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Effects of Cinnamaldehyde, Ocimene, Camphene, Curcumin and Farnesene on *Candida albicans*

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Abstract

Efficacy of five plant molecules against thirty three clinical isolates and two standard strains of *C. albicans*, differentially susceptible to fluconazole (FLC) is tested in this study. Effect on biofilm (adhesion, development and maturation) formation, morphogenesis and synergy with fluconazole (FLC) against a FLC resistant strain of *Candida albicans* ATCC 10231 is also evaluated. All the plant molecules tested were equally effective against isolates and strains of *C. albicans* (N = 35) tested in this study. Cinnamaldehyde was found most effective against planktonic growth followed by ocimene. Both the molecules exhibited fungicidal activity and killed 99.9% of inoculum within 80 and 20 min of exposure respectively at 0.62 mM and 176.8 mM concentrations. Curcumin (5 - 20 mM), camphene (8 - 32 mM) and farnesene (25 - 100 mM), although inhibited planktonic growth, were fungistatic. All the five plant molecules tested in this study inhibited morphogenesis significantly and exhibited considerable activity against biofilm formation. Inhibition of biofilm was found to be stage specific *i.e.* efficacy was more against adhesion followed by developing and mature biofilm. Plant molecules tested exhibited excellent synergy with fluconazole. However FIC index values 0.155, 0.062 and 0.046 indicate that ocimene was the most effective synergistic molecule inhibited planktonic growth, developing biofilm and mature biofilm growth respectively at very low concentrations. This is the first report of anti-*Candida* activity of three terpenoids viz. ocimene, farnesene and camphene against planktonic & biofilm growth, morphogenesis as well as synergy with FLC. Plant molecules tested in this study may find use in antifungal chemotherapy individually and or in a combination with FLC.

Keywords

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1. Introduction

Fungal pathogens are infecting billions of people every year and deaths due to fungal infections are almost equal to that of Tuberculosis, Malaria etc. [1] [2]. *Candida albicans* is one of the most important opportunistic fungal pathogens responsible for superficial mucocutaneous infections of non-genital (Oropharyngeal) and genital (VVC, balanitis and balanoposthitis and candiduria) tracts of healthy individuals to life threatening, invasive infections in immunocompromised patients [1]-[3]. Oral candidiasis may be of pseudomembranous, hyperplastic, erythematous, angular cheilitis and Denture stomatitis type [2] [3]. It was also reported to be associated with caries in children and dental loss [1]-[3]. It is one of the most frequent agent associated with almost 80% of total fungal bloodstream infections commonly called as candidemia and fourth most common agents associated with nosocomial bloodstream infections with about 40% attributable mortality [2]-[5]. Candidemia leads to colonization of intravascular medical devices like catheters, heart valve, pacemakers etc., in addition to denture in the form of biofilm, a highly complex micro-ecosystem extremely resistant to antifungal agents [2]-[5]. Dramatic increase in incidences of *C. albicans* infections in recent years is associated with the factors like prolonged dosage of broad-spectrum antibiotics, immunosuppressive therapy, extended stay at ICU and patients with AIDs, burns, surgery as well as malignancy etc., that affects either balance in body microflora and or paralyze host immune system [1]-[3] [5].

C. albicans is a polymorphic yeast which can exist in various morphological forms that facilitate its survival under extreme microenvironments by forming biofilms or invading and destructing target tissues [1] [2] [6]. Unlike other pathogenic organisms, morphogenic plasticity helps *C. albicans* in evading host immune responses and confers differential responses towards antifungal agents [1] [2] [6]. Being eukaryotic organisms, fungal specific drug targets are very few and thus limited numbers of antifungal agents like azoles, polyenes, allylamines, and echinocandins etc., are available in the market [7]. Among these, azoles and polyenes were the most promising ones but emergence of azole resistance among the *C. albicans* isolates and severe toxicities of polyenes in hosts has limited their use especially in difficult-to-treat infections like biofilms and invasive candidaisis [8] [9]. This situation has compelled scientific community to expedite the search for novel, potent and host friendly antifungal agents as well as look for alternative options like combination antifungal therapy [7]-[10]. Combination therapy is already successful and being used against several diseases [10]-[12].

Cinnamaldehyde, ocimene, farnesene, curcumin and camphene were tested in this study as earlier studies have shown that essential oils rich in these molecules (except curcumin) exhibits excellent anti-*Candida* activity [13]-[19]. Curcumin was included to evaluate its potential against biofilm formation as it was reported to inhibit morphogenesis in *C. albicans* as well as shown excellent apoptosis inducing activity [20]. We have tested efficacy of these plant molecules against planktonic growth, morphogenesis and biofilm formation (adhesion, development and maturation) of *C. albicans*. Synergistic potential with FLC against planktonic and biofilm growth of *C. albicans* is also evaluated in this study.

2. Materials and Methods

2.1. Plant Molecules and Growth Media

Plant molecules viz. ocimene (≥90% v/v), cinnamaldehyde (≥95% v/v), farnesene (≥90% v/v), curcumin and camphene were purchased from Sigma-Aldrich India Pvt. Ltd., Bangalore (India).

Yeast Extract Peptone Dextrose broth, RPMI 1640 medium, Horse Serum and MTT were purchased from Hi-media Laboratories, Pvt. Ltd. Mumbai (India). Polystyrene make 96 well micro titer plates were procured from Tarson India Ltd.

2.2. *Candida albicans* Isolates and Strains

Thirty-three clinical isolates of *Candia albicans*, differentially susceptible to fluconazole (FLC) (Resistant − N = 11, S-DD − N = 02 and susceptible − N = 22) used in this study were received from Swami Ramanand Teerth Culture Collection (SRTCC) of School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded (MS) India. Out of thirty-three, 22 isolates were from oral candidiasis (15 from HIV positive and seven from healthy individuals), 6 from respiratory, 3 from genitourinary and 2 from gastroenteritis infections. A FLC resistant strain of *C. albicans* ATCC 10231 (Member of CaDR MP-8 Panel developed by ATCC for drug testing) [28] and a susceptible strain ATCC 90028 were procured from the Microbial Type Culture Collection, Institute

of Microbial Technology (IMTECH), Chandigarh (India) and included as a quality control in this study. All the cultures were maintained on yeast extract peptone dextrose (YPD) agar slants at 4°C.

2.3. Preparation of Working Stocks of Plant Molecules

Concentrations of ocimene, cinnamaldehyde, and farnesene procured in liquid form were found to be 5.824, 7.545 and 3.580 molar, respectively. Considering the solubility issues, 10% (v/v) ethanol was added to ocimene and farnesene to improve the solubility. Cinnamaldehyde was needed in very less concentration and thus diluted with ethanol by two fold. Stock solution of camphene (1 M) was prepared in ethanol. Curcumin stock solution (1 M) was prepared by solubilizing in minimum volume of 0.5 M NaOH and diluting immediately with YPD.

2.4. Inhibition of Planktonic Growth of *Candida albicans*

Anti-*Candida* activity of five plant molecules against thirty-three clinical isolates and two standard strains of *C. albicans* was tested by broth micro dilution assay (CLSI M27-A3) as mentioned previously [14] [15] [29]. Briefly, 100 µL YPD broth containing 24 h grown *C. albicans* yeast phase cells (2×10^3 cfu/mL) and 0.001% (v/v) tween 20 was transferred aseptically to each well of the 96 well micro titer plate. However double volume (200 µL) was added in the 12th well of each row, highest concentration of plant molecules were added to this well and serially diluted up to 8 fold. Well number 3 and 2 of each row served as respective solvent controls and well number 1 as control without solvent. Doubling dilutions of plant molecules used were as follows: ocimene (352 to 1.37 mM), cinnamaldehyde (5 to 0.019 mM), farnesene (300 to 1.17 mM), curcumin (20 to 0.078 mM) and camphene (32 to 0.125 mM). Plates were observed visibly after 24 h of incubation at 35°C and concentrations causing complete growth inhibition were considered as minimum inhibitory concentration (MIC). Triplicates were used for each concentration and experiment was repeated thrice.

Minimum fungicidal concentrations (MFCs) of plant molecules were determined by plating 5 µl culture from these wells on YPD agar plates. Number of colonies appeared on the agar plates after 48 h of incubation at 35°C were counted. Lowest concentration killing of 99.9% inoculums was considered as MFCs [14] [15].

2.5. Time Kill Assay

Time dependant killing of a Fluconazole resistant strain of *C. albicans* ATCC10231 inoculum by MFCs of ocimene and cinnamaldehyde was studied as done previously by Zore *et al*. [14] [15]. In brief, *C. albicans* cells (2.5×10^3 cfu/ml) in 100 µl YPD broth, were exposed to MFCs of ocimene and cinnamaldehyde for different time intervals viz. 0, 5, 10, 20, 40, 80 min. After different time intervals, cells were washed twice, re-suspended in 50 µl of YPD broth and inoculated on YPD agar plates. Number of colonies appeared on these plates were counted after incubation at 30°C for 48 h and compared with that of the control. Wells lacking plant molecule but containing respective solvent (ethanol) were served as growth controls. Triplicates were used for each time interval and experiment was repeated thrice.

2.6. Inhibition of Morphogenesis in *Candida albicans* (ATCC 10231)

Effect of all the five plant molecules on serum induced morphogenesis of *C. albicans* (ATCC 10231) was studied by using micro titer plate based morphological assay as done previously by Zore *et al*. [14] [15]. In brief, 100 µl of YPD supplemented with 25% horse serum and 24 h grown *C. albicans* yeast phase cells (10^5 cfu/ml), were treated with different concentrations of plant molecules viz. ocimene (176 to 1.37 mM), cinnamaldehyde (5 to 0.031 mM), farnesene (100 to 0.78 mM), curcumin (20 to 0.15 mM) and camphene (16 to 0.12 mM). Three wells were used for each concentration and wells lacking plant molecule but containing respective solvents were used as control. Plates were incubated at 37°C for 90 minutes, cells of different morphological types (Budded, un-budded, hyphae and pseudohyphae) were counted after incubation microscopically using hemocytometer and percentage inhibition of hyphae induction was calculated (**Figure 2**).

2.7. Inhibition of Biofilm (Adhesion, Development and Maturation) Formation

2.7.1. Inoculum Preparation

C. albicans cells grown in YPD for 24 h at 30°C were harvested, washed with sterile distilled water and re-suspended in phosphate buffered saline (PBS). Cell density was adjusted to 1×10^7/ml, aseptically.

2.7.2. Biofilm Assay

Effect of plant molecules viz. ocimene (352 to 23.1 mM), cinnamaldehyde (5 to 0.312 mM), farnesene (300 to 18.52 mM), curcumin (20 to 7.5 mM) and camphene (32 to 3 mM) was evaluated by measuring adhesion (90 min), development (24 h) and maturation (48 h) of *C. albicans* (ATCC 10231) biofilms by MTT assay after removing non adhered cells [4] [27].

In brief, adhesion was performed by dispensing 100 µl inoculum without and with plant molecules (concentrations mentioned above) aseptically to the wells and incubated at 37˚C for 90 min. Non adhered cells were removed by washing the wells thrice with PBS and adhesion was measured and compared with the respective solvent controls. To test efficacy against biofilm development, cells were allowed to adhere (adhesion) as mentioned above. 100 µl of fresh RPMI 1640 medium with and without plant molecules were added to these wells and incubated further. Growth was measured after 24 h incubation at 37˚C and compared with respective solvent controls. To test effect against mature biofilm, 24 h old biofilms developed as mentioned above were used. 100 µl of fresh RPMI 1640 medium with and without plant molecules were added to these wells with developed biofilms after removing non adhered cells and incubated further at 37˚C. Growth was measured after 48 h incubation and compared with respective solvent controls. Triplicates were used for each concentration and experiment was repeated three times. MTT assay was performed as described previously [27].

2.7.3. Synergistic Activity of Plant Molecules and Fluconazole against Planktonic and Biofilm Growth (Development and Maturation) of a FLC Resistant Strain of *Candida albicans* (ATCC 10231)

Effect of plant molecules on fluconazole susceptibility in planktonically growing *C. albicans* (ATCC 10231) was studied by micro dilution checkerboard assay [21]-[30]. Concentration of plant molecules used with FLC (2 to 128 g/ml) were as follows: ocimene (88 - 5.5 mM), cinnamaldehyde (0.625 - 0.037 mM), farnesene (100 - 6.25 mM), curcumin (20 - 1.25 mM) and camphene (16 - 1 mM). Similarly synergistic activity of FLC ($\geq$256 - 8 g/ml) with ocimene (22 to 5.5 mM), cinnamaldehyde (0.31 - 0.07), farnesene (18.7 - 4.65 mM), curcumin (3.75 - 0.93 mM) and camphene (3 - 0.75 mM) was evaluated against development and maturation of *C. albicans* biofilms. Biofilm development and maturation was performed as mentioned above (biofilm assay). Fractional inhibitory concentrations (FICs) and FIC index values were calculated for each plant molecules [27] [30]. FIC is inhibitory concentration in combinations divided by the concentration that has same effect when used individually while FIC index values are sum of the FICs [26]. FIC index ($\sum$FIC) shows type of interaction between two compounds viz. value 0.5 to 4.0 shows additive interaction, value < 0.5 exhibit synergistic interaction and value > 4.0 exhibit antagonistic interactions [4] [26] [27] [30].

3. Results

3.1. Inhibition of Planktonic Growth

All the five plant molecules tested, inhibited planktonic growth and were equally effective against FLC resistant as well as susceptible isolates of *C. albicans* (**Table 1**). *C. albicans* (ATCC 10231) is a FLC resistant strain (MIC is >128 µg/ml) used as a FLC resistant member in a *C. albicans* Drug resistant Panel MP-8 developed by ATCC for drug testing (CaDR MP-8 Panel, ATCC). In our study, MIC of FLC for planktonic growth could not be achieved up to 128 µg/ml and thus MIC is denoted as >128 µg/ml.

Cinnamaldehyde was found to be most effective, inhibited all the isolates at or less than 0.62 mM concentration (**Table 1**). MICs of remaining four molecules were ranged from 5 - 20 mM (curcumin), 8 - 32 mM (camphene), 25 - 100 (farnesene) and 44.1 - 176.4 (ocimene) (**Table 1**). Cinnamaldehyde and ocimene exhibited fungicidal activity while remaining three were fungistatic (**Table 1**, **Figure 1**, **Supplementary Figure 1(a)**, **Figure 1(b)**). In general, MFCs of cinnamaldehyde and ocimene were greater than MICs (**Table 1**, **Supplementary Figure 1(a)**). Time dependant kill curve analysis showed that MFCs of cinnamaldehyde (0.62 mM) and ocimene (88.2 mM) kills 99.9% inoculums within 80 and 20 minutes of exposure respectively (**Table 1**, **Supplementary Figure 1(b)**).

