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Anti-candidal biofilm potential of solvent extracts of *Aeollanthus cucullathus* (Ryding) and its chemical analysis

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ABSTRACT

The antifungal drugs available and mostly used for the treatment of candidiasis are facing fungal resistance, toxicity phenomena and they highlight limited antifungal cell targets. The aim of this study is to assess the antibiofilm potential and to determine the chemical content of *Aeollanthus cucullathus*. The chemical composition was determined using gas chromatography coupled with mass spectrometry and the bioguided fractionation was performed through silica gel column chromatography. The inhibition parameters were determined by micro-dilution technique. The antibiofilm activity was evaluated using 24 wells macroplate antibiofilm inhibition assay. The interpretation of the biofilm inhibition was performed using an Ultra Violet-spectrophotometer and a Confocal Laser Scanning Microscopy for quantitative and qualitative biofilm analysis. The extraction of the secondary metabolites produced a yield ranging from 1.52 up to 11% respectively for organic solvent and water solvent extracts. The chemical analysis revealed the presence of four major compounds; tetratetracontane (17.02% and 28.72%), dodecane, 6-phenyl (10.49%) and 1,2-Benzenedicarboxylic acid, mono (2-ethyl) (7.36%). Tetratetracontane were found in Hexane and Hexane ethyl acetate extracts respectively with an amount of 17.02% and 28.71%. The minimum inhibitory concentrations ranged from 0.625 up to 5 mg/mL both for *Candida albicans* and *Candida glabrata*. The extract inhibited the biofilm formation up to 0.0625 mg/mL for *C. albicans* and up to 0.10 mg/mL against *C. glabrata*. Ascorbic acid (17%) and its derivatives (50.76%) were identified in *Aeollanthus cucullathus* fractions. The results obtained highlighted the preventive potential of *Aeollanthus cucullathus* against biofilm formation and the presence of potent biomolecules.

1. Introduction

Candida genus is an opportunistic microorganism, constituted by commensal yeasts which become pathogen when the biological equilibrium of the body is broken (Güdücüo et al., 2005). This is mainly due to the immunodepressed status of the target population, who are mainly represented by HIV/AIDS and cancer patients, those admitted to intensive care unit (ICU), submitted to transplantation or by those who possess a natural weak immune system (Dean and Burchard, 1996; Caugant and Sandven, 1993). *Candida* infection is an opportunistic disease and its rate is growing with the number of immunocompromised people around the world especially in Africa where HIV/AIDS infection percentage remains higher (Unaids and Who, 2009) than other world regions. Among *Candida* genus, many species are pathogenic. *Candida glabrata* (*C. glabrata*) and *Candida albicans* (*C. albicans*) are the most pathogenic ones. *C. albicans* causes 64% of infections, with a mortality burden exceeding 40% despite the use of antifungal drugs (Mensa et al., 2008). *C. glabrata* is the second human opportunistic and pathogenic yeast fungi. Although, it does not form any filaments, but can cause many infections (Flevari et al., 2013; Kucharikova et al., 2015). The main virulence attribute of *Candida* to resist to antifungal drugs is its property to form biofilms, densely

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packed communities of cells adhered to a surface. These biofilms are subsequently resistant to conventional antifungal therapeutics and to the host immune system defence (Gulati and Nobile, 2017). Further, host membranes are made up of lipids and proteins. These metabolites constitute also the target of some pathogenic enzymes attack. *Candida* secretes extracellular enzymes which are considered to be integral to their pathogenesis. These enzymes are categorized into two main types, proteases which hydrolyse peptide bonds, and phospholipases, which hydrolyse phospholipids. Secreted aspartyl proteases are produced during infection for digesting host proteins and host cells to acquire nutrients, spread within the host tissues and to avoid immune system response (Naglik et al., 2003). This enzyme produced by sap or yep gene expression, is the most virulent factor implied in the pathogenicity of the *Candida* strains (Shahla et al., 2016).

However, antifungal drugs that are available and mostly used for the treatment of these infections including amphotericin B as polyene, fluconazole as azole and the most recent class echinochandin mainly highlighted by caspofungin/amidulafungin (Ostrosky-zeichner et al., 2010) are already facing fungal resistance, toxicity phenomena and they highlight limited antifungal cell targets (Sanguinetti et al., 2015; Spampinato et al., 2013). That resistance issue, makes urge the investigation for new, effective and none toxic drugs. Medicinal plants could represent the best source for obtaining a wide variety of drugs most efficient and none toxic. More than 80% of the world's population depends on traditional medicine for primary health care (WHO, 2002). In some African countries, there is a considerable medicinal knowledge in herbal medicines to cure diseases. Moreover, *Aeollanthus* genus were traditionally used to treat some infectious diseases while highlighting in vitro antifungal potential (Bouquet, 1969; Gradé et al., 2009; Ngo Mback et al., 2016).

