_____Original Article_____

# The Evaluation of the virulence factors of clinical *Candida* isolates and the anti-biofilm activity of *Elettaria cardamomum* against multi-drug resistant *Candida albicans*

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## Abstract

**Background and Purpose:** Today, treatment of life-threatening fungal infections, caused by *Candida* species, has become a major problem. In the present study, we aimed to evaluate the antifungal susceptibility patterns of different clinical *Candida* isolates, determine the virulence factors in multi-drug resistant (MDR) *Candida* species, and assess the anti-biofilm activity of *Elettaria cardamomum* against MDR *Candida* species.

**Materials and Methods:** A total of 202 isolates from different *Candida* species were obtained from three governmental hospitals in Senthamangalam, Tiruchengode, and Namakkal, Tamil Nadu, India. The isolates were identified, using conventional methods. *Candida* species were tested for virulence factors such as biofilm, protease, and phospholipase activity. The minimum inhibitory concentration (MIC) of *Elettaria cardamomum* against MDR biofilm-forming *C. albicans* was determined, using plate and tube methods.

**Results:**The identified *Candida* isolates (n=202) were *C. albicans* (74/202), *C. glabrata* (53/202), *C. parapsilosis* (44/202), *C. tropicalis* (15/202), and *C. dubliniensis* (16/202). The isolates were subjected to antifungal susceptibility testing and the virulence factors were determined. In terms of biofilm production, non-*C. albicans* species such as *C. dubliniensis* showed 75% activity. Also, regarding protease activity, *C. parapsilosis* (75%) showed the highest percentage of protease production. In addition, *Candida* species showed strong positivity for phospholipase activity (62.87%). In the MIC method, the acetonic extract completely inhibited biofilm production at a concentration of 125 µl (56.25 µg). In comparison with the ethanolic extract, the acetonic extract showed major activity against biofilm production.

**Conclusion**: Based on the findings, pathogenic *C. albicans* species were inhibited by the ethanolic and acetonic extracts of *E. cardamomum*. In recent years, MDR and biofilm-forming pathogenic *Candida* species have been increasingly detected in clinical settings. Therefore, herbal derivatives might contribute to the treatment of infections without causing any side-effects and prevent the associated mortality.

**Keywords:** Biofilm inhibition, *Candida* species, *Elettaria cardamomum*, Virulence factors

## Introduction

Today, *Candida* species are emerging as the major agents of hospital-acquired infections. These species are ranked as the third or fourth most commonly isolated bloodstream pathogens. Although *C. albicans* is recognized as the predominant etiological agent of candidiasis, other Candida species such as *C. krusei, C. glabrata, C. lusitaniae,* and *C. dubliniensis* (the most recently identified *Candida* species), which tend to be less susceptible to common antifungal drugs, have also emerged as substantial opportunistic pathogens [1, 2].

Infectious diseases account for various health problems in developing countries such as India. Microorganisms have developed resistance to many antibiotics, and as a result, immense clinical problems have emerged in the treatment of these diseases [3]. The resistance of organisms has increased due to the indiscriminate use of commercial antimicrobial drugs, commonly used for the treatment of infectious diseases. Consequently, researchers have been encouraged to seek new antimicrobial compounds from various sources, including medicinal plants [4].

*Candida* strains possess a number of virulence factors (such as slime production), which enable the organisms to cause hematogenously disseminated infections in susceptible hosts. Biofilms are structured microbial communities, which are attached and encased in a matrix of exopolymeric materials [5] and are essential to the development

of clinical infections.

A typical laboratory fungal model of biofilm formation involves two operational steps: 1) adhesion, and 2) biofilm growth and maturation [6]. The presence of virulence factors determines the pathogenic potential of any microorganism. *Candida* species have been shown to possess virulence factors such as haemolysin, coagulase, and biofilm formation, phospholipase activity, phenotypic switching, surface hydrophobicity, adherence to vaginal epithelial cells, and proteinase production [7, 8].

*Elettaria cardamomum* has been traditionally used in the treatment of various gastrointestinal, cardiovascular, and neural disorders. This herb is used as a powerful aromatic, carminative, diuretic, and stimulant agent. In India, *E. cardamomum* is used for many conditions, including asthma, bronchitis, kidney stones, anorexia, general debility, and urinary tract disorders. Previous studies have revealed the effectiveness of this herb as an anti-carcinogenic, anti-ulcerogenic, antimicrobial, and anticonvulsant agent [9-11].

A number of studies have been conducted on the chemical and antimicrobial properties of *E. cardamomum* [12, 13]. However, the present study focuses on the antimicrobial activity of different solvent *E. cardamomum* extracts against infection-causing pathogens. The objective of our study was to evaluate the virulence factors and antifungal susceptibility patterns of different *Candida* species and validate the antimicrobial potential of *E. cardamomum* extracts against *Candida* species causing infection.

## Materials and Methods
### Study design

The present study was performed to analyze pathogenic *Candida* species and the virulence factors. The devices were collected from patients who were infected and admitted to the intensive care unit. After the *Candida* species were isolated from the devices, they were identified using selective media, standard biochemical tests, and microscopic observation.

Then, the identified *Candida* species were assessed in terms of their antibiogram activity against azoles and amines via disc diffusion method. Multi-drug resistant (MDR) *Candida* species were evaluated in terms of virulence factors such as protease, hemolysin, biofilm, and phospholipase. Considering the presence of different biotype characteristics, *Candida* species

were confirmed. Then, MDR biofilm-forming *Candida* species were inactivated, using *E. cardamomum* extracts through disc diffusion and tube methods.

### Collection of Candida species

A total of 202 *Candida* isolates were obtained from three governmental hospitals in Senthamangalam, Tiruchengode, and Namakkal, Tamil Nadu, India during August 2011 and June 2012.

### Identification of Candida species

All the isolates were streaked on Bromocresol Green Agar (Hi-Media, Mumbai, India) for genus-level identification and further streaked on HiCrome *Candida* Differential Agar (Hi-Media, Mumbai, India) for species-level confirmation. Biochemical tests were performed via sugar fermentation and assimilation methods. Among *Candida* species, *C. albicans* showed the ability to produce germ tubes in human serum.

### Antifungal susceptibility test

Antifungal susceptibility test was performed via disc diffusion method. The used discs included amphotericin B (100 units), fluconazole (10 μg), clotrimazole (10 μg), nystatin (100 μg), itraconazole (10 μg), and ketoconazole (10 μg). The inhibition zone was measured and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [14]. The control *C. albicans* ATCC 10231, *C. glabrata* ATCC 15126, and *C. tropicalis* ATCC 10610 strains were also used in this study.

### Determination of virulence factors Biofilm production

Biofilm production was determined by visual methods. 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 (+) [15].

### Determination of protease activity

Protease activity of *Candida* species was determined in the present study. A loopful of colonies 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, using the standard formula [16]:

Pz= Colony diameter/(colony diameter + zone of precipitation)

### Phospholipase production

The phospholipase activity of *Candida* species was determined, using egg yolk agar medium. Approximately 5μl of the standard inoculum of the test strain, containing 108 cells/ml *Candida*, 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 indicated phospholipase enzyme production [17].

### Adherence of Candida species by cell surface hydrophobicity test

The hydrophobicity of *Candida* isolates was measured by assessing the adhesion of the yeast to hydrocarbons, such as cyclohexane or xylene. The tested strains were grown overnight in 5 ml of yeast peptone dextrose or yeast nitrogen base broth at 28°C. The cells were washed with phosphate-buffered saline (PBS) and concentrated to obtain a solution corresponding to an optical density of 1 in 600.

For adhesion assays, 3 ml of the cell suspension was mixed with 150 μl of cyclohexane or xylene in acid-washed glass tubes. The sample was vigorously mixed, using a vortex mixer for 1 min. After being placed at room temperature for 20-60 min, absorbance at 600 nm in the aqueous phase (A1) was measured and compared with the value obtained prior to the mixing procedure (A0). A percentage of cells in the cyclohexane/xylene layer (adhered cells) was used to estimate hydrophobicity, using the following formula [18]:

Percentage of cell adhesion= (A1/A0) × 100

### Preparation of ethanolic and acetonic extracts of E. cardamomum

For this purpose, 10 g of each powdered plant sample was mixed with 250 ml of 96% ethanol and 100% acetone in a separate conical flask. The mixture was kept for 2-5 days in tightly sealed containers at room temperature and shaken several times every day. This mixture was filtered through a filter paper to remove the coarse plant materials. Further extraction of the residue was repeated 3-5 times until a clear supernatant liquid was obtained. The filtrates of each tested plant were evaporated to dryness, using a rotary evaporator at 40°C. The final dried samples were weighed and stored at -20°C until further use. The MIC of *E. cardamomum* was determined using the following two methods:

### Plate method

The wells were punctured in plates containing SDA, and the ethanolic and acetonic extracts were loaded in different concentrations, i.e., 20, 50, 70, and 100 μl. Afterwards, the plate was incubated and the results were reported [19].

