained separately.
- Centrifuge the cells at 6000rpm for 15 min at 4°C.
- Give twice washing with the milli Q water to the pellet down cells at 6000rpm for 15 min at 4°C.
- Take the weight of the cells by removing the moisture.
- Add 2.5 ml MeOH, 1.5ml KOH (60% [wt/vol]) and 1 ml pyrogallol dissolved in CH_3OH (0.5% [wt/vol]) in the cell suspension and then vortexing it to mix the solution
- Further refluxing the cell suspension at 80°C for 2 hrs
- Organic solvents like *n*-heptane are used for extracting the sterols and this process is repeated 2-3 times by adding known volume of *n*-heptane.
- The upper layer is collected into another glass vial.
- Four-peak spectral absorption patterns were shown by the extracted sterol which is produced by ergosterol and 24(28)-dehydroergosterol (24(28)-DHE) contents.
- Absorbance of ergosterol+24(28)-DHE is seen at 281.5 nm ($A_{281.5nm}$), whereas absorbance of only 24(28)-DHE is seen at 230 nm (A_{230nm}).

3.3.3. Morphogenic studies of *Candida albicans*

3.3.3.1. On Spider media:

- Spider media is used for the hyphal morphogenic study of *C. albicans* cells **(T.Prasad, 2005).**
- The cells grown in YEPD media were inoculated in spider media in the absence (control) and presence of Clove and Cardamom and then they were allowed to grow at 37ºC for the hyphal growth.
- The images are captured in *FluoView™ FV1000* confocal microscope.

3.3.3.2. On n-acetyl glucosamine media:

- N-acteyl glucosamine media is used for studying hyphal morphogenesis of *C. albicans* cells **(Prasad T, 2005).**

- The cells grown in YEPD media were inoculated in n-acteyl glucosamine media in the absence (control) and presence of clove and cardamom and then they were allowed to grow at 37ºC for hyphal growth.
- The images are captured in *FluoView™ FV1000* confocal microscope.

RESULTS & DISCUSSION

4. RESULTS AND DISCUSSION

5. 4.1. RESULTS AND DISCUSSION

4.1. *C. albicans* cells showed sigmoidal growth curve

C. albicans cells grown on YEPD media were monitored in spectrophotometer by measuring OD at 600nm. The values obtained were plotted against time (**Fig: 6**.) and the curve obtained by this plotting is sigmoidal. For carrying out further experiments mid-exponential time was recorded.

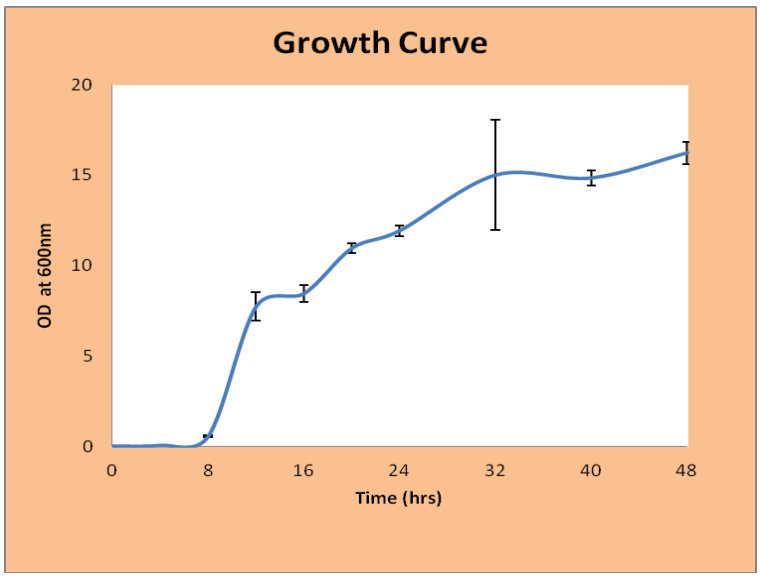


Fig 6: Growth curve of *C. albicans*

4.2. Spot assay helped to determine the MIC of plant extracts (Clove and Cardamom)

In this assay, 5 µL spot of yeast culture (OD_{600}=0.1) was spotted onto YEPD plates in the absence (Growth control) and presence of plant extract (Cardamom (20µl/ml) and clove (2µl/ml)) by fivefold serial dilution. Growth difference was checked after incubating the spotted plates for 48 hrs at 30°C.