3.2. Inhibition of Morphogenesis by Plant Molecules

All the plant molecules tested exhibited excellent anti-morphogenic activity and inhibited hyphae as well as

Table 1. Anti-*Candida* potential of plant molecules against clinical isolates (N = 33) and standard strains of *Candida albicans* (n = 2).

Assay	Compounds	MIC (mM)	*Candida albicans* strains and isolates (N = 35)		
			Susceptible (N = 21)	S-DD (N = 2)	Resistant (N = 12)
Broth Micro Dilution Assay	Ocimene	44.1	05 (**0**)	-	03
		88.2	08 (**11**)	01	03 (**03**)
		176.4	08 (**10**)	01 (**02**)	06 (**09**)
	Cinnamaldehyde	0.15	04 (**0**)	-	02
		0.31	04 (**07**)	-	03 (**01**)
		0.62	13 (**14**)	02 (**02**)	07 (**11**)
	Farnesene	25	13	-	05
		50	02	-	-
		100	06	02	07
	Curcumin	05	06	-	02
		10	05	-	04
		20	10	02	06
	Camphene	8	08	-	05
		16	10	-	06
		32	03	02	01

Bold values in parentheses indicate the number of isolates wherein 99.9% inoculum was killed by respective concentration (MFC), S-DD, dose-dependent susceptible to fluconazole, (According to CLSI M27 A3, 2007). All the compounds were tested in triplicates (for each concentrations) and assay was repeated three times.
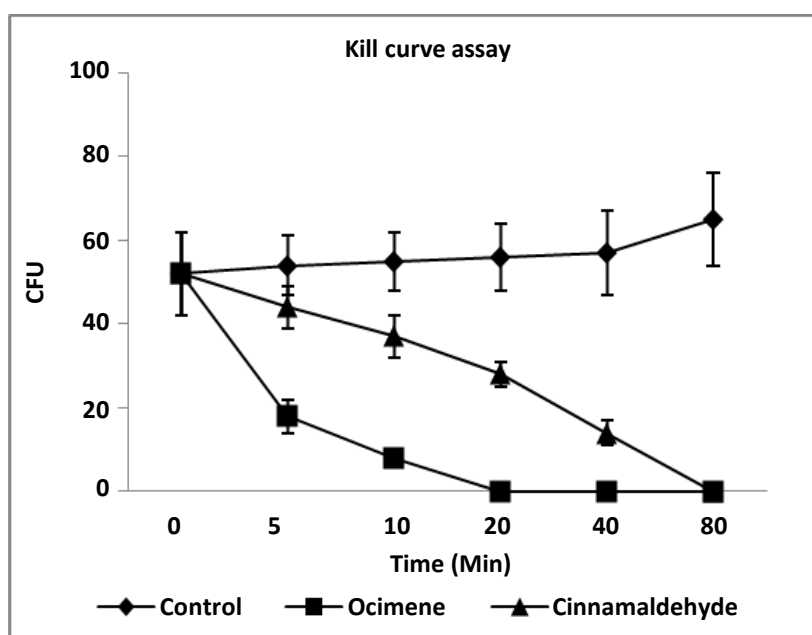
Figure 1. Time kill curve at MFCs of ocimene and cinnamaldehyde in *Candida albicans* (ATCC 10231).

pseudohyphae formation completely at the MICs of growth (**Figure 2**). Compared to planktonic growth, hyphae formation seems to be more sensitive as 50% inhibition of morphogenesis was achieved at very low concentrations viz. 22 mM (ocimene), 0.078 mM (cinnamaldehyde), 9.1 mM (farnesene), 1.25 mM (curcumin) and 3 mM (camphene) (**Figure 2**). Inhibition of hyphae formation by cinnamaldehyde, farnesene, curcumin and camphene lead to pseudohyphae formation initially but higher concentrations resulted in to un-budded cells (**Figure 2**).

3.3. Inhibition of Biofilm Formation by Plant Molecules

Biofilm assay (adhesion, development and maturation) showed that all the five plant molecules inhibit adhesion in a concentration dependent manner (**Table 2**, **Supplementary Figures 1(c)-1(e)**). Farnesene (300 mM) and ocimene (352 mM) inhibited adhesion completely while curcumin (60 mM) and cinnamaldehyde (5 mM) caused 97% and 91% inhibition respectively (**Table 2**, **Supplementary Figures 1(c)-1(e)**). Camphene was least effective, could inhibit adhesion by 64% at 48 mM concentration (**Table 2**, **Supplementary Figures 1(c)-1(e)**).

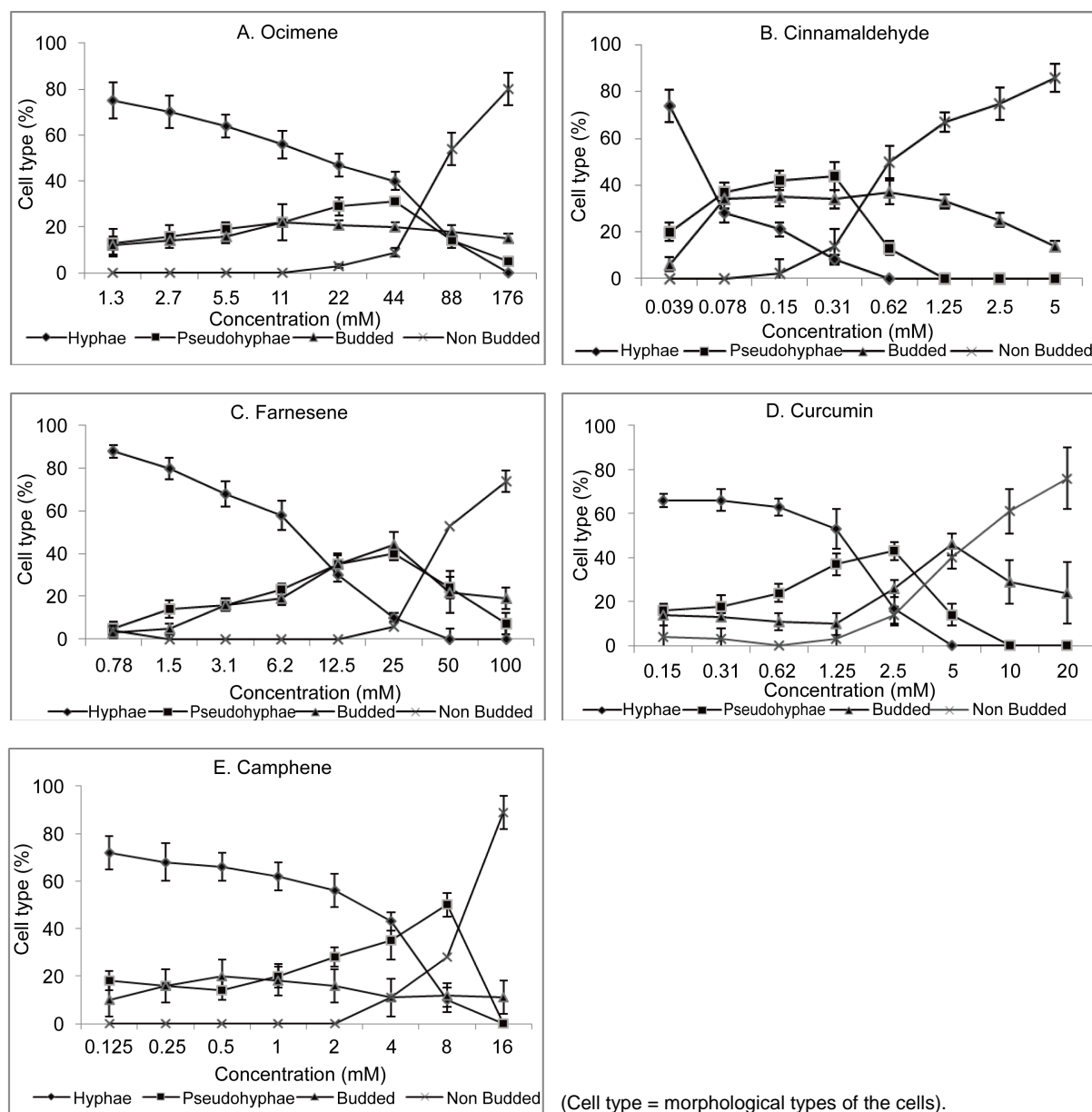(Cell type = morphological types of the cells).

Figure 2. Effect of Plant Molecules on Morphogenesis in *Candida albicans* (ATCC 10231).

Table 2. Effect of Plant Molecules on *Candida albicans* biofilm progression: Adhesion, development and maturation.

Plant molecules/Developmental Stage	Biofilm Formation (%)					
	Concentrations (mM)					
Ocimene	**Control**	**23.1**	**44.2**	**88.4**	**176.8**	**352**
Adhesion	100 ± 0	60 ± 2	5 ± 1	4 ± 1	4 ± 0	0 ± 0
Developing Biofilm	100 ± 0	54 ± 4.28	27 ± 10.24	22 ± 0.25	18 ± 0.64	14 ± 0.31
Mature Biofilm	100 ± 0	80 ± 14.3	69 ± 7.12	49 ± 8.80	41 ± 5.12	25 ± 6.77
Cinnamaldehyde	**0**	**0.312**	**0.625**	**1.25**	**2.5**	**5**
Adhesion	100 ± 0	100 ± 0	67 ± 3	26 ± 7	23 ± 6	21 ± 1
Developing Biofilm	100 ± 0	25 ± 19	22 ± 1.0	20 ± 0.4	17 ± 0.5	13 ± 0.6
Mature Biofilm	100 ± 0	40 ± 5.6	37 ± 8	36 ± 7.61	35 ± 12.63	35 ± 14.9
Farnesene	**0**	**18.52**	**37.5**	**75**	**150**	**300**
Adhesion	100 ± 0	70 ± 2	62 ± 9	5 ± 2	4 ± 1	0
Developing Biofilm	100 ± 0	42 ± 18	34 ± 16	33 ± 5.97	30 ± 4	28 ± 6
Mature Biofilm	100 ± 0	64 ± 15	50 ± 7.94	47 ± 7.66	33 ± 1.77	32 ± 9.2
Curcumin	**0**	**7.5**	**15**	**15**	**30**	**60**
Adhesion	100 ± 0	82 ± 1	61 ± 5	5 ± 2	4 ± 2	3 ± 1
Developing Biofilm	100 ± 0	100 ± 1.36	100 ±	87 ± 1.71	39 ± 5.1	39 ± 10.2
Mature Biofilm	100 ± 0	86 ± 3.74	82 ± 0.75	72 ± 6.05	65 ± 0.82	62 ± 1.63
Camphene	**0**	**3**	**6**	**12**	**24**	**48**
Adhesion	100 ± 0	75 ± 1	55 ± 1	41 ± 1	39 ± 1	36 ± 1
Developing Biofilm	100 ± 0	48 ± 23	34 ± 14	18 ± 5.33	10 ± 0.36	9 ± 0.97
Mature Biofilm	100 ± 0	80 ± 8.11	68 ± 13	65 ± 12	48 ± 11.5	30 ± 3.3
Fluconazole	**0**	**16**	**32**	**64**	**128**	**256**
Developing Biofilm	100 ± 0	52 ± 5	39 ± 3	38 ± 4	38 ± 3	38 ± 3
Mature Biofilm	100 ± 0	62 ± 14	54 ± 10	46 ± 14	45 ± 10	45 ± 11

Numbers represent biofilm (adhesion, developing and mature) growth% ± Standard deviation. * Stage of development (Duration): Adhesion = 0 - 90 min, Developing biofilm = 90 min - 24 h, Mature biofilm = 24 - 48 h.

All the five plant molecules inhibited developing biofilm considerably but could not achieve complete inhibition (**Table 2**, **Supplementary Figures 1(c)-1(e)**). Cinnamaldehyde (5 mM), camphene (48 mM), ocimene (352 mM) and farnesene (300 mM) inhibited biofilm development by 87%, 90%, 86% and 72% respectively (**Table 2**, **Supplementary Figures 1(c)-1(e)**). Curcumin caused 60% inhibition at 30 mM but further increase in concentration could not increase inhibition significantly (**Table 2**, **Supplementary Figures 1(c)-1(e)**). 256 µg/ml concentration of FLC could cause 62% inhibition of biofilm development (**Table 2**).

Mature biofilm seems to be more tolerant to plant molecules tested in this study as MIC of planktonic growth *i.e.* ocimene (88.4 mM), cinnamaldehyde (0.31 mM) and farnesene (37.5 mM) could inhibit mature biofilm by 50% (**Table 2**, **Supplementary Figures 1(c)-1(e)**). A maximum of 75, 65 and 68% inhibition was observed by ocimene (352 mM), cinnamaldehyde (5 mM) and farnesene (300 mM) respectively (**Table 2**, **Supplementary Figures 1(c)-1(e)**). Camphene (48 mM) and FLC (256 µg/ml) inhibited mature biofilm by 70% and 55% respectively while curcumin concentration up to 60 mM, could not achieve 50% inhibition (**Table 2**, **Supplementary Figures 1(c)-1(e)**).

3.4. Synergistic Activity of Plant Molecules with FLC against *Candida albicans* ATCC 10231

Plant molecules tested in this study showed very good synergistic activity against planktonic and biofilm

(developing and mature) growth. As *C. albicans* (ATCC 10231) is a FLC resistant strain, 128 µg/ml FLC could not inhibit planktonic growth completely and thus MIC is considered as >128 µg/ml in synergy assay (**Table 3**). Similarly, fluconazole (256 µg/ml) could inhibit developing biofilm by 62% and mature biofilm by 55% and thus concentration required for more inhibition (>62% and >55%) is considered as >256 µg/ml in synergy assay (**Table 3**).

Table 3. Synergistic activity of plant molecules and fluconazole against A. Planktonic growth and B. Developing and C. Mature Biofilm of a FLC-resistant strain of *Candida albicans* (ATCC 10231).

Combinations	MIC		FICs		FIC index	Interaction
	FLC (µg/ml)	Comp. (mM)	FLC	Comp.		
A. Planktonic Growth (MIC100)						
FLC alone	>128	0	1	0	1	NI
Ocimene + FLC	4	22	0.031	0.124	0.155	synergistic
Ocimene alone	0	176.4	0	1	1	NI
Cinnamaldehyde + FLC	4	0.15	0.031	0.241	0.273	synergistic
Cinnamaldehyde	0	0.62	0	1	1	NI
Farnesene + FLC	8	12.5	0.062	0.125	0.187	synergistic
Farnesene alone	0	100	0	1	1	NI
Curcumin + FLC	8	2.5	0.062	0.125	0.187	synergistic
Curcumin alone	0	20	0	1	1	NI
Camphene + FLC	8	4	0.064	0.25	0.289	synergistic
Camphene alone	0	16	0	1	1	NI
B. Developing Biofilm (MIC90)						
FLC alone	>256	0	1	0	1	NI
Cinnamaldehyde + FLC	8	0.152	0.031	0.030	0.061	Synergistic
Cinnamaldehyde	0	5	0	1	1	NI
Farnesene + FLC	8	18.7	0.031	0.062	0.093	Synergistic
Farnesene	0	300	0	1	1	
MIC80						
Ocimene + FLC	8	5.5	0.031	0.031	0.062	Synergistic
Ocimene	0	176.8	0	1	1	NI
Camphene + FLC	8	1.5	0.031	0.125	0.156	Synergistic
Camphene	0	12	0	1	1	
MIC70						
Curcumin + FLC	8	1.85	0.031	0.030	0.062	Synergistic
Curcumin	0	60	0	1	1	NI
C. Mature Biofilm (MIC 90)						
FLC alone	>256	0	1	0	1	NI
Cinnamaldehyde + FLC	8	0.152	0.031	0.030	0.061	Synergistic
Cinnamaldehyde	0	5	0	1	1	NI
MIC80						
FLC alone	>256	0	1	0	1	NI
Ocimene + FLC	8	5.5	0.031	0.015	0.046	Synergistic
Ocimene alone	0	352	0	1	1	NI
MIC50						
FLC alone	256	0	1	0	1	NI
Farnesene + FLC	8	9.35	0.031	0.249	0.280	Synergistic
Farnesene	0	37.5	0	1	1	
Camphene + FLC	8	0.75	0.031	0.031	0.062	Synergistic
Camphene	0	24	0	1	1	NI

FICs = fractional inhibitory concentrations, FIC index = ΣFICs, FLC = fluconazole, Comp. = compounds tested. (FICI ≤ 0.5 = Synergy, FICI > 0.5- ≤ 4.0 = No interaction and FICI > 4 = Antagonistic; Odds 2003). FLC inhibited developing and mature biofilms by 62% and 55% respectively at 256 µg/ml and thus MIC 90, 80 and 70 of FLC is considered as >256 µg/ml. Synergy assays were repeated three time.

Plant molecules tested sensitized *C. albicans* cells and brought down MIC of FLC significantly (**Table 3**). Ocimene (22 mM) and cinnamaldehyde (0.15 mM) brought down MIC of FLC by 32 fold >128 µg/ml to 4 µg/ml) while farnesene (12.5 mM), curcumin (2.5 mM) and camphene (4 mM) lowered down MIC by 16 fold (>128 µg/ml to 8 µg/ml) (**Table 3**). FIC index value, 0.155 shows that ocimene is the most synergistic molecule followed by 0.187 (farnesene and curcumin), 0.273 (cinnamaldehyde) and 0.289 (camphene) (**Table 3**).

All the five plant molecules tested, inhibited both developing and mature biofilm significantly at very low concentrations (sub toxic) when used in combinations with FLC, indicating excellent synergistic potential (**Table 3**). Cinnamaldehyde (0.152 mM) and farnesene (18.7 mM) inhibited developing biofilm by 90%; ocimene (5.5 mM) and camphene (1.5 mM) by 80% while curcumin (1.85 mM) by 70% when used with FLC (8 µg/ml) (**Table 3**). FICI values indicate that cinnamaldehyde (0.061) exhibited more synergistic activity with FLC against developing biofilm followed by ocimene and curcumin (0.062), farnesene (0.093) and camphene (0.156) (**Table 3**).

Fluconazole (8 µg/ml) inhibited mature biofilm by 90% synergistically with cinnamaldehyde (0.152 mM) while ocimene (5.5 mM), farnesene (9.7 mM) and camphene (0.75 mM) could cause 80% inhibition (**Table 3**). FIC index value showed that ocimene (0.046) was the most synergistic molecule against mature biofilm followed by cinnamaldehyde (0.061), camphene (0.062) and farnesene (0.280) (**Table 3**).

4. Discussion

Search for novel and potent antifungal agents are intensified in recent years because currently available drugs like azoles and polyenes etc., are not very promising against increasing incidences of difficult-to-treat fungal infections [7]-[10]. Plants produces structurally diverse group of molecules in response to various biotic and abiotic stresses as a defense mechanism in addition to signaling and attracting pollinators [31]. These molecules reported to exhibit excellent biological activities since they are synthesized against various biological agents (pests) [31]. Terpene the largest group of plant secondary metabolite being used for flavouring and fragrance from centuries is being exploited in recent years for their multifarious biological activities [31]. Essential oils rich in ocimene, farnesene, camphene and cinnamaldehyde reported to exhibit various biological activity including anti-*Candida* activity [13] [21]-[25] [31]-[33]. However no studies are available on biological activities of ocimene but recent reports suggests ocimene could be associated with antioxidant, antifungal, anti-parasitic, anticancer, antibacterial and wound healing activities of ocimene rich essential oils [13] [21]-[25]. Farnesene produced by plants in response to insect attack reported to inhibit growth of eukaryotic (like insects, parasites) as well as prokaryotic pathogens and exhibit anticancer, anti-plasmodial, hepatoprotective, antioxidant, anti-inflammatory activity [34]-[40]. Camphene a monoterpene used in flavour and fragrance, reported to inhibit sterol biosynthesis by inhibiting sterol regulatory element-binding protein (SREBP)-1c, SREBP-2, 3-hydroxy-3 methyl-glutaryl-CoA reductase (HMGCR) in eukaryotic cells [41]-[50]. Semicarbazide derivatives of camphene showed good antifungal activity against *Trichophyton mentagrophytes* [41]-[50]. Camphene also reported to exhibit antioxidant, antinociceptive, antibacterial, expectorant, broncholytic, antiulcer activity and also helpful in expulsion of urolithiasis [41]-[50]. Ocimene, farnesene and camphene could be inhibiting *C. albicans* growth by affecting membrane integrity as terpenoids are known to cause cell wall and membrane damage [14] [15] [21]-[25]. However inhibition of sterol biosynthesis could also be associated with anti-*Candida* activity of camphene [50]. Cinnamaldehyde was also known to inhibit growth of various microorganisms by affecting membrane integrity, energy generation and extended-spectrum beta-lactamase (ESBL) protein dependant drug resistance [17]-[19]. Curcumin is known to induce apoptosis in eukaryotic cells and also reported to inhibit planktonic and biofilm growth of *C. albicans* [20] [51]. Thus membrane integrity damage and apoptosis inducing activity of cinnamaldehyde and curcumin could be associated with anti-*Candida* activity in our study.

Anti-morphogenic activity supports membrane integrity damage (by terpenoids and cinnamaldehyde) mediated inhibition as it led to inhibition of morphogenesis through modulation of signaling pathways involved [7] [12] [14] [15] [17]-[24]. Curcumin is reported to up regulate TUP1, a negative regulator of hypahe induction [20] [51]. Membrane damage reported to affect membrane functions including signaling and transport [14] [15] [19] [22]-[24]. Morphogenesis is considered as a survival strategy of *C. albicans* under extreme conditions that facilitate adhesion, colonization, invasion and destruction of host tissues by evading host immune responses and confers differential response towards antifungal agents [2]-[6]. Inhibition of morphogenesis is considered as a good strategy to avoid invasive infections and biofilm formation and plant molecules tested in this study exhibited

excellent activity against morphogenesis (**Figure 2**). Cinnamaldehyde and curcumin inhibited hyphae induction significantly compared to earlier reports while it is the first report of anti-morphogenic activity of camphene, farnesene and ocimene (**Figure 2**).

Anti-morphogenic activity of plant molecules could be contributing to biofilm inhibition as hyphae induction is the most important prerequisite for adhesion and colonization of denture as well as intravascular medical devices like catheters, heart valves, pacemakers, stents etc., in the form of biofilms [3]-[5] [27]. Hyphae specific traits like HWP1, ALS3 etc., are essential in establishing cell-cell interaction, providing necessary support and integrity to the cells in biofilms that allows survival, proliferation, intake of nutrients, efflux of metabolic wastes, evasion of host immune responses and maintaining pool of dormant persister cells [3]-[5] [27]. All the plant molecules tested inhibited biofilm formation and activity was found to be stage dependent *i.e.* adhesion was more susceptible followed by developing and mature biofilms (**Table 2**, **Supplementary Figures 1(c)-1(e)**). It could be due to the differential accessibility of the *C. albicans* cells in addition to differential expression of drug resistant traits during adhesion, development and maturation of biofilm [3]-[5] [27]. Ocimene and farnesene were most effective, inhibited adhesion completely but failed to inhibit developing and mature biofilm completely (**Table 2**, **Supplementary Figures 1(c)-1(e)**). Plant molecules could be inhibiting adhesion and developing biofilm by inhibiting hyphae induction and elongation respectively. As cells exposed to plant molecules at 0 h failed to induce hyphae and thus adhesion while cells exposed after adhesion (90 min), inhibited hyphae elongation (hyphal length did not increase further after addition of plant molecules) and biofilm development further supporting the mechanism of membrane integrity damage (**Supplementary Figures 1(c)-1(e)**). Mature biofilms were more tolerant as cells were less accessible to plant molecules and FLC (**Table 2**, **Supplementary Figures 1(c)-1(e)**).

Combination therapy found promising against various difficult to treat diseases in recent years and various plant molecules showed excellent synergistic activity with existing drugs [9]-[12] [14]-[16]. Plant molecules showed synergistic activity with antibiotics against bacterial and fungal pathogens and mechanisms of synergy was reported to be associated with enhancing penetration and bioavailability of antibiotics [12] [14] [15]. All the five plant molecules tested, exhibited excellent synergy with FLC against planktonic and biofilm growth of a recalcitrant strain of *C. albicans* (ATCC 10231) [28]. It indicates that plant molecules tested could find use in combination therapy against difficult-to-treat infections of *C. albicans* [9] [14]-[16]. It is significant as it could lower the dosage further reducing risk of side effects associated with higher dosage and prolonged use of antifungal agents [8] [9] [14]-[16]. Synergistic activity was found to be dependent on accessibility of the cells *i.e.* very active against planktonic growth (accessible), followed by developing biofilm (less accessible) and mature biofilm (inaccessible) [3]-[5] [27] [51].

Thus ability to cross the barriers of biofilm matrix could be associated with the differential synergistic activity of plant molecules with FLC against biofilm growth of *C. albicans* in addition to earlier hypothesized mechanism of membrane fluidization mediated modulation of signaling, transport and cell cycle arrest [14] [15] [23] [24].

5. Conclusions

Our study showed that all the five plant molecules tested exhibited excellent activity against planktonic growth and morphogenesis. Membrane destabilizing activity could be associated with anti-*Candida* activity as modulation of membrane functions like cell signalling reported to inhibit morphogenesis and cell cycle. Activity against biofilm formation was found to be differential and it could be due to differential accessibility of the cells in biofilm. Inhibition of hyphae induction and elongation affects adhesion and biofilm development, respectively. All the five plant molecules exhibited excellent synergy with FLC even against recalcitrant biofilms, strengthening their candidature as possible anti-*Candida* agents in combination therapy.

Based on our findings, we conclude that compounds tested in this study may find use in antifungal chemotherapy alone or in combination with FLC. Considering the high intake of these molecules in the form of flavouring agents in food products, toxicity concerns (at MICs) may not arise however detailed toxicity study is needed.

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the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

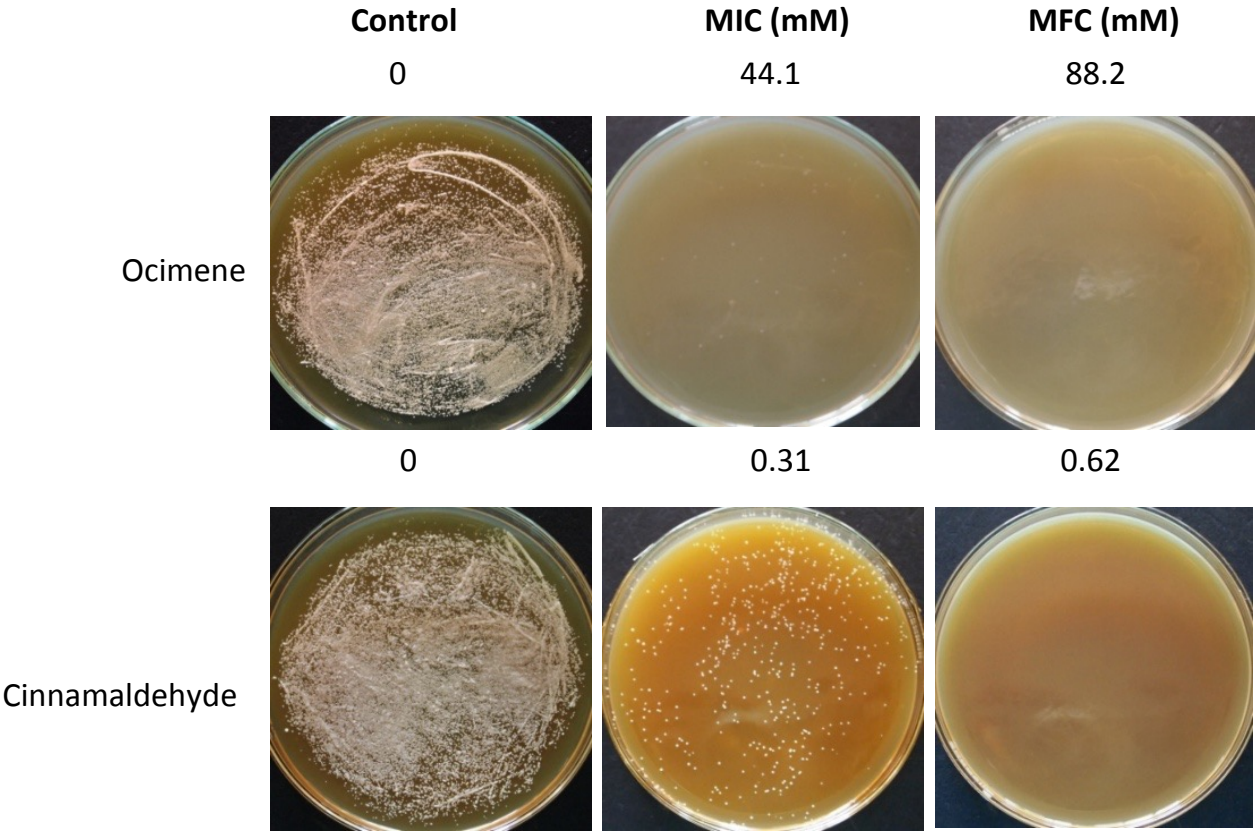## Conflict of Interest

No conflict of interest to disclose.

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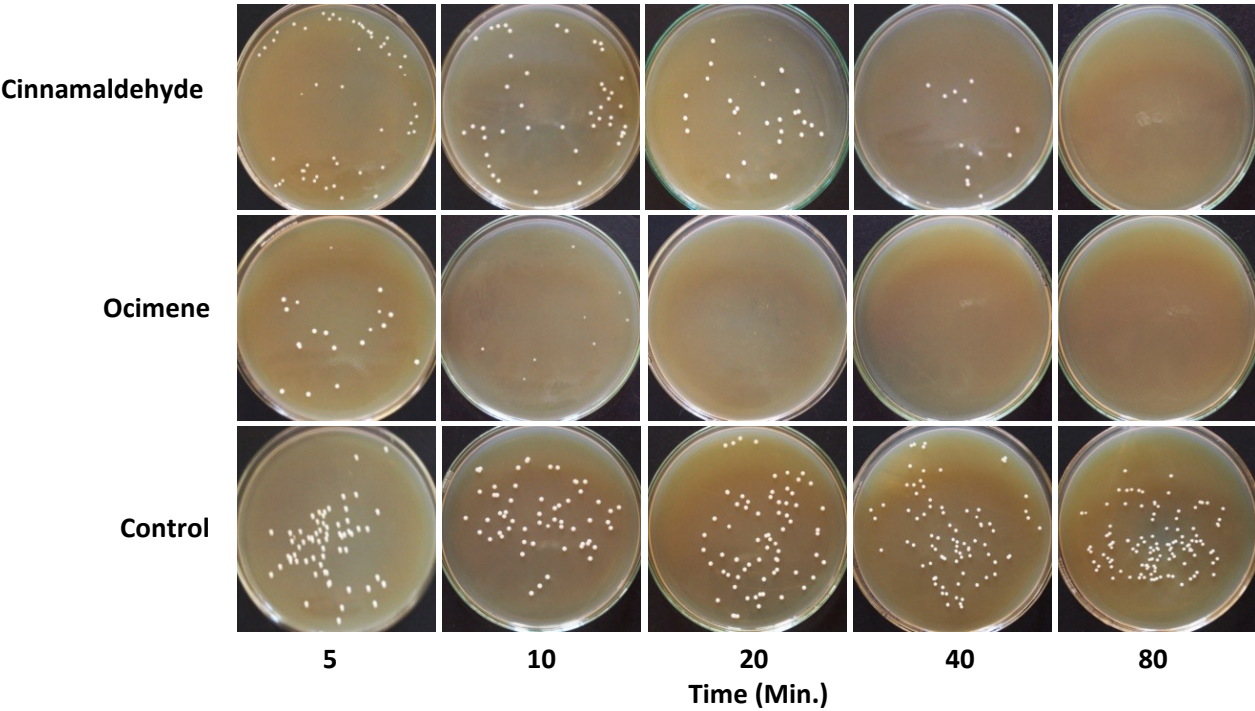
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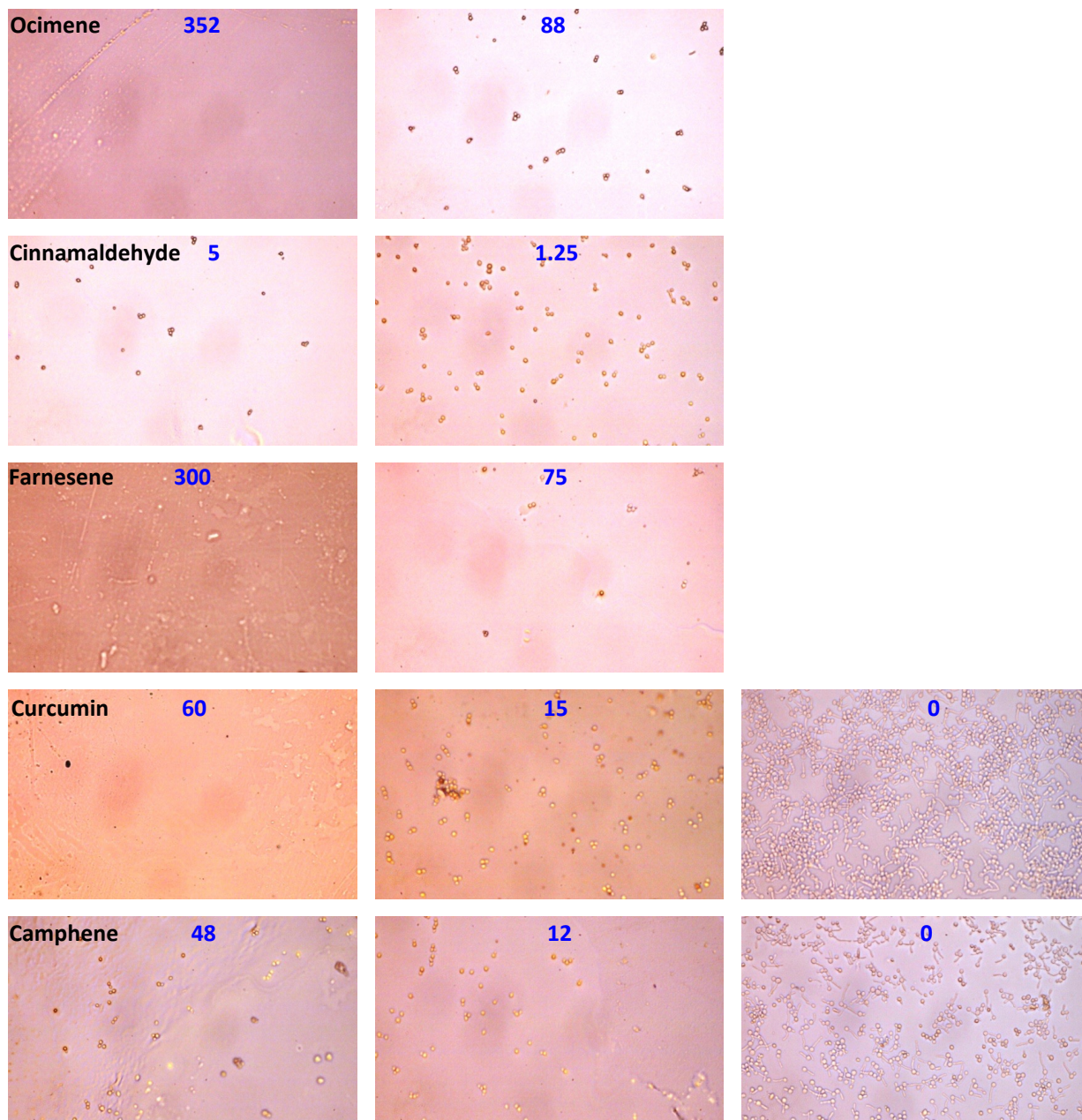http://dx.doi.org/10.1016/j.ijantimicag.2014.05.017

Supplementary

	Control	MIC (mM)	MFC (mM)
	0	44.1	88.2

Ocimene

	0	0.31	0.62

Cinnamaldehyde

(a)

Cinnamaldehyde

Ocimene

Control

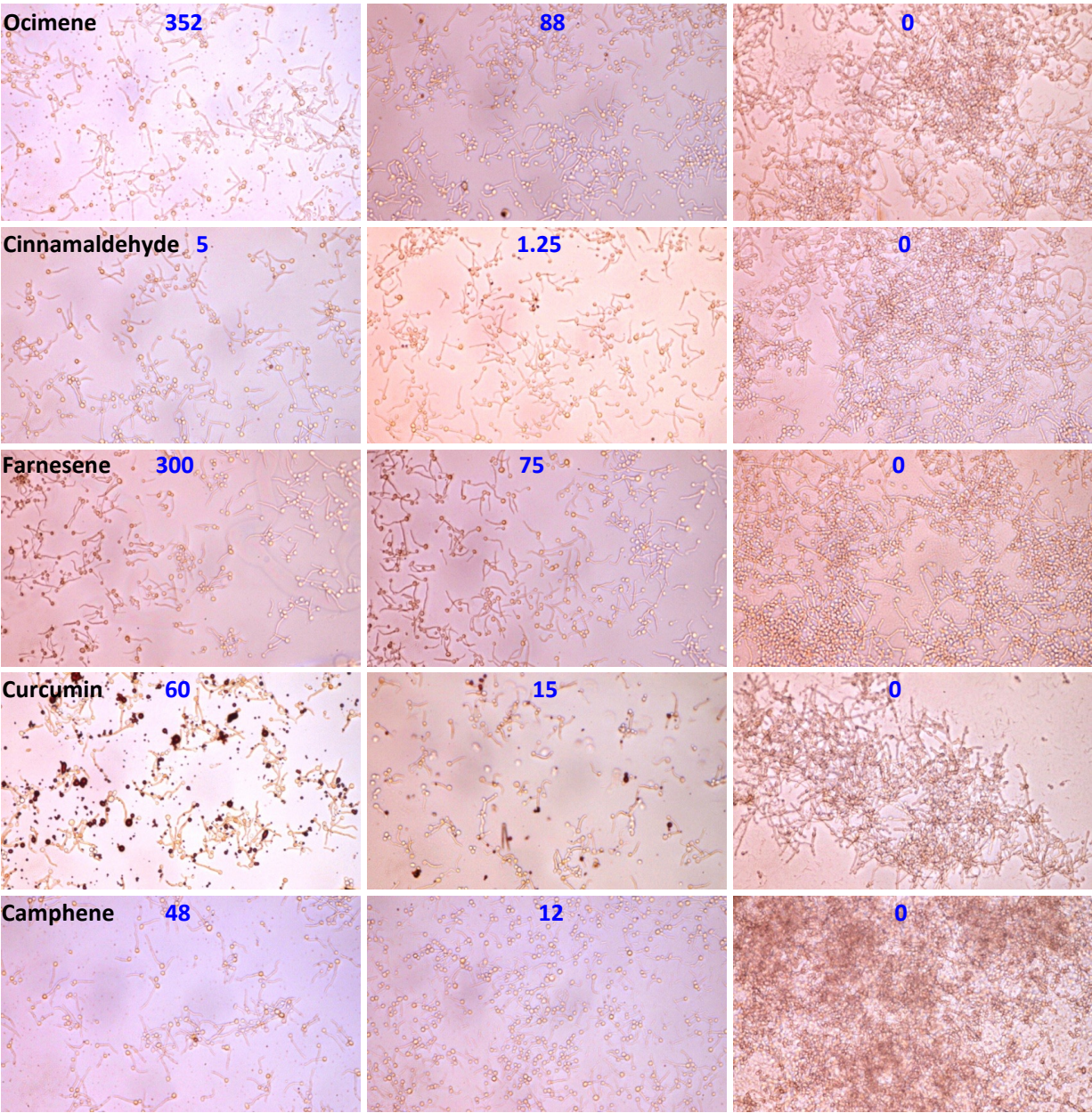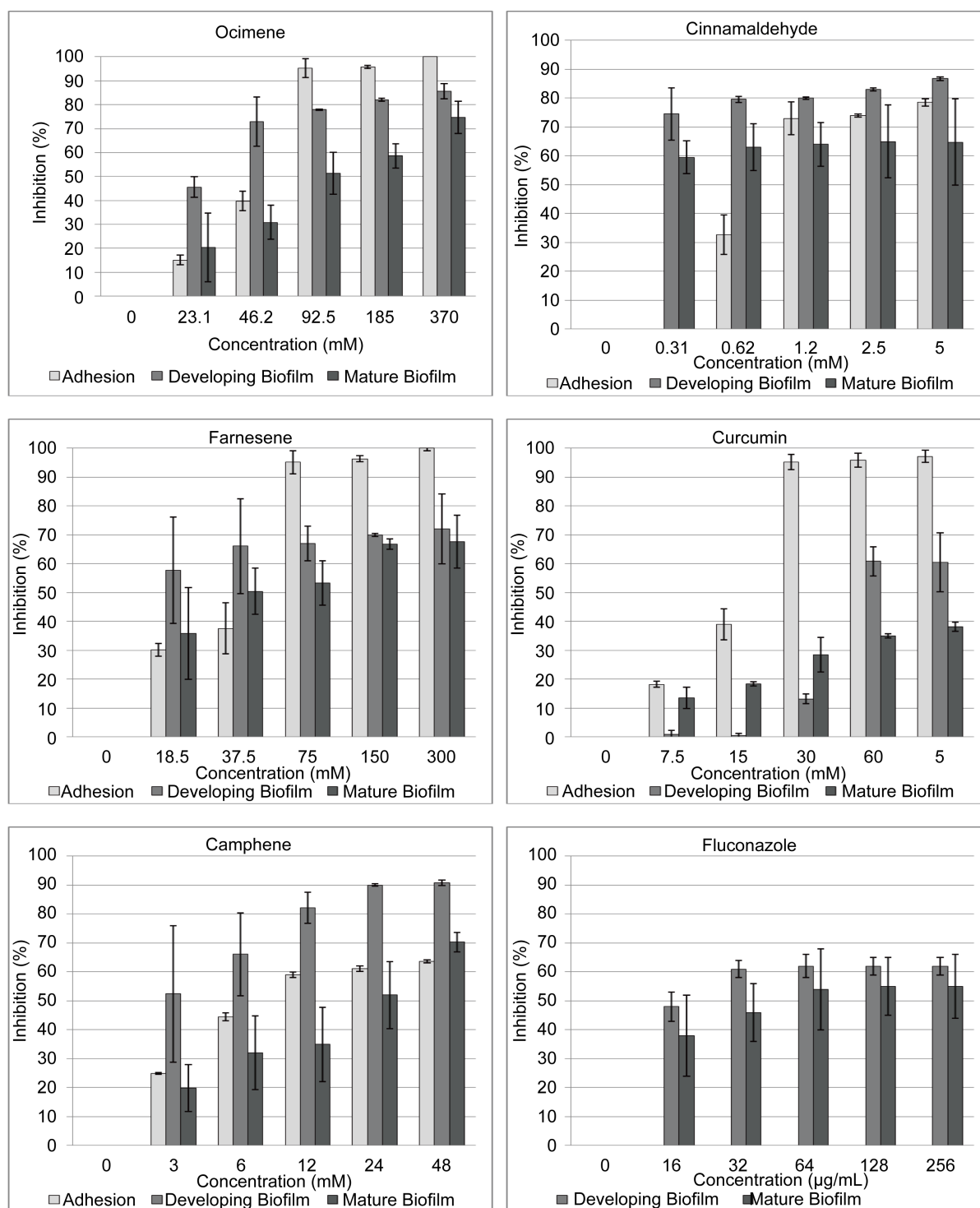| 5	10	20	40	80

Time (Min.)

(b)

(c)

(d)

(e)

Supplementary Figure 1. (a) Determination of MFCs; (b) Time dependant Killing Candida albicans inoculums by MFCs; (c) Effect of Terpenoids (concentrations in mM) on Adhesion of *Candida albicans* ATCC 10231. (Values indicate concentrations of plant molecule tested in mM; 0 mM represents respective controls); (d) Effect of Terpenoids (Concentrations in mM) on developing Biofilm of *Candida albicans* ATCC 10231. (Values indicate concentrations of plant molecule tested in mM; 0 mM represents respective controls); (e) Effect of Plant Molecules on Adhesion, Developing Biofilm and Mature Biofilm of a Fluconazole Resistant Strain of *Candida albicans* (ATCC 10231).