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Original article

A study on *Candida* biofilm growth characteristics and its susceptibility to aureobasidin A

Komathy Munusamy, Jamuna Vadivelu, Sun Tee Tay*

Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

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ABSTRACT

Background: Biofilm is known to contribute to the antifungal resistance of *Candida* yeasts. Aureobasidin A (AbA), a cyclic depsipeptide targeting fungal sphingolipid biosynthesis, has been shown to be effective against several *Candida* species.
Aims: The aim of this study was to investigate *Candida* biofilm growth morphology, its biomass, metabolic activity, and to determine the effects of AbA on the biofilm growth.
Methods: The biofilm forming ability of several clinical isolates of different *Candida* species from our culture collection was determined using established methods (crystal violet and XTT assays). The determination of AbA planktonic and biofilm MICs was performed based on a micro-broth dilution method. The anti-biofilm effect of AbA on *Candida albicans* was examined using field emission scanning electron microscope (FESEM) analysis.
Results: A total of 35 (29.7%) of 118 *Candida* isolates were regarded as biofilm producers in this study. *Candida parapsilosis* was the largest producer, followed by *Candida tropicalis* and *C. albicans*. Two morphological variants of biofilms were identified in our isolates, with 48.6% of the isolates showing mainly yeast and pseudohyphae-like structures, while the remaining ones were predominantly filamentous forms. The biofilm producers were divided into two populations (low and high), based on the ability in producing biomass and their metabolic activity. *Candida* isolates with filamentous growth, higher biomass and metabolic activity showed lower AbA MIC_{50} (at least fourfold), compared to those exhibiting yeast morphology, and lower biomass and metabolic activity. The observation of filament detachment and the almost complete removal of biofilm from AbA-treated *C. albicans* biofilm in FESEM analysis suggests an anti-biofilm effect of AbA.
Conclusions: The variability in the growth characteristics of *Candida* biofilm cultures affects susceptibility to AbA, with higher susceptibility noted in biofilm cultures exhibiting filamentous form and high biomass/metabolic activity.

Estudio de las características de biopelículas de *Candida* y su sensibilidad a la aureobasidina A

Palabras clave:
Candida
Biopelícula
Concentración mínima inhibitoria
Aureobasidina

RESUMEN

Antecedentes: La biopelícula de las levaduras del género *Candida* contribuye a la resistencia de este género a los antifúngicos. La aureobasidina A (AbA), un depsipéptido cíclico cuya diana es la biosíntesis de los esfingolípidos en los hongos, ha mostrado tener cierta acción antifúngica frente a diferentes especies de *Candida*.
Objetivos: El objetivo de este estudio fue investigar el desarrollo de las biopelículas de *Candida*, su biomasa y su actividad metabólica, y determinar el efecto de AbA en su desarrollo.

* Corresponding author.
E-mail address: tayst@um.edu.my (S.T. Tay).

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Métodos: Se utilizaron los métodos de cristal violeta y XTT para determinar la formación de biopelícula en diferentes especies de *Candida* de nuestra colección. La determinación de los valores MIC de AbA sobre cultivos planctónicos o con biopelícula fue realizada mediante un método de microdilución en caldo. El efecto anti-biopelícula de AbA sobre *Candida albicans* fue analizado mediante el uso de un microscopio electrónico de barrido de emisión de campo (FESEM).
Resultados: Treinta y cinco aislamientos de *Candida* (29,7%) de un total de 118 produjeron biopelícula. *Candida parapsilosis* fue la especie más productora, seguida de *Candida tropicalis* y *C. albicans*. Se observaron dos variantes morfológicas en las biopelículas, con un 48,6% de los aislamientos con estructuras formadas fundamentalmente por levaduras y seudohifas, mientras que los restantes aislamientos mostraron mayoritariamente formas filamentosas. Los aislamientos productores de biopelícula fueron divididos en dos grupos en función de su capacidad de producir biomasa y de la actividad metabólica de la biopelícula. Aquellos aislamientos de *Candida* con estructuras filamentosas, mayor cantidad de biomasa y de actividad metabólica mostraron menores valores CMI_{50} (de hasta cuatro veces) respecto a aquellos aislamientos con morfología levaduriforme, y menor biomasa y actividad metabólica. El desprendimiento y casi completa eliminación de la biopelícula del aislamiento de *C. albicans* estudiado mediante FESEM y tratado con AbA sugiere el efecto antifúngico de esta molécula.
Conclusiones: Las distintas particularidades de las biopelículas del género *Candida* condicionan la sensibilidad de los aislamientos a la AbA; la sensibilidad es mayor en aquellas biopelículas con estructura filamentosa, y mayor biomasa y actividad metabólica.

Candidiasis is a significant cause of morbidity and mortality among hospitalized immunocompromised patients. Among the *Candida* species, *Candida albicans* is the most frequently isolated yeast from clinical settings.[1,15,17] However, in recent years, there has been an increase in the incidence of candidiasis caused by other species of the genus including *Candida parapsilosis*, *Candida tropicalis* and *Candida glabrata*.[3,6,16] *Candida* is known to produce biofilms on medical devices. Biofilms are defined as structured microbial communities that are attached to a surface and encased in a matrix of exopolymeric material. Yeast cells can be released from biofilms, migrate through the bloodstream and cause a systemic infection.[15,19] Hence, mortality is greater in patients infected by biofilm-producing isolates than those infected by non-biofilm-forming isolates.[26] Biofilm-associated infections are generally difficult to treat due to the decreased susceptibilities of the biofilm cultures to antimicrobial therapy.[2,14,15,20] Removal of catheter has been recommended for management of biofilm-associated infections in catheterized patients with candidemia.[5] Although candins and liposomal-amphotericin B have been reported to have good antibiofilm activity,[2,19] the difficulty in completely recover from biofilm-associated infections has prompted studies to search for alternative therapies.[18–19]

Candida biofilms are different depending on the species, morphology and metabolic activity. It has been reported that up to 30% of *C. albicans* isolates exhibiting high metabolic activity in preformed biofilms showed the most marked antifungal effect (lower MICs), as seen in the case of micafungin.[13] Aureobasidin A (AbA) is a cyclic depsipeptide antifungal produced by *Aureobasidium pullulans* with a strong fungicidal activity against a variety of fungi, including *Candida*.[7,23,24] The antifungal inhibits inositol phosphorylceramide (IPC) synthase, a key enzyme absent in mammals responsible for sphingolipid biosynthesis in fungi.[27] AbA has low toxicity and has shown effectiveness when given orally to mice with candidiasis.[24] A study in our laboratory reported *in vitro* susceptibility of several *Candida* species to AbA, and demonstrated that biofilms formed by 0, 1, 2, and 4 h adherent populations were more susceptible to AbA treatment when compared with mature biofilms (represented by 24 h yeast-adherent populations).[25] However, studies are still lacking on the effects of AbA on the candidal biofilm morphology, biomass and metabolic activity. It has been reported that the antifungal activity of micafungin against *C. albicans* is dependent on biofilm metabolic activity; therefore isolates with lower metabolic activity were less susceptible to micafungin and isolates with higher biofilm metabolic activity were significantly more susceptible to micafungin.[13]

In this study, the biofilm morphology, biomass and metabolic activity of Malaysian isolates of several *Candida* species were studied and the effects of AbA on the biofilm growth were determined. The antibiofilm effect of AbA on *C. albicans* was also investigated using field emission scanning electron microscope (FESEM) analysis.

Methods

Clinical isolates. A total of 118 clinical isolates from six *Candida* species were obtained from a culture collection at the Diagnostic Microbiology Laboratory, University Malaya Medical Centre (UMMC), Malaysia. Forty-eight isolates were obtained from blood cultures, and 70 isolates recovered from non-sterile sites (respiratory tract, vagina, urine, etc.) were included in this investigation. The isolates were identified as *C. albicans* ($n = 48$, 40.7%), *C. tropicalis* ($n = 38$, 32.2%), *C. parapsilosis* ($n = 16$, 13.6%), *C. glabrata* ($n = 12$, 10.2%), *Candida nivariensis* ($n = 3$, 2.5%), and *Candida kefyr* ($n = 1$, 0.8%) using routine mycological procedures (Table 1). *C. albicans* SC5314 strain was included as a control strain.

Determination of minimum inhibitory concentrations (MICs). For planktonic MIC determination, a microbroth dilution method was performed in accordance with the guidelines of the Clinical and Laboratory Standards Institute M27-A3 document.[4] A stock solution of 10 μg/ml of AbA (Clontech, Mountain View, CA, USA) dissolved in dimethyl sulfoxide (Fluka Chemika, USA) was used to prepare a dilution range from 8 to 0.0156 μg/ml. The planktonic MIC is defined as the lowest concentration of AbA inhibiting the growth of the tested yeast after incubating for 48 h.

Assessment of biofilm biomass and metabolic activity. The formation of *Candida* biofilm was performed using a modified protocol of Jin et al.[9] Briefly, several yeast colonies were suspended in RPMI 1640 medium and adjusted to approximately 10^7 cells/ml ($OD_{520} = 0.38$) using a Genesys spectrophotometer (Thermo Scientific, USA). The yeast suspension (100 μl) was then inoculated into each designated well in a flat-bottomed polystyrene microtiter plate (Corning, USA) and incubated at 37 °C for 1.5 h with constant shaking (75 rpm). After the attachment phase, unattached cells were removed and the wells were washed thrice with sterile

Table 1
Growth morphology, biomass, and metabolic activity of the biofilm developed by different *Candida* isolates.

Biofilm growth characteristics	*Candida* species	OD readings	
		Biofilm biomass	Biofilm metabolic activity
Predominantly yeast/pseudohyphae (n = 17, 48.6%)	*C. albicans* (n = 6, 35.3%)	0.162 ± 0.06	0.510 ± 0.15
	C. tropicalis (n = 7, 41.2%)	0.212 ± 0.07	0.705 ± 0.09
	C. parapsilosis (n = 4, 23.5%)	0.297 ± 0.07	0.909 ± 0.11
Average		0.224 ± 0.07	0.707 ± 0.20
Predominantly filamentous form (n = 18, 51.4%)	*C. albicans* (n = 4, 22.2%)	0.202 ± 0.14	0.787 ± 0.32
	C. tropicalis (n= 10, 55.6%)	0.169 ± 0.04	0.728 ± 0.13
	C. parapsilosis (n = 4, 22.2%)	0.283 ± 0.16	0.774 ± 0.15
Average		0.217 ± 0.06	0.763 ± 0.03

phosphate buffered saline (PBS). Fresh RPMI 1640 medium was then added into each well and incubation continued for 24 h.

For determining the biofilm biomass, crystal violet (CV) assay was performed as described by Reisner et al.[22] Biofilm cultures (after 24 h of growth) were stained with 0.1% (v/v) CV solution for 15–20 min. After washing thrice with PBS, the CV stain was dissolved thoroughly in 96% (v/v) ethanol. The absorbance (A_{585nm}) of the resultant solution was measured using an automated microplate reader (Epoch, BioTek, USA). Isolates giving absorbance readings of ≥0.1 at 585 nm in the CV assay were regarded to have good adherence and biofilm forming ability.[22] The experiments were repeated in quadruplicate. For determining the biofilm metabolic activity, XTT (2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) reduction assay was used. Biofilm cultures were incubated with XTT-menadione-PBS solution in the dark for 1 h at 37 °C and the absorbance of the resultant solution (100 μl) in each well was determined at 490 nm. The experiment was performed in duplicate for each isolate. The absorbance readings of the negative control wells (containing no cells) were subtracted from the absorbance readings of the test wells.

Determination of biofilm MICs. For MIC determination, 24 h-biofilm cultures in a microtiter plate were incubated with a series of AbA solutions ranging from 128 to 0.25 μg/ml at 37 °C for 48 h. Antifungal-free wells and biofilm-free wells were included to serve as positive and negative controls, respectively. The biofilm MIC is defined as the lowest concentration of AbA which causes 50% reduction in the biofilm metabolic activity.