### Tube method

A loopful of organisms from the surface of SDA plates was inoculated into the tubes (tubes were taken in pairs for each *Candida* strain), containing 6 ml of Sabouraud broth, supplemented with 8% glucose to the final concentration. Also, a control tube with broth, without cell suspension, was used for the comparisons. The tubes were incubated at 37°C for 48 h. Afterwards, various concentrations of acetonic and ethanolic *E. cardamomum* extracts (25-300 μl) were added to *Candida* broth tubes. They were maintained for 30 min, and then, the cell suspension in the tubes was poured or aspirated out. The walls of the tubes were stained with safranin 1%, and the adhesive layer, produced on the tube walls, was interpreted for biofilm or slime inhibition [20].

## Results

### Identification of Candida species

In SDA, the organisms produced smooth, diploid colonies and were confirmed by the Bromocresol Green Agar medium. Chlamydospore production was confirmed by using corn meal agar. Species were distinguished by germ tube production. *C. albicans* showed a light green color in HiCrome *Candida* Differential Agar. Also, *C. Glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. dubliniensis* showed pink, cream, metallic blue, and dark green colors, respectively. Biochemical characteristics were confirmed by sugar fermentation. Sugar assimilation test results are presented in Figures 1, 2, and 3. A total of 202 isolates were identified

**Figure 1.** Bromocresol Green Agar medium



**Figure 2.** Sabouraud Dextrose Agar medium



**Figure 3.** *Candida* Differential Agar medium

as *C. albicans* (n=74), *C. glabrata* (n=53), *C. parapsilosis* (n=44), *C. tropicalis* (n=15), and *C. dubliniensis* (n=16).

On the antifungal sensitivity test, *C. albicans* showed 95.94% resistance to fluconazole, and *C. glabrata* showed 94.33% resistance to fluconazole, clotrimazole, and ketoconazole. Based on the findings, *C. parapsilosis* showed 95.45% resistance to fluconazole and clotrimazole, *C. dubliniensis* exhibited 100% resistance to clotrimazole and itraconazole, and *C. tropicalis* showed 100% resistance to fluconazole and itraconazole. The results are presented in Table 1.

In terms of biofilm production, *C. dubliniensis* (75%) showed strong biofilm-producing activity, followed by *C. glabrata* (67.9%), *C. albicans* (58.11%), *C. tropicalis* (53.33%), and *C.* *parapsilosis* (45.45%) (Figure 4). Regarding protease activity, among *Candida* species, *C. parapsilosis* (75%) showed the highest percentage of protease production, followed by *C. albicans* (68.92%), *C. glabrata* (64.15%), *C. tropicalis* (40.0%), and *C. dubliniensis* (37.5%) (Figure 5). In terms of phospholipase activity, 62.87%, 14.35%, 8.91%, 17.82%, and 0.99% of *Candida* species showed strong positive, moderate positive, weak positive, poor positive, and no enzymatic activity, respectively (Figure 6).

In total, 70 isolates of MDR biofilm-producing *Candida* species were evaluated in terms of adherence activity. The absolute adherence percentage of *Candida* species ranged between 20% and 100%. Among *Candida* species, the isolates of *C. glabrata* (98.33%), followed by *C. albicans*,

**Table 1.** *In vitro* antifungal susceptibility of 202 clinical *Candida* isolates against six antifungal agents

| *Candida species* | Fluconazole (%) | Clotrimazole (%) | Amphotericin (%) | Nystatin (%) | Ketoconazole (%) | Itraconazole (%) |
|---|---|---|---|---|---|---|
| *Candida albicans* | 95.94 | 94.59 | 68.91 | 89.18 | 90.54 | 93.24 |
| *Candida glabrata* | 94.33 | 94.33 | 73.58 | 90.56 | 94.33 | 88.67 |
| *Candida parapsilosis* | 95.45 | 95.45 | 72.72 | 81.81 | 86.36 | 93.18 |
| *Candida dubliniensis* | 87.50 | 100 | 00.00 | 87.50 | 93.75 | 100 |
| *Candida tropicalis* | 100 | 80 | 60 | 93.33 | 93.33 | 100 |
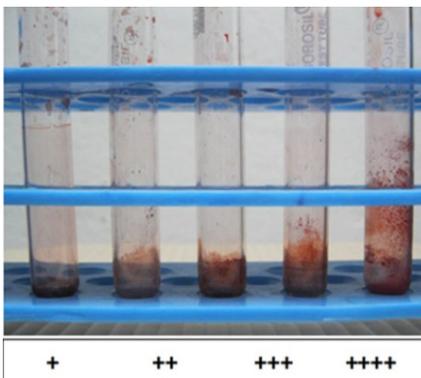


**Figure 4.** Tube biofilm formation



**Figure 5.** Protease activity



**Figure 6.** Phospholipase activity

**Table 2.** Adherence percentage of multi-drug resistant biofilm-producing *Candida* species

| *Candida species* | $A_1/A_0 \times 100$ range | |
|---|---|---|
| | From | To |
| *Candida albicans* | 0.0 | 90.90 |
| *Candida glabrata* | 0.0 | 98.33 |
| *Candida parapsilosis* | 0.0 | 90.00 |
| *Candida dubliniensis* | 0.0 | 51.28 |
| *Candida tropicalis* | 30.00 | 51.28 |



**Figure 7.** Anti-biofilm activity of *E. cardamomum* against MDR *Candida albicans*

*C. parapsilosis*, *C. dubliniensis*, and *C. tropicalis*, showed the most significant adherence (Table 2).

Based on the assessment of the anti-biofilm activity of *E. cardamomum* against MDR *Candida* species, the inhibition zone ranged between 10 and 15 mm in diameter. In the disc diffusion method, *E. cardamomum* extracts showed greater activity. With 100 µl of acetonic *E. cardamomum* extracts, more anti-biofilm activities were observed against *C. albicans* isolates (inhibition zone: 15 mm), compared to the ethanolic extracts (10 mm).

In the tube method, both ethanolic and acetonic extracts completely inhibited biofilm formation at 200 µl (90 µg) and 125 µl (56.25 µg) concentrations. The acetonic extract showed the greatest biofilm inhibition at a low concentration, as indicated by the reduction in biofilm production in test tubes (Figure 7).

## Discussion

Over the past few years, yeasts of the genus *Candida* have been recognized as one of the most important etiological agents of nosocomial infections [21]. Fungal infections are most commonly caused by pathogenic *Candida* species, particularly *C. albicans*, *C. tropicalis*, and *C.*

*parapsilosis*. These organisms are regarded as increasingly important nosocomial pathogens [22]. Based on the phenotypic, biochemical, germ tube, and microscopic observations, five different *Candida* species were identified in the present study.

Azoles exert direct effects on the fatty acids of cell membranes [23] and inhibit ergosterol biosynthesis through their interactions with lanosterol demethylase, which is responsible for the conversion of lanosterol to ergosterol in fungal cell membranes, leading to the depletion of ergosterol in the membrane [24, 25]. Overall, biofilm-forming *Candida* cells have the ability to develop resistance against azoles, especially fluconazole and clotrimazole [26].

In the present study, more than 80% of the isolates showed resistance to all drugs, except amphotericin (73.58%). In contrast, in a previous study, *C. dubliniensis*, *C. parapsilosis*, *C. lusitaniae*, and *C. kefyr* were susceptible to all the tested azoles, whereas *C. tropicalis* strains were susceptible to fluconazole and voriconazole [27]. In another study, resistance rates to fluconazole and itraconazole were 2.8% and 36.8%, respectively [28].

Biofilm formation is one of the most extensively

investigated virulence factors of *Candida* species [29]. In the current study, a higher proportion of *C. dubliniensis* isolates was able to form biofilms (75%), followed by *C. glabrata* (67.9%), *C. albicans* (58.11%), *C. tropicalis* (53.33%), and *C. parapsilosis* (45.45%) as strong biofilm producers, respectively. Considering the biofilm-forming capacity of these isolates, their pathogenicity could result in severe co-infection with other diseases.

In the present study, biofilm production rate was high in *C. dubliniensis* and *C. glabrat*a, as these isolates were obtained from immunocompromised patients. However, it should be noted that the patient's body condition had been already adapted for these pathogens. Therefore, the virulence factors of the isolates were easily expressed, as the immunity level was very low in these patients. Nevertheless, in another study, contradictory results were reported [30].

In the current study, the ability of proteinase production was reported in 52.4% of *Candida* isolates. Maximum proteinase production was observed in 46 *C. albicans* isolates (56.7%), followed by non-*C. albicans* species (n=18, 43.9%). These results were in agreement with the proteinase-producing ability, detected in 38 (76%) *Candida* isolates. Maximum proteinase production was observed in 31 *C. albicans* isolates (79.5%), followed by non-*C. albicans* species (n=7, 63.63%) [31].

