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Evaluating the Antifungal Potential of Cinnamaldehyde: A Study of its Efficacy against *Candida* Species

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Abstract

***Candida* species exist as commensals in nature, colonizing the mucous membranes, gastrointestinal tract, vagina as well as the skin and usually cause infections in immunocompromised patients. *C. albicans* are known to be the most prevalent *Candida* species associated with infections, while there has been a significant surge in the incidence of Non-*Candida albicans Candida* species (NCAC) recently. The recent occurrences of the antifungal resistance in *Candida*, especially in NCAC species are quite alarming which raises the need for a safe and efficient alternative antimycotic drug. This study analyses the efficacy of cinnamaldehyde against *Candida* species, which is known to cause the majority of the fungal infections in humans. Cinnamaldehyde is a natural antimicrobial compound derived from cinnamon and has demonstrated significant antimycotic properties. Antifungal susceptibility profiles of cinnamaldehyde against *Candida* species were studied by disc diffusion as well as by broth microdilution assays. The mean diameter of the inhibition zone (IZ) formed by direct contact and disc volatilization assays were 61.26 mM and 65.20 mM, respectively. Both the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of cinnamaldehyde ranged from 16-256 mg/L with mean MIC of 60.61 mg/L and a mean MFC of 81.94 mg/L. Co-incubation of *Candida* cells with cinnamaldehyde resulted in the loss of viable cells within 4 hours of incubation. Cinnamaldehyde was found to exhibit both fungistatic and fungicidal properties, making it a potent natural alternative for conventional antifungal agents.**

Keywords: Antimycotic Agent, Disc Diffusion Assay, Disc Volatilization Assay, Minimum Fungicidal Concentration (MFC), Minimum Inhibitory Concentration (MIC)

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INTRODUCTION

Candidal infections present considerable global health concerns, impacting millions of individuals and frequently resulting in morbidity as well as mortality.[1] *Candida* species belong to Ascomycota and are found to colonize the skin, gastrointestinal tract, mucous membranes and vagina of humans.[2] *Candida* species can exist as unicellular yeasts, form pseudohyphae or filamentous hyphae exhibiting polymorphism.[3] They can switch between various morphological forms, produce various hydrolytic enzymes and express adhesion proteins, which establish the infection in the host as well as aid in the invasion and dissemination of the pathogen.[4] They are responsible for a wide spectrum of infections varying from superficial mycotic infections like oral and vulvovaginal candidiasis (VVC) to severe systemic disorders like candidemia.[5] Further, colonization of the gastrointestinal tract (GI) in humans is especially significant as most of the *Candida* infections are known to arise from the commensals, including VVC and candidemia.[6] In various diseases of the GI, including colorectal cancer and inflammatory bowel disease (IBD), overgrowth of *Candida* species is observed.[7] They are also known to slow down the healing process of gastric ulcers by colonizing them.[8] *C. albicans* is known to be the causative agent in most of the *Candida* infections, making it the most widespread *Candida* species. However, there are other *Candida* species known as Non-*Candida albicans Candida* species (NCAC) such as *C. glabrata* (now *Nakaseomyces glabrata*), *C. tropicalis, C. parapsilosis*, *C. krusei* (now *Pichia kudriavzevii*), *C. kefyr, C. guillermondii* and *C. dubliniensis* are becoming more widely recognised as significant opportunistic pathogens, especially in patients with compromised immune systems.[9-12] Other frequently encountered *Candida* species include *C. auris, C. famata*, *C. rugosa, C. lusitaniae, C. lipolytica, C. ciferrii, C. pelliculosa, C. intermedia* and *C. utilis*.[13] The increasing antifungal resistance among the *Candida* species, especially in NCACs, suggests the need to develop novel and effective antifungal therapeutic agents instead of relying completely on the traditional antifungal drugs.[14,15] And further, the use of traditional antifungal drugs often results in certain disadvantages, such as toxicity and drug interactions, apart from antifungal resistance.[16] It is therefore crucial to examine alternative antifungal agents, especially those from natural sources, as they might provide unique modes of action along with enhanced safety profiles.[17] For instance, cinnamaldehyde is a prime ingredient of cinnamon essential oil (90%), exhibiting a wide array of antibacterial and antifungal activity.[18] The antifungal properties of cinnamaldehyde are attributed to its ability to disrupt the fungal cell membranes and interfere with the development of the fungal cell walls, causing the lysis of the fungal cells when treated with cinnamaldehyde. It also interferes with various metabolic pathways in fungi and disturbs the biofilm formation in *Candida* species.[18] Extensive studies have demonstrated that cinnamaldehyde possesses both fungistatic and fungicidal activities against *C. albicans*.[19,20] Even though the preliminary data are sufficient to establish the efficacy of cinnamaldehyde, there is a lack of studies that examine the antifungal activity of cinnamaldehyde in NCAC species.[21,22] Such comparative studies become essential as NCAC species often exhibit characteristics that are different from the *C. albicans* in their pathogenicity and often exhibit intrinsic resistance to commonly prescribed antifungal drugs.[23] The complete range of antifungal activity of cinnamaldehyde has to be explored to facilitate the therapeutic applications of cinnamaldehyde and promote targeted treatments. This study assesses the antifungal properties of cinnamaldehyde against *C. albicans* and NCAC species. This study would contribute to the existing body of evidence that supports the use of antifungal drugs extracted from natural compounds in combating *Candida* infections and thereby, address the challenges imposed by increasing antifungal resistance.