Spot assay is generally done to evaluate the drug sensitivities of *C. albicans* cells on solid YEPD media in presence and absence of plant extracts (Clove and Cardamom). The obtained results confirmed the sensitivity of *Candida* cells towards Clove and Cardamom. MIC of Clove and cardamom were also determined by Spot assay (**Fig: 7**).

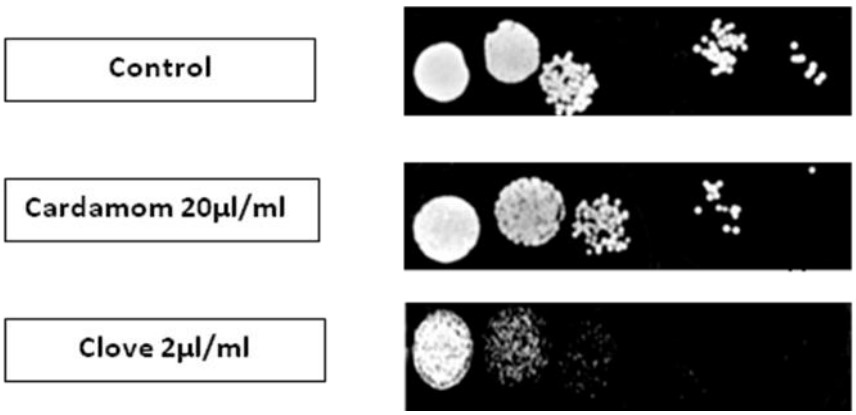
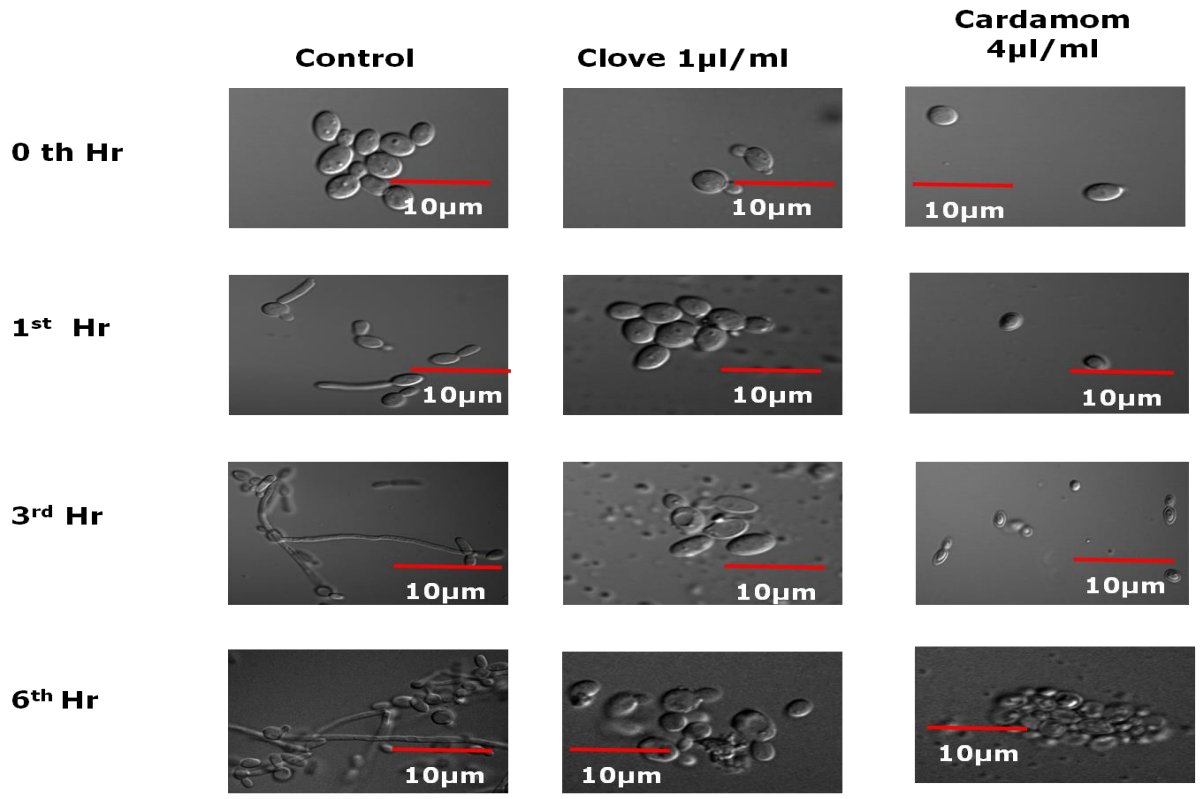