So, extracts from *Aeollanthus* sp. contain new biomolecules to face antibiofilm activity against *Candida albicans* and *Candida glabrata*. The main objective of this study is to determine the chemical content and the inhibitory effect of *Aeollanthus* plant extracts against *Candida genus* resistance factor. The step by step specific objectives are to: (1) screen the anticandidal activity against planktonic *Candida* cells (2) determine the antibiofilm potential of the active plant extracts and (3) perform a bio-guided fractionation followed by a chemical analysis using a GC-MS method.

2. Material and methods

2.1. Plant material and microbial strains

Plant samples were harvested from Cameroon regions during the annual second semester. The sample species were constituted of herbaceous plants in occurrence *Aeollanthus heliotropiodes* (*A. heliotropiodes*) and *Aeollanthus cucullathus* (*A. cucullathus*), with respective Voucher numbers 42756HNC and 24622SRFCAM by comparison to the previous recorded species. *Candida* strains and isolates were obtained from the Laboratory of Phytobiochemistry, University of Yaoundé I; Bei Resources and Laboratory of Microbiology, University of Bharathidasan. The antimicrobial tests were performed using Yeast Nitrogen Base (YNB), Sabouraud Dextrose Agar (SDA) and Sabouraud Dextrose Broth (SDB).

2.2. Plant secondary metabolites extraction

2.2.1. Aqueous extracts

Aeollanthus cucullathus and *Aeollanthus heliotropiodes* were harvested in the month of September during the raining season respectively in the Western and Centre regions of Cameroon. The extracts were extracted as described by Oliveira et al. (2013) with some modifications. The aerial parts were dried and crushed. The obtained dried material was macerated with distilled water during 3 days. Then, the filtrate was kept in − 80 °C during 12 h, and then lyophilised. The yields of extraction were calculated according the following formula:

$$\text{Yield of Extraction } (\%\ W/W) = ((\text{Weight of Extract}) / (\text{Total vegetal weight})) \times 100$$

2.2.2. Organic solvent extracts

The residual dried powder obtained from the previous aqueous extraction was then used for organic solvents extraction. The dried material was macerated respectively with hexane and hexane: ethyl acetate (70:30) during 3 days. Then, the filtrate was dried using soxhlet apparatus (Azwanida, 2015). The yields of extraction were calculated according the following formula:

$$\text{Yield of Extraction } (\%\ W/W) = ((\text{Weight of Extract}) / (\text{Total vegetal weight})) \times 100$$

2.3. Determination of MIC of plant extracts

The Minimum Inhibitory Concentrations (MIC) were determined as described by the Clinical Laboratory Standard Institute recommendations (CLSI, 2008). The Sabouraud Dextrose Broth (SDB) medium was poured in 96 wells microplate. Then, plant samples were serially diluted from 0.005 to 5 mg/mL and fluconazole from 0.006 to 6.25 mg/mL. A volume of *Candida* inoculum was added to reach a final concentration of 2.5×10^3 CFU/mL (Colony Forming Units) in each well. The inoculum solution was poured in the whole microplate except wells containing only SDB (blank). All experiments were performed in triplicate. The plates were incubated at 37 °C during 48 h. The lowest concentration at which no visible growth was observed was considered as the MIC. The extracts with the best activity were used for the inhibition of resistance factors.

2.3.1. In vitro antibiofilm activity of plant extracts

The effect of *Aeollanthus* extracts at sub inhibitory concentrations of 1/2, 1/6 and 1/10 MIC was assessed against biofilm-forming ability using macroplate biofilm assay as described with some modifications by Lewisoscar et al. (2015). Briefly, 30 µL (10^6 CFU/mL) of a *Candida* inoculum was added into 500 µL of fresh Yeast Nitrogen Base (YNB) supplemented with 5 g of glucose and cultivated in the presence and absence of plant extracts for 72 h at 37 °C. The wells containing YNB + cells served as control. After incubation, the wells were washed with distillated water to remove planktonic cells. The remaining yeast cells were subsequently stained with 0.1% of crystal violet solution for 20 min at room temperature. After staining, the cells were washed with distilled water to remove crystal violet solution. The plates were dried at room temperature for 2 h. Then, a volume of 1 mL of 33% glacial acetic acid was added into the wells. After 20 min, the optical density (OD) of each well content was read at a wavelength of 590 nm using a Thermal Scientific Multiskan FC (Vantaa, Finland). The proportional optical densities were recorded at 590 nm and the inhibition percentages were calculated according the following equation:

$$\text{Biofilm Inhibition } (\%) = ((\text{ODcontrol} - \text{ODtest})/ (\text{ODtest})) \times 100$$

For the microscopic analysis using Confocal Laser Scanning Microscopy (CLSM) and fluorochrome microscope, the same experiment were repeated. Glass slides were added in each well. After incubation, the slides were removed, washed with distilled water to discard planktonic cells. