Presumptive Identification of *Candida* spp. from Clinical Isolates and Anticandidal Effect of Essential Oils

S. S. Khandare^{1*}, A.P. Moon², M. G. Ingale³

P.G.Department of Microbiology, Bajaj College of Science, Wardha. Maharashtra-442001.

Corresponding author email- ksuhas21@gmail.com

Abstract

Candida is normal flora of human skin, mouth, vagina, colon and causes mycosis worldwide. *Candida* causes oral thrush, vaginitis and other potentially life-threatening diseases. This study assesses anticandidal potential of essential oils against *Candida* species isolated from clinical samples obtained from Mahatma Gandhi Institute of Medical Sciences, Sewagram, Wardha, Maharashtra. On the basis of morphological characterization on Sabouraud dextrose agar, CHROMagar, Germ tube test and biochemical characterization, *Candida* species identified as *Candida albicans*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis*. Clove, thyme, cinnamon and eucalyptus essential oils with concentrations of 6.2, 12.5, 25, 50 and 100 µg/ml were used to study their anticandidal activity. *C. albicans* demonstrated zone of inhibition (ZOI) 29, 20, 32, 29 mm, *Candida krusei* 25, 25, 30, 21 mm, *Candida glabrata* 24, 22, 31, 32mm, *Candida parapsilosis* 33,31,33, 29mm towards clove, thyme, cinnamon and eucalyptus oil (each at 100 µg/ml) respectively which are highest ZOI as compared with antifungal agent Amphotericin-B, which showed 16, 13, 12, 8 mm against *C. albicans*, *C. krusei*, *C. glabrata* and *C. parapsilosis* respectively. All *Candida* spp. produced biofilms and enzyme lipase. This study will facilitate the development of novel broad-spectrum key molecules against a range of *Candida* species.

Key words: *Candida* spp., Essential oils, zone of inhibition, biofilm, lipase.

1. Introduction:

Candida is dimorphic fungus that exists as a commensal of warm-blooded animals including humans (Ashman et al., 1995). It is oval yeast with a single bud. It is a part of a normal flora of mucous membrane of the upper respiratory, gastrointestinal and female genital tracts (Levinson, 2008). Many changes in the internal and external environment induce this harmless saprophyte to become a true pathogen.

Fungi, once considered non-pathogenic microbiological curiosities have emerged as an important cause of community acquired and health care associated infections (Chakrabarti et al., 2000). Factors like HIV/AIDS, treatment with broad spectrum antibiotics and immunosuppressive drugs increase the vulnerability to fungal infections (Deorukhkar et al., 2012). Among various pathogenic species of fungi, *Candida* is the most prominent cause of fungal infections (Sullivan et al., 1996). *Candida albicans* is an opportunistic fungal pathogen that exists as a harmless commensal in the gastrointestinal and genitourinary tracts in about 70% of humans and about 75% of women suffer from *Candida* infection at least once in their lifetime (Ruhnke et al., 2002). Although a part of normal microbiota, *Candida* is capable of causing various clinical manifestations ranging from mucocutaneous overgrowth to disseminated infections like candidemia (Eggimann et al., 2003). Virulence factors like tissue adhesion, phenotypic switching, biofilm formation and production of extracellular hydrolytic enzymes play an important role in colonization and invasion of host tissues (Sachin et al., 2012). The genus *Candida* is composed of heterogeneous group of organisms and more than 17 different *Candida* spp. are implicated in human infections (Pfaller et al., 2007). Advances in medical practice have increased the frequency of fungal infections in hospitalized patients. Fungal pathogens, now account for almost 10% of all nosocomial blood stream infections (BSIs) (Warnock et al., 1996). Among various fungal pathogens, *Candida* spp. is the important cause of substantial

morbidity and mortality in hospitalized patients. It is fast becoming a very important pathogen among critically ill hospitalized patients (Rajkumari *et al.*, 2014).

Essential oils are natural products produced by aromatic plants and are mainly composed of terpenes and terpenoids (Nazzaro *et al.*, 2013). Being lipophilic, these oils typically integrate into membrane structures causing increased cell permeability, leaching of intracellular components and inactivation of enzymes (Sikkematb *et al.*, 1994). Essential oils can act against *Candida* by inhibiting ergosterol synthesis (Adil *et al.*, 2014), altering cell wall morphology (de Castro *et al.*, 2013), inhibiting enzymes involved in cell wall synthesis (Bang *et al.*, 2000), changing cell membrane permeability (Rajkowska *et al.*, 2016) and producing oxygen reactive species (Singh *et al.*, 2016). Antimicrobial, anti-aseptic, anti-inflammation and anti-oxidant activity of essential oils, alone and in combination with commercial agents is well known (Santos *et al.*, 2012). The aim of this study was therefore to investigate the antifungal potential of four commercial essential oils ie. clove, thyme, cinnamon and eucalyptus oil against *Candida* species. Antifungal activity of commercial essential oils was compared to Amphotericin-B, Terbinafine, Ketoconazole and Fluconazole. These findings highlight the in vitro efficacy of certain essential oils against *Candida* species.

2. Methodology

Sample collection:

For the isolation of *Candida* species, various clinical specimens like urine, sputum, endo-cervical and vaginal swabs (VS), urethra swabs, wound and eye swabs were collected from department of Microbiology, Mahatma Gandhi Institute of Medical Sciences, Sewagram, Wardha, Maharashtra. All the specimens were preserved until use according to the standard microbiological procedures (Jay *et al.*, 2013).

Procurement of essential oils

Selected essential oils (Clove, Thyme, Cinnamon and Eucalyptus) were purchased from the local market of Wardha, Maharashtra. These oils were selected based on their use in traditional medicine.

Isolation of *Candida* species from clinical samples

For isolation of *Candida* species from clinical samples, Sabouraud's Dextrose Agar (SDA) supplemented with chloramphenicol (0.05 mg/ml) and cyclohexamide (0.5 mg/ml) was used. Samples were inoculated on the sabourauds dextrose agar plates. Plates were incubated at 37°C at 24 hrs. White to cream-colored, pasty and smooth colonies appeared within 24 hours to 48 hrs. on SDA. The samples were sub cultured and were maintained on SDA slants at 4°C. (Samyuktha *et al.*, 2017).

Identification of *Candida* species

The colonies morphologically resembling to the members of genus *Candida* were subjected to Gram staining and Lactophenol cotton blue staining. Isolates were also characterized biochemically by performing indole test, tellurite reduction test, tetrazolium reduction test and also by performing germ tube test. Once the colonies were confirmed, speciation was done by inoculating *Candida* spp. on CHROMagar and incubated at 37°C for 24 hrs. The species were identified by morphology of the colonies on CHROMagar. CHROMagar candida is a novel, differential culture medium that is claimed to facilitate the isolation by colorimetric presumptive identification (Kashid *et al.*, 2011 & Vijaya *et al.*, 2011).

Determination of virulence factors of *Candida* sp.

The various virulence factors of *candida* species are determined as follows

A) Determination of Phospholipase production

The phospholipase activity of *Candida* species was determined by using egg yolk agar medium. Approximately 5 µl of the inoculum of the *Candida* strain was aseptically inoculated onto the egg yolk agar. The plates were dried at room temperature and then incubated at 37°C for 48 h. The plates were examined in terms of the presence of precipitation zone around the colony. The presence of precipitation zone indicates phospholipase enzyme production (Samaranayake *et al.*, 1984).

B) Determination of protease activity

For determination of Protease activity of *Candida* species, a loopful of culture was introduced to a sterile filter paper disc and placed on the surface of bovine serum albumin agar (BSA; pH: 5.0). The inoculated plates were incubated at 37°C for two days, and the diameter of the inhibition zone around the discs was measured to determine protease activity (Gokce *et al.*, 2007).

C) Determination of Lipase activity

A precipitation test using Tween 80 agar plates was carried out to confirm lipolytic activity. Tween 80 substrate plates were prepared as follows (g/L); 10 g peptone, 5 g NaCl₂, 0.1 g CaCl₂·2H₂O, 20 g agar and 10 ml (v/v) Tween 80. This method is based on the principle of calcium salt precipitation. Due to action of lipase enzyme, hydrolysis of tween 80 releases fatty acids which bind with the calcium in the medium to form insoluble crystals around the point of inoculation. Tween 80 is used for the detection of lipases as it contains esters of oleic acid. The organisms were inoculated onto the plates and incubated at 37 °C for 2–4 days. A white precipitation around the boundary of the colony was indicative of lipase activity (D. Kumar *et al.*, 2012).

D) Determination of Biofilm production

Biofilm formation was determined by visual method. In this method a loopful of colonies from the surface of Sabouraud Dextrose Agar (SDA) plate was inoculated into a polystyrene tube (Falcon conical tube with a screw cap), containing 10 ml of Sabouraud Dextrose Broth (SDB), supplemented with glucose (final concentration of 8%). After incubation at 35°C for 48 h, broth in the tubes was gently aspirated. The tubes were washed with distilled water twice and then stained with safranin 2% for 10 min. Afterwards, they were tested to determine the presence of an adherent layer. With respect to adherence to the surface of the tube, biofilm production was scored as strong (+++), moderate (++) and weak (+). Similarly Biofilm production was also assessed on Tryptic soy broth & Congo red agar medium (Yigit *et al.*, 2011).

Evaluation of Anticandidal activity of essential oils by Agar well diffusion assay

The anticandidal activities of the selected essential oils were determined by Agar well diffusion assay techniques (Reeves *et al.*, 1989). In this method, 100 µl of standardized inoculum of each test fungus were spread onto sterile Sabouraud Dextrose Agar. Essential oils with concentrations of 6.2, 12.5, 25, 50 and 100 µg/ml were used to study their anticandidal activity. Well with 8 mm diameter was cut from the agar using a sterile cork-borer and subsequently each well was filled with the appropriate concentrations of essential oils. The plates were kept at room temperature for 1 hr to allow proper diffusion of the oil into agar and then incubated at 30 °C for 3–5 days. Triplicates were prepared for each sample. The essential oils having antimicrobial activity inhibit the fungal growth and the clear zones were formed. The zone of inhibition was measured in millimeters (Raid *et al.*, 2014).

Evaluation of Anticandidal activity of standard antifungal drugs

For comparative analysis of sensitivity of *Candida* sp. to essential oils and antifungal drugs, a disc diffusion assay as recommended by Clinical and Laboratory Standards Institute (CLSI) was performed to determine the susceptibility of the test isolates to standard antifungal drugs. Briefly, 100 µl of candida suspension adjusted to a 0.5 McFarland standard was streaked onto SDA plates and then discs of antifungal drugs (Amphotericin-B (AMB), Terbinafine (TRF), Ketoconazole (KTC), Fluconazole (FLC) (100 µg/disc)), were placed over the agar surface and incubated at 37 ° C for 24 h. The diameter of the zone of inhibition around the discs was recorded in mm. Each experiment was conducted in triplicate and the average zone size was calculated (Clinical & Laboratory standards Institute USA 2009).

3. Results and Discussion:

In the present study, four *candida* species were isolated from clinical samples obtained from Mahatma Gandhi Institute of Medical Sciences, Sewagram, Wardha. On the basis of morphological & biochemical characteristics & growth on differential culture medium CHROMagar these *candida* species were identified as *Candida albicans*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis*. All the four species were found to be gram positive & also showed positive results for germ tube test, tellurite reduction test & tetrazolium reduction test. Among the four isolates, *Candida albicans* and *Candida parapsilosis* were also found to produce indole. The speciation of isolates was done by studying their growth on CHROMagar. On this medium, *Candida albicans*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis* produce Green, purple and white colour colonies respectively (Table 1 & Fig 1). Chromogenic agar is a newer and more rapid method to speciate candida, which contains enzymatic substrates that are linked to chromogenic compounds. When specific enzyme cleaves the substrate, chromogenic substances produce colour. The action of different enzymes produced by yeast species results in color variation which is useful for the presumptive identification of some yeasts (Odds et al., 1994). But we found that CHROMagar is an effective and faster screening method which can be used for speciation of *Candida*. In the present study CHROM agar could identify, *Candida albicans*, *Candida tropicalis* and *Candida glabrata*. Similar findings have been reported by different studies (Golia et al., 2013).

All the isolates were found to produce lipase enzyme as marker of their virulence (Table 2). As far as the biofilm formation is concern, all the isolates were found to form the biofilm. *Candida albicans* was found to produce strong (+++), moderate (++) & strong (+++) biofilm on Tryptic soy broth, Sabouraud Dextrose Broth (SDB) & Congo red agar medium respectively. *Candida krusei* was found to produce moderate (++) , weak (+) & moderate (++) biofilm on Tryptic soy broth, SDB & Congo Red agar medium respectively. *Candida glabrata* showed strong biofilm production on Tryptic soy broth and SDB, but no biofilm production was observed on Congo red medium. Percent biofilm production by *Candida parapsilosis* was found to be weak (+), strong (+++) and weak (+) on Tryptic soy broth, SDB & Congo red agar medium respectively (Table 3).

Essentials oils have been tested for in vitro anticandidal activity and demonstrated to have potential anticandidal effect. Essential oils with concentrations of 6.2, 12.5, 25, 50 and 100 µg/ml were used to study their anticandidal activity. *C. albicans* demonstrated zone of inhibition (ZOI) 29, 20, 32, 29 mm, *Candida krusei* 25, 25, 30, 21 mm, *Candida glabrata* 24, 22, 31, 32mm, *Candida parapsilosis* 33, 31, 33, 29 mm towards clove, thyme, cinnamon and eucalyptus oil (each at 100 µg/ml) respectively which are highest ZOI as compared with antifungal agent Amphotericin-B which showed 16, 13, 12, 8 mm against *C. albicans*, *C. krusei*, *C. glabrata* and *C. parapsilosis* respectively. Anticandidal activity shown by clove and cinnamon oils (ZOI 33 mm each) against *Candida parapsilosis* was found to be more than antifungal agent Terbinafine (ZOI of 31 mm). Anticandidal activity of essential oils was also found to be more as compared to ketoconazole and fluconazole. Activity of clove and cinnamon oil was found to be more (29 and 32 mm each) against *C. albicans* as compared to fluconazole (28