Fig 7: Spot assay of *C. albicans* showing growth in the absence (control) and in the presence of Cardamom and Clove

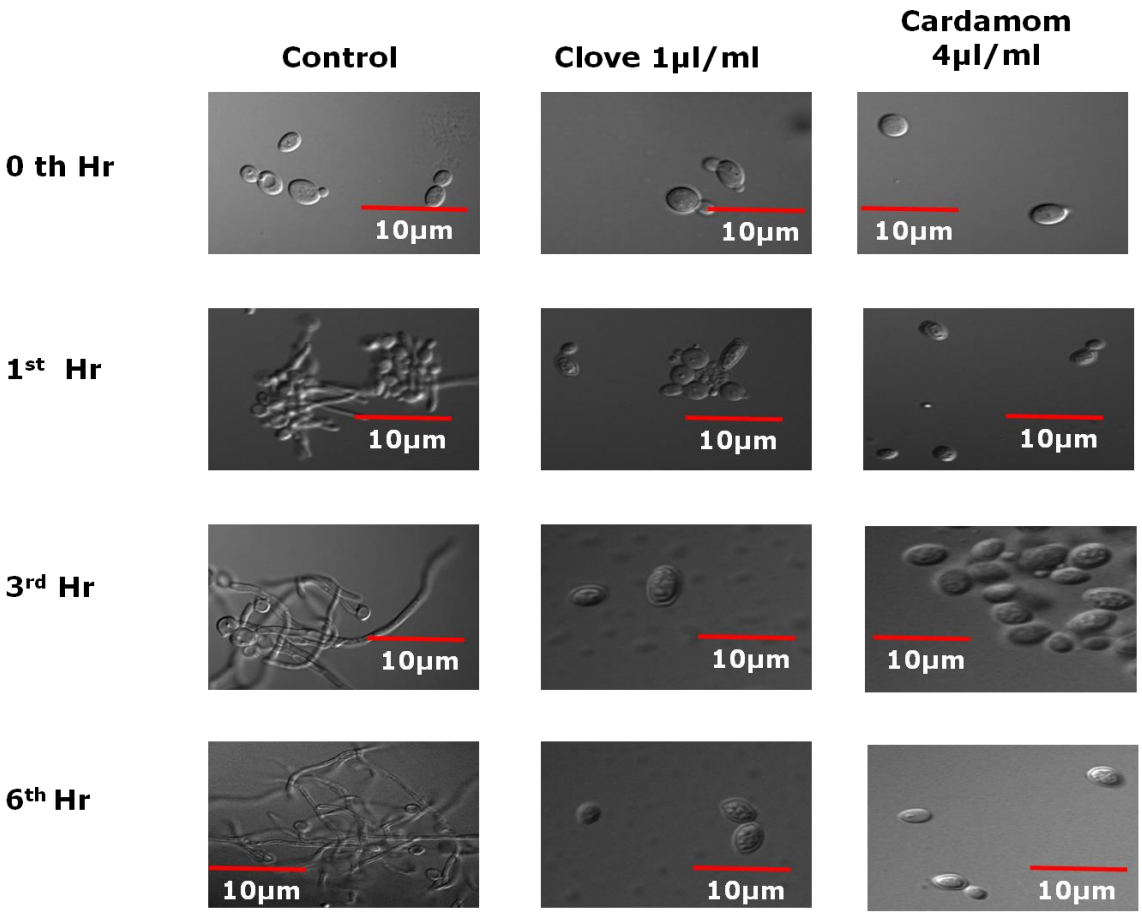
4.3. On spider media plant extracts showed inhibition in hyphal Morphogenesis

In this study the effect of plant extracts is used analyze the growth of hyphae in spider media. The results of spot assay of *Candida* cells are used to calculate MIC of Clove and Cardamom. The cells grown in YEPD media were inoculated in spider media in the presence of Clove and Cardamom and then they were allowed to grow at 37ºC for the hyphal growth. The images are captured in *FluoView™ FV1000* confocal microscope. The retrieved results showed complete inhibition of hyphal morphogenesis upon treatment with both Clove and Cardamom (Fig.8).



4.4. On N-Acetyl glucosamine media plant extracts showed inhibition in hyphal Morphogenesis

In this study the effect of plant extracts is used analyze the growth of hyphae in n-acetyl glucosamine media. The results of spot assay of *Candida* cells are used to calculate MIC of Clove and Cardamom. The cells grown in YEPD media were inoculated in n-acetyl glucosamine media in the presence of Clove and Cardamom and then they were allowed to grow at 37ºC for the hyphal growth. The images are captured in *FluoView™ FV1000* confocal microscope. The retrieved results showed complete inhibition of hyphal morphogenesis upon treatment with both Clove and Cardamom (Fig.9).



4.5. After treating with Clove and Cardamom significant reduction in levels of ergosterol was seen in *Candida* cells on spider media

The ability of Clove and Cardamom to inhibit hyphal morphogenesis was very well seen in different susceptibility assay but the process behind both the plant extracts inhibition on hyphal morphogenesis was still not clear. So this study was done to prove the function of sterol on morphogenesis of hyphae on spider media. Cell membranes of *Candida* cells are mainly made up of Ergosterol. Cells induced on YEPD media were grown on spider media and incubated at 37ºC to induce hyphal cells. Sterols were extracted after harvesting the cells. Cells treated with clove and cardamom showed marked reduction in the level of sterol (Fig. 10). Clove and cardamom showed 6% and 4% respective reduction in spider media when they are compared with the control.

Fig 10: Graph showing the estimation of sterol of *C. albicans* by using Spider media in the absence (control) and presence of Clove and Cardamom

4.6. After treating with Clove and Cardamom significant reduction in levels of ergosterol was seen in *Candida* cells on n-acetyl glucosamine media

The ability of Clove and Cardamom to inhibit hyphal morphogenesis was very well seen in different susceptibility assay but the process behind both the plant extracts inhibition on hyphal morphogenesis was still not clear. So this study was done to prove the function of sterol on morphogenesis of hyphae on n-acetyl glucosamine media. Cell membranes of *Candida* cells are mainly made up of Ergosterol. Cells induced on YEPD media were grown on n-acetyl glucosamine media and incubated at 37ºC to induce hyphal cells. Sterols were extracted after harvesting the cells. Cells treated with clove and cardamom showed marked reduction in the level of sterol (Fig. 10). Clove and cardamom showed 20% and 80% respective reduction in n-acetyl glucosamine media when they are compared with the control

Fig 11: Graph showing the sterol estimation of *Candida albicans* by using n-acetyl glucosamine in the absence (control) and presence of plant extracts (Clove and Cardamom)

CONCLUSION

5. CONCLUSION

Isopropanol is used for the extraction of the active components of both clove and cardamom whereas they are emulsified by using ethyl acetate. Minimum inhibition concentration of clove and cardamom in *Candida* cells was determined by Spot assay. Clove and cardamom showed complete inhibition of hyphal morphogenesis on both spider and N-Acetyl glucosamine supplemented media. Furthermore, the sterol content analysis was done to determine the molecular mechanism behind the inhibition of hyphal morphogenesis by the plant extracts. Marked reduction in sterol content was seen in clove and cardamom treated cells grown on spider and N-acetyl glucosamine supplemented media. The retrieved results were compared with untreated (control) cells. Cell membranes of *Candida* cells are mainly made up of Ergosterol; so when the sterol content of the membrane is altered it may show inhibition of the hyphal morphogenesis of *C. albicans* towards clove and cardamom. Thus, it appears from the results that the plant extract used for this study have clinical relevance since they are effective against hyphal morphogenesis because of the important trait of *Candida* cells during pathogenesis. It is evident from the results that the plant extracts used for this study are also effective in interfering with the bio-synthetic pathway of ergosterol. It is important to find that plant extracts other cellular targets which appear to have the potential to contribute to development of new therapeutic strategies for clinical applications.

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