The mentioned results were in contrast with those reported by Dan et al. (2002) [32], who showed proteinase activity in 34 (85.0%) *Candida* isolates; this rate reached 100% in non-*C. albicans* species. In another study by Sachin et al. (2012), proteinase production was found in 65 (59.1%) *Candida* isolates [33]. Also, in a study by Tsang et al. (2007), maximum proteinase production was reported in *C. albicans* (82.1%), followed by non-*C. albicans* species (80%) [34].

In a previous study, phospholipase production was reported in 85 (62.9%) isolates. A total of 49 (81.6%) *C. albicans* isolates showed phospholipase activity. Among non-*C. albicans* species, maximum phospholipase activity was reported in *C. tropicali*s and *C. glabrata*. Also, 62.8%, 17.82%, and 14.35% of *Candida* species showed strong, weak, and moderate positivity, respectively [35]. In line with these findings, we also revealed strong positivity for phospholipase activity in *C. parapsilosis* (72.72%, 32 out of 44), *C. albicans* (69.81%, 37 out of 53), *C. glabrata* (64.86%, 48 out of 74), *C. dubliniensis* (37.50%, 6

out of 16), and *C. tropicalis* (33.33%, 5 out of 16).

Based on a previous study, the hydrophobicity of *Candida* strains constitutes the measurement of yeast adherence to hydrocarbons [36]. In this study, optimal adherence was observed in the presence of xylene (82.68% for *C. albicans*, 85.23% for *C. parapsilosis*, 76.45% for *C. glabrata*, 79.07% for *C. kefyr*, 74.3% for *C. holmii*, and 40.2% for *C. sake*). On the contrary, in the present study, *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. dubliniensis* showed 89.28%, 88.88%, 62.5%, 100.0%, and 80.00% adherence in the presence of xylene, respectively.

Nosocomial fungal infections caused by *Candida* species are an important cause of morbidity and mortality, especially in immunocompromised patients. Use of available treatment options for invasive mycoses is limited due to their narrow spectrum of activity, drug resistance, toxicity, and drug-drug interactions [37]. Therefore, it is essential to develop more effective and less topical agents for the treatment of common, drug-resistant fungal infections.

Plants, as a source of medicinal compounds, have played a dominant role in the maintenance of human and animal health since ancient times. According to the World Health Organization reports, plant extracts or their active constituents are used in traditional medicine by 80% of the world's population [38]. Over 50% of all modern clinical drugs have a natural origin [39]. Despite the gradually increasing popularity of medicinal plants in clinical fields, their anti-fungal properties have been reported to be minor in most previous studies; as a result, new antifungal agents should be developed in the future [40]. In the present study, we focused on the *in vitro* effects of a number of local medicinal plants against the growth of *C. albicans* and compared their results with the antifungal activity of standard anti-*Candida* drugs (clotrimazole and nystatin) in the culture media.

In a previous study, the greatest antimicrobial activity of *E. cardamomum* was reported against *S. aureus* (19.3 mm) in the acetonic extracts with an MIC of 25 mg/ml. This study revealed that the organic fruit extract of *E. cardamomum* showed good antimicrobial activity and could be used in the development of novel herbal ear drops [41]. In the present study, similar results were reported. Compared to the ethanolic extract, the acetonic extract majorly inactivated pathogenic *C. albicans*. Overall, *E. cardamomum* is an alternative therapeutic option for the inhibition of

biofilm-forming *Candida* species.

## Conclusion

*Candida* species, which are normally found in human hosts, can cause infections in case the immune system is compromised; as a result, they are known as opportunistic pathogens. In addition, these species can survive the hospital environment. In general, those who continuously use antifungal drugs for infection become resistant to them. Therefore, a new generation of more potent drugs needs to be developed to prevent the increasing threat of emerging azole and amine-resistant *Candida* species. Overall, a better understanding of the mechanism of *Candida* species, resistant to azoles and amines, is essential. Consequently, modified medicines, such as plant materials and dry fruits, are used for the treatment of these infections. *E. cardomomum* is a dry fruit, used as an alternative medicine for the treatment of infections and biofilm inhibition. Therefore, use of alternate medicines is required to treat infections and reduce the associated side-effects.

## Acknowledgments

## Author's contribution

V.P. designed and supervised the study. R.P. performed all the experiments, and T.S. drafted the manuscript. The final version of the manuscript was revised by V.P.

## Conflicts of interest

The authors declare no conflicts of interest.

## Financial disclosure

There was no financial interest related to the materials of the manuscript.

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