mm). The highest zone of inhibition was found to be against *Candida parapsilosis* ie.33 mm each shown by clove and cinnamon oil respectively. The highest zone of inhibition shown by clove, thyme and eucalyptus oil was found to be against *C. parapsilosis* (33 mm), *C. parapsilosis* (31 mm), *C. parapsilosis* (33 mm), *C. glabrata* (32 mm) respectively. While the lowest activity was recorded against *C. albicans* for thyme oil (ZOI 20 mm). Our result showed that cinnamon oil has higher anticandidal activity against all the tested *candida* species. The antimicrobial properties of *Cinnamon* are thought to be due to eugenol and cinnamaldehyde (Matan et al., 2006). Cinnamon bark has cinnamaldehyde as its major component, while cinnamon leaf oil has eugenol rather than cinnamaldehyde. Eugenol is a phenolic compound, and several authors have reported its high antimicrobial activity in phenolic structured compounds (Suhr et al., 2003). Many publications have reported about significant antimicrobial effects of cinnamon and clove essential oils against important fungal pathogens: These results was in conformity with the previous studies, which reported that clove, thyme and cinnamon essential oils exhibited an inhibitory effect against a wide range of pathogenic fungi. Radwan et al., 2014 tested antifungal activities of the essential oils against the fungi & his findings revealed that thyme oil completely inhibited the growth of different fungal isolates at concentrations of 0.25, 0.5 and 1%. Clove and cinnamon oils completely inhibited the growth of different fungal isolates at a concentration of 6%.

Table 1: Morphological and Biochemical Characterization of *Candida* sp.

Tests	<i>C. albicans</i>	<i>C. krusei</i>	<i>C. glabrata</i>	<i>C. parapsilosis</i>
Grams staining	+	+	+	+
Germ tube test	+	+	+	+
Indole test	+	-	-	+
Tellurite reduction	+	+	+	+
Tetrazolium reduction	+	+	+	+
Colonies on SDA	White	White	Cream	Cream
CHROMagar	Green	Purple	White	White

Table 2: Studies on virulence markers

Virulence factors	<i>C. albicans</i>	<i>C. krusei</i>	<i>C. gabrata</i>	<i>C. parapsilosis</i>
Phospholipase	-	-	-	-
Lipase	+	+	+	+
Proteinase	-	-	-	-

Table 3: Biofilm formation

Medium	<i>C. albicans</i>	<i>C. krusei</i>	<i>C. glabrata</i>	<i>C. parapsilosis</i>
Tryptic soy broth	Strong (+++)	Moderate (++)	Strong (+++)	Weak (+)
SDA	Moderate (++)	Weak (+)	Strong (+++)	Strong (+++)
Congo red agar	Strong (+++)	Moderate (++)	-	Weak (+)

Table 4: Anticandidal activity of essential oil & antifungal agents

Candida sp.	Essential oils (100 µg/ml)				Antifungal agents (100 µg/disc)			
	Zone of inhibition (in mm)				Zone of inhibition (in mm)			
	Clove	Thyme	Cinnamon	Eucalyptus	AMB	TRF	KTC	FLC
<i>C. albicans</i>	29 ± 2	20 ± 1	32 ± 1	29 ± 1	16 ± 1	32 ± 1	28 ± 2	28 ± 2
<i>C. krusei</i>	25 ± 2	25 ± 2	30 ± 2	21 ± 1	13 ± 2	33 ± 2	29 ± 1	35 ± 1
<i>C. glabrata</i>	24 ± 2	22 ± 2	31 ± 1	32 ± 1	12 ± 2	32 ± 1	28 ± 2	26 ± 2
<i>C. parapsilosis</i>	33 ± 1	31 ± 1	33 ± 1	29 ± 2	08 ± 1	31 ± 2	27 ± 2	22 ± 2

Fig 1: Growth of Candida species on CHROMagar *C. albicans* *C. krusei* *C. glabrata* *C. parapsilosis***4. Conclusion:**

Essential oils like Clove, Thyme, Cinnamon & Eucalyptus oils inhibited the growth of *Candida* species more efficiently than other antifungal agents. This comprehensive study will benefit researchers who wish to explore the potential of essential oils in the development of novel broad-spectrum key molecules against a broad range of *Candida* sp. Future scope of antifungal activity of essential oil will help to control *Candida* pathogens as well as diseases in humans.

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