MATERIALS AND METHODS

This study included 33 vaginal *Candida* isolates obtained from SRM Medical College Hospital and Research Centre, Kattankulathur. Identification of *Candida* species was performed by standard biochemical and microbiological techniques including KOH wet mount (presence of yeast cells and germ tube), sugar fermentation, sugar assimilation assay and colony morphology

on Sabouraud dextrose agar (SDA) and cornmeal agar (chlamydospore formation). Further, Hicrome *Candida* differential agar (Himedia, India) was employed to differentiate *Candida* species based on the color produced by the colonies produced on the agar. The cinnamaldehyde (purity ≥ 98%) used in this study is obtained commercially from Himedia, India.

Antifungal susceptibility assays using disc diffusion

Diffusion by direct contact

The method developed by Hammer *et al.* was employed to evaluate the anti-*Candidal* activity of cinnamaldehyde.[21] In brief, a yeast cell suspension was created using normal saline and adjusted to a concentration of 0.5 McFarland standard (1.5 x 10^8 CFU/mL). The culture was swabbed on SDA in three directions to form lawn culture. The SDA plates were allowed to dry for some 5 minutes, following which sterile filter paper discs loaded with 5 µl of cinnamaldehyde at concentration of 1 mg/µl were placed on the centre of the SDA petri dish. The petri dishes were further incubated at 35°C for 48 hours. Following the incubation duration, the diameter of the inhibition region formed was measured.[24]

Disc volatilisation assay

The antifungal activity of cinnamaldehyde in vapor phase was examined by disc volatilisation technique suggested by Boukhatem *et al.* 100 µl of yeast cells were spread on SDA plates, followed by placing sterile filter paper discs on the upper lid of the plates. These filter paper discs were impregnated with 5 µl of Cinnamaldehyde at 1 mg/µl concentration. The petri dishes were then incubated at 35°C for 48 hours. Post incubation, the zone of inhibition (IZ) formed was measured and recorded in millimeters.[25]

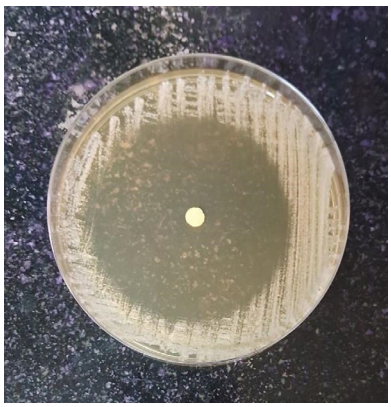

Figure. Inhibition zone formed by cinnamaldehyde against *Candida* species

Broth microdilution method

Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC)