Field emission scanning electron microscope (FESEM) analysis. The preparation of the biofilm cultures of *C. albicans* SC5314 strain (biofilm MIC, 32 μg/ml) for FESEM analysis was performed as described by Khan and Ahmad[10] and Jayatilake et al.[8] Biofilm cultures were developed on nucleopore membranes (0.2 μm pore size, 13 mm in diameter; Whatman, USA) which had been pre-sterilized by UV irradiation and placed in different flat-bottom wells of a 24-well plate (SPL Life Sciences, Korea). Briefly, yeast suspension (10^7 cells/ml) was inoculated onto each membrane, and the cells were adhered on the surface for 1.5 h. The cells were washed and incubated with fresh SD minimal medium at 37 °C for 24 h with shaking (75 rpm). The biofilm cultures were then exposed to different concentrations of AbA (0, 8, 32 μg/ml) for 2.5 h. For FESEM analysis, the biofilm cultures were fixed overnight with 4% glutaraldehyde in cacodylate buffer, and treated with 2% (w/v) osmium tetroxide for 1 h. The membranes were then subjected to dehydration in a series of ethanol solutions (10–100%), ethanol: acetone mixtures (3:1, 1:1 and 1:3), and acetone. After drying in CO_2 (CPD 7501, Polaron, UK), each membrane was mounted onto an aluminum stub and coated with gold (Biorad E5100 Series 11, USA) for observation under a FEI Quanta 450 FEG (USA) field emission scanning electron microscope (FESEM). The diameters of the hyphae and yeast cells were determined using the measurement tools of FESEM.

Statistical analysis. All statistical analyses were performed using Statistical Package for the Social Sciences ver. 23.0 (SPSS Inc., Chicago, IL). Due to the small sample size, Mann Whitney test[12] was used to compare the biofilm biomass and the metabolic activity among *Candida* species. A p-value lower than 0.05 was considered as statistically significant.

Results and discussion

A total of 35 (29.7%) of 118 *Candida* isolates were regarded as biofilm producers based on the CV assay, of which the majority were isolates of *C. parapsilosis* (8/12 isolates, 66.7%), followed by *C. tropicalis* (17/38 isolates, 44.7%), and *C. albicans*

Table 2
In vitro susceptibilities to AbA of the planktonic and biofilm cultures of *Candida* isolates.

Yeast isolates	Planktonic MIC (μg/ml)			Biofilm MIC (μg/ml)[a]		
	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
C. albicans (n = 48)	0.5 to ≥8	2	4	1 to ≥128	32	≥128
Non-C. albicans Candida spp. (n = 70)						
C. parapsilosis (n = 16)	0.5 to 4	1	2	2 to ≥128	≥128	≥128
C. tropicalis (n = 38)	0.25 to ≥8	1	2	0.5 to ≥128	32	≥128
C. glabrata (n = 12)	1–4	1	2		ND	
C. nivariensis (n = 3)	4 to ≥8	ND	ND		ND	
C. kefyr (n = 1)	1	ND	ND		MD	

[a] The biofilm MICs were determined for 10 isolates of *C. albicans*, 17 isolates of *C. tropicalis* and 8 isolates of *C. parapsilosis*.
Planktonic MIC: The lowest concentration of AbA which inhibits the visible growth of the yeast after incubation at 37 °C for 48 h.
Biofilm MIC: The lowest concentration of AbA which causes 50% reduction in the metabolic activity.
MIC_{50} and MIC_{90} values were only calculated for *Candida* species with five or more isolates.
ND: not determined.

Table 3
Effect of AbA on the biofilm growth morphology, biomass, and metabolic activity.

Biofilm growth characteristics	Susceptibility to AbA	
	MIC_{50} (μg/ml)	Range (μg/ml)
Morphology		
Mainly yeast growth (n = 17)	≥128	1 to ≥128
Mainly filamentous growth (n = 18)	32	0.5 to ≥128
Biomass		
Low (n = 17)	≥128	0.5 to ≥128
High (n = 18)[a]	32	1 to ≥128
Metabolic activity		
Low (n = 17)	≥128	0.5 to ≥128
High (n = 18)[b]	32	1 to ≥128

[a] OD reading >0.175.
[b] OD reading >0.71.

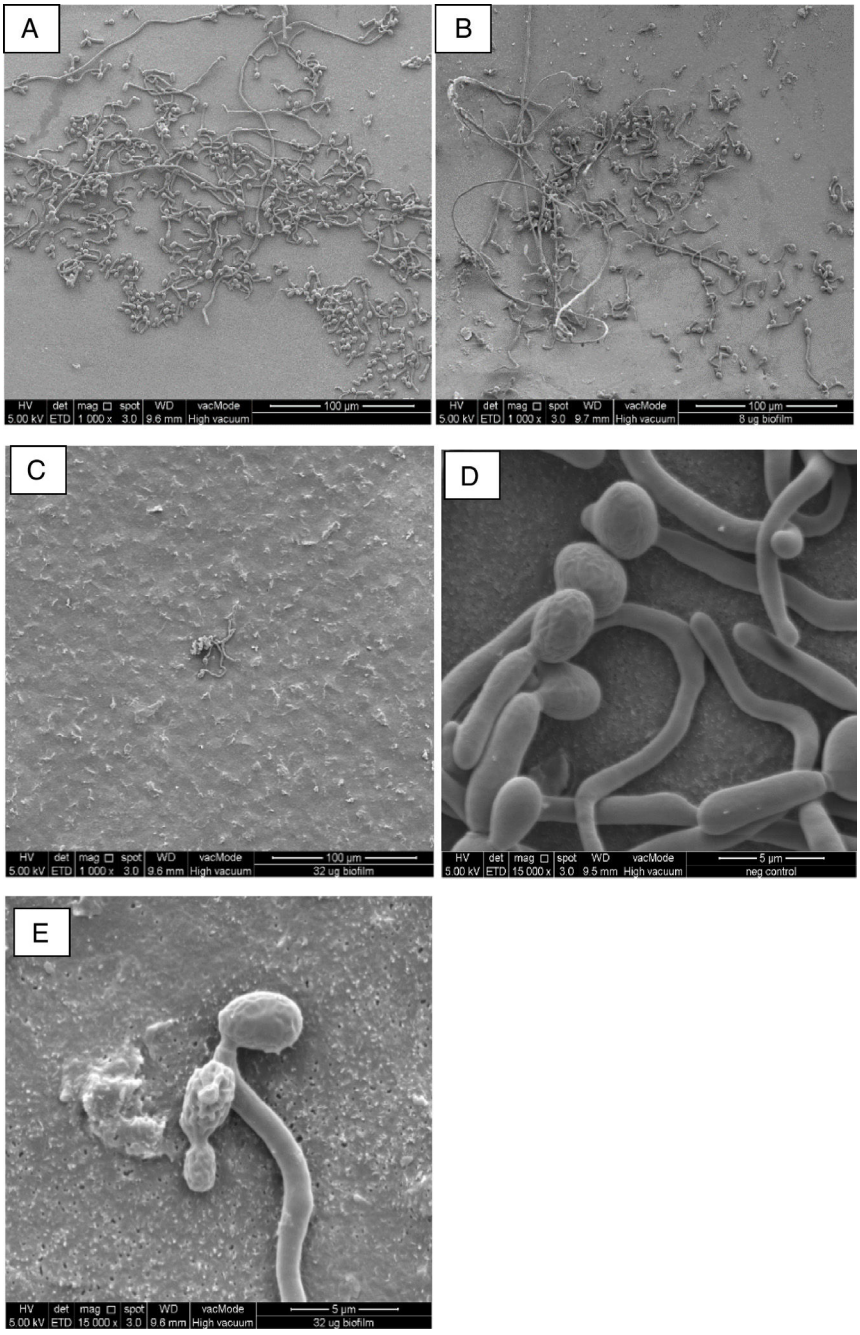

Fig. 1. FESEM images of *C. albicans* biofilm cultures upon exposure to AbA for 2.5 h. (A) Untreated biofilm culture (1000×); (B) biofilm culture treated with 8 μg/ml AbA (1000×); (C) biofilm culture treated with 32 μg/ml AbA (1000×); (D) untreated biofilm culture (15,000×); (E) biofilm culture treated with 32 μg/ml AbA (15,000×).

(10/48 isolates, 20.8%). Interstrain and interspecies variability in biofilm morphology was observed microscopically. Amongst the 35 biofilm-producing isolates, 17 (48.6%) developed only yeast/blastospores or pseudohyphae, while the remaining 18 (51.4%) isolates exhibited a dense network of biofilms consisting mainly of filamentous cells (Table 1). Almost half (55.6%) of the biofilm producers showing filamentous growth were *C. tropicalis*. The results of CV assay correlated with microscopic observations, as *C. glabrata*, *C. nivariensis*, and *C. kefyr* isolates were poor biofilm producers, exhibiting no filamentation and producing low biofilm biomass. Amongst the biofilm-producing isolates, *C. parapsilosis* isolates showed the highest biofilm biomass, as assessed by CV assay; however, no significant difference was noted when compared to *C. albicans* and *C. tropicalis* ($p > 0.05$). Nevertheless, the biofilm metabolic activity of *C. parapsilosis* was significantly higher compared to that of *C. albicans* ($p = 0.014$) and *C. tropicalis* ($p = 0.012$).

Table 2 shows the *in vitro* susceptibilities of the planktonic and biofilm cultures of *Candida* isolates to AbA. The planktonic MICs for 118 *Candida* isolates ranged from 0.25 to ≥8 μg/ml, with the MIC_{50} and MIC_{90} being 1 and 4 μg/ml, respectively. Eleven (22.4%) isolates of *C. albicans* out of 49, two out of 38 isolates of *C. tropicalis*, one out of 16 isolates of *C. parapsilosis*, and one out of 12 *C. glabrata* isolates had MICs of ≥4 μg/ml. *C. albicans* isolates had apparently a twofold increase in MIC_{50} (2 μg/ml) and MIC_{90} (4 μg/ml) in the planktonic form when compared to the rest of the species. Three *C. nivariensis* isolates exhibited high planktonic MICs (ranging from 4 to ≥8 μg/ml). Overall, the AbA planktonic and biofilm MICs of *Candida* yeasts in this study (Table 1) were higher than those reported by Tan and Tay in a previous study.[25] This is probably

due to the inclusion of a larger number of *Candida* isolates in this study.

The biofilm MICs for 35 biofilm-producing *Candida* isolates ranged from 1 to ≥128 μg/ml, with the MIC_{50} and MIC_{90} being 32 and ≥128 μg/ml, respectively (Table 2). Overall, the AbA biofilm MICs for all *Candida* isolates in this study were at least twofold higher when compared with their respective planktonic MICs. *C. parapsilosis* had the highest biofilm MIC_{50}. The reduced susceptibility to several antifungal drugs of *Candida* biofilm cultures in comparison to planktonic cultures has been related to several mechanisms of resistance such as reduced growth rate, expression of resistance genes, efflux pumps, and the existence of 'persister' cells.[14,21]

Table 3 shows the effects of AbA on biofilm growth morphology, biomass, and metabolic activity. *Candida* isolates exhibiting filamentous growth, high biomass and high metabolic activity showed at least fourfold lower MIC_{50} (32 μg/ml) in comparison to those exhibiting yeast growth, lower biomass and metabolic activity (≥128 μg/ml). In a recent study, Marcos-Zambrano et al.[13] reported higher susceptibility of *Candida* isolates with high biofilm metabolic activity to micafungin. The authors explained that since a high degree of fungal wall biosynthesis was involved in biofilm formation, this may cause the yeasts to become more susceptible to the antifungal drug. A similar explanation may be applied to this study, as higher susceptibility to AbA was noted in biofilm cultures exhibiting high metabolic activity (Table 2). The high metabolic activity in *Candida* biofilm cultures is often correlated with filamentous growth of *Candida* biofilm (and thus higher biomass in comparison to yeast growth), thus it is not surprising a higher susceptibility to AbA in biofilm cultures exhibiting filamentous growth and high biomass (Table 3).

In comparison to the negative control (Fig. 1A), FESEM analysis revealed filament detachment in *C. albicans* SC5314 biofilm culture when treated with 8 μg/ml AbA (0.25× of its biofilm MIC, Fig. 1B). Upon treatment with 32 μg/ml AbA (1xMIC), almost the complete elimination of filamentous cells was shown as only scanty yeast cells were seen on the nucleopore membrane (Fig. 1C). Untreated yeast cells were mostly intact with well-defined and smooth surfaces (Fig. 1D), while rough and distorted cell surfaces were observed in the AbA-treated cells (Fig. 1E). Smaller diameter was also noted in the filaments of treated *C. albicans* biofilm cultures; however, the difference was not significant when compared to the untreated culture, ($p > 0.05$). The precise mechanism for this phenomenon is not known but susceptibility to AbA has been related to lipid rafts which are important for *Candida* biofilm formation.[11]

In conclusion, our results show that in comparison with planktonic cultures, AbA seems to be less active against *Candida* biofilms. The antifungal effects of AbA against *Candida* biofilm cultures should be further explored at the molecular level since little is known about its mechanism of action. The mechanism behind the higher susceptibility to AbA of *Candida* biofilm cultures exhibiting filamentous form and higher biomass/metabolic activity should also be explored.

Conflict of interest

The authors report no conflict of interest.

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