The microdilution assay was performed to assess the MIC and MFC of cinnamaldehyde, using the approach outlined by Bona *et al.* with specific alterations.[26] Briefly, the culture from SDA was diluted in sterile saline to obtain a final concentration of 10^3 CFU/mL. Cinnamaldehyde was added to DMSO (80% Cinnamaldehyde and 20% DMSO) followed by dilution in Sabouraud dextrose broth (SDB) to obtain a final concentration range of 2-1024 mg/L. Amphotericin B (initial range 32-0.032 mg/L, final concentration of 16-0.016 mg/L) was employed as positive control. Serial dilutions of Amphotericin B and cinnamaldehyde were performed on the microtiter plate (MTP). 100 µl of cinnamaldehyde/ 100 µl of Amphotericin B from each dilution were added to 100 µl of culture suspension respectively. Growth controls were also determined with SDB and DMSO. The MTPs were incubated at 35°C for 48 hours. The MIC is denoted by the concentration of cinnamaldehyde/Amphotericin B in the first well without turbidity. MFC is the lowest concentration of cinnamaldehyde/Amphotericin B which shows complete absence of *Candida* growth when subcultured on agar.[24]

Time kill assay

The time kill analysis was performed according to the methodology proposed by Canton *et al.* A *Candida* isolate (*C. albicans* strain 3) with the MIC similar to the mean MIC and the MFC being 2x of respective MIC was chosen to perform this assay. *C. albicans* strain 3 diluted in SDB was incubated at 37°C with cinnamaldehyde and Amphotericin B at their respective MFC to reach a final concentration of 5 × 10^4 CFU/mL. The initial concentrations of Cinnamaldehyde and Amphotericin B were 0, 1/4, 1/2 and 1×MIC. The viability of the yeast cells co-incubated with cinnamaldehyde and Amphotericin B were

examined at 0, 1, 2, 4, 6, 8, 12 and 24 hours using the viable plate count method.[24]

RESULTS

The antifungal activity of cinnamaldehyde was tested against all 33 clinical isolates (16 isolates of *C. albicans*, 9 isolates of *C. glabrata* (*N. glabrata*), 5 isolates of *C. tropicalis* and 3 isolates of *C. krusei* (*P. kudriavzevii*)). The inhibitory property of cinnamaldehyde was confirmed by the formation of an IZ equal or larger than the inhibition zone formed by Amphotericin B (positive control). Cinnamaldehyde inhibited all the clinical isolates tested with mean IZ of 61.26 mm (Figure and Table 1).

Following disc diffusion assays, the antifungal activity of cinnamaldehyde was assessed by broth microdilution assay. MIC and MFC of cinnamaldehyde were evaluated for the clinical isolates. DMSO was used as negative control and it did not inhibit any *Candidal* growth. Mean MIC was found to be 60.61 mg/mL and mean MFC was 81.94 mg/mL. Cinnamaldehyde can be considered fungicidal as their MFC/MIC ratio is less than 4 (Table 2).[27]

The time kill curve was plotted by co-incubating *C. albicans* strain 3 with cinnamaldehyde and the total viable count (TVC) was determined at 0, 1, 2, 4, 6, 8, 12 and 24 hours. The cells were found to be viable up to 2 hours of co-incubation and by 4 hours of incubation, no more cells were found to be viable.

Table 1. Inhibition zone (IZ) formed by disc diffusion assays against *Candida albicans* and Non-*Candida albicans Candida* species (NCACs)

	Inhibition zone (IZ)	Percentage of inhibited isolates
Amphotericin B	Disc diffusion assay (Direct contact)	
Candida albicans	14.94 mm	Not applicable
Non-*albicans* *Candida* species	12.44 mm	
Mean	13.65 mm	Not applicable
Range	10-19 mm	
Standard deviation	2.41	
Cinnamaldehyde	Disc diffusion assay (Direct contact)	
Candida albicans	64.37 mm	100%
Non-*albicans* *Candida* species	58.32 mm	
Mean	61.26 mm	100%
Range	50-76 mm	
Standard deviation	6.75	
Cinnamaldehyde	Disc diffusion assay (Disc volatilization)	
Candida albicans	57.10 mm	100%
Non-*albicans* *Candida* species	62.74 mm	
Mean	65.20 mm	100%
Range	55-77 mm	
Standard deviation	6.37	

DISCUSSION

Candida species are opportunistic pathogens capable of causing different types of infections that range from superficial infections to systemic mycotic infections.[28] These clinical conditions caused by *Candida* species are collectively called as candidiasis and these impose serious concerns in the immunocompromised individuals.[29] The extensive utilization of broad-spectrum antibiotics and immunosuppressive therapies has resulted in the surge of candidiasis cases worldwide.[14] The efficacy of the routinely

Table 2. Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) of Cinnamaldehyde and Amphotericin B against *Candida albicans* and Non-*Candida albicans Candida* species (NCACs)

	MIC (mg/mL)	MFC (mg/mL)	MFC/ MIC
Amphotericin B			
Candida albicans	0.051	0.096	1.88
Non-*albicans* *Candida* species	0.076	0.13	1.71
Mean	0.062	0.114	0.256
Range	0.016-0.128	0.032-0.256	
Standard deviation	0.04	0.085	
Cinnamaldehyde			
Candida albicans	51	88	1.72
Non-*albicans* *Candida* species	69.45	76.24	1.10
Mean	60.61	81.94	1.78
Range	16-256	16-256	
Standard deviation	46.56	47.88	

used antimycotic drugs is questionable given the emergence of antifungal resistant *Candida* strains.[30,31] Further, the conventional antifungal drugs are more prone to toxicity and side effects associated with the drug use facilitating the need to explore alternative therapeutic agents, especially the naturally occurring antifungal drugs might offer a best option.[17]

Cinnamaldehyde, a primary component of cinnamon essential oil, possesses wide-ranging antifungal effects.[18] Various studies have highlighted the potential of cinnamaldehyde as a potent antibacterial and antifungal agent, especially against *Candida* species.[32-34] Even though a reasonable number of studies are available in the literature, suggesting the antimycotic activity of cinnamaldehyde against *C. albicans*, the studies on its effects on the NCAC are limited.[35] This study thus included both *C. albicans* and NCAC and the antifungal activity of cinnamaldehyde is elucidated.

Disc diffusion method was performed by both direct contact and disc volatilization to evaluate the fungistatic potential of cinnamaldehyde and the results attained are similar to the previously reported results. The inhibition of *Candida* by cinnamaldehyde is considered successful only if the inhibition zone formed by cinnamaldehyde is equal or wider than the zone of inhibition of Amphotericin B. Cinnamaldehyde resulted in a wider IZ (mean 61.26 mm) in almost all the *Candida* clinical strains tested compared to the zone of inhibition formed by Amphotericin B (13.65 mm) in disc diffusion assay (direct contact). Cinnamaldehyde demonstrated a mean inhibition zone of 64.37 mm against *C. albicans*. Additionally, it exhibited an average inhibition zone of 58.32 mm against NCACs, with specific measurements of 54.77 mm for *C. glabrata* (*N. glabrata*), 66.1 mm for *C. tropicalis*, and 56 mm for *C. krusei* (*P. kudriavzevii*). A recent study on antifungal activity of various natural compounds including cinnamaldehyde, by Saracino *et al.* reported almost similar results where the mean diameter of IZ formed was 69 mm.[36] Another study made on the antifungal activity of cinnamaldehyde against *C. albicans* reported IZ of 60 mm diameter.[22] Cinnamaldehyde in the vapor phase resulted in IZ of 65.20 mm (mean), which corresponds to the previous study made on Cinnamomum essential oils.[25]

Fungicidal activity of cinnamaldehyde was demonstrated by performing microdilution assay. Cinnamaldehyde showed reasonably lower mean MICs and MFCs values (60.61 mg/mL and 81.94 mg/mL respectively) which correlates with the previous studies made on the antifungal activity of cinnamaldehyde.[36,37] A study reported MIC and MFC of cinnamaldehyde against *C. albicans* to be as low as 125 µg/mL. [24] A study by Miranda *et al.* revealed the MIC of *C. albicans* to be 59.7 mg/mL, *C. glabrata* (*N. glabrata*) to be 68.6 mg/mL, *C. krusei* (*P. kudriavzevii*) to be 50.8 mg/mL and *C. tropicalis* mean MIC to be 64 mg/mL.[37] Previously discussed study by Saracino *et al.* revealed the MIC and MFC of cinnamaldehyde against *Candida* species to be 50.05 mg/mL and 109.26 mg/mL, respectively.[34] Our study demonstrated the mean MIC of *C. albicans* to be 51 mg/mL, *C. glabrata* (*N. glabrata*) to be 85.33 mg/mL, *C. krusei* (*P. kudriavzevii*) to be 74.67 mg/mL and *C. tropicalis* to be 38.4 mg/mL. Although the results of *C. albicans* are not varying greatly with reference to the previous studies, the discrepancies in the results of NCACs might be attributed to the small sample size.

The kinetics of the fungicidal activity was determined by the time kill curve assay. The test strain when incubated with cinnamaldehyde were viable till 2 hours and by 4 hours, all the cells were killed. This result is consistent with the previous study made on the kinetics of fungicidal activity of cinnamaldehyde.[36] A different study suggested the loss of viable cells by 12 hours of incubation.[24]

Cinnamaldehyde suppresses the ergosterol biosynthetic pathway as well as constantly interacts with the plasma membrane to bind with ergosterol, acting as a potent antifungal agent.[38] Cinnamaldehyde strongly inhibits plasma membrane ATPase, as a result the ATPase dependent efflux mechanisms are consequently inhibited.[38,39] The phospholipase and proteinase activity are significantly suppressed as a result leading to the reduced expression of hydrolytic enzymes.[36,37] Dimorphism of *Candida* species is also regulated by plasma membrane ATPase,

suppression of which leads to altered phenotypic switch.[40,41] This results in reduction in the number of germ tubes formed and the invasion of *Candida* species is also diminished leading to reduced pathogenicity making cinnamaldehyde a suitable candidate to suppress *Candidal* infections.[42,43]

CONCLUSION

Cinnamaldehyde exhibits significant antifungal activity against both *C. albicans* and NCAC, offering a promising alternative to conventional antimycotic drugs in the face of emerging antifungal resistance in *Candida* species, especially in NCACs. The MIC and MFC for *C. albicans* were measured at 51 µg/mL and 69.65 µg/mL, respectively. In comparison, the MIC and MFC for NCAC species were determined to be 88 µg/mL and 76.24 µg/mL, respectively, using the microdilution method which depicts the antifungal activity of cinnamaldehyde. Cinnamaldehyde is capable of inhibiting the growth as well as the proliferation of *Candida* species, acting as a potent fungistatic and fungicidal agent. Further, cinnamaldehyde belonging to the natural origin, when combined with its antifungal efficacy, positions itself as a viable adjunct or alternative to the available conventional antimycotic drugs. The effectiveness of cinnamaldehyde can be optimized by combining it with conventional drugs, thus providing novel antifungal formulations. *In-vivo* studies can be performed to study the toxicity profile of cinnamaldehyde in order to incorporate the same in the treatment regimen.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Institutional Ethics Committee, SRM Medical College Hospital and Research Centre, India, with clearance number 8379/IEC/2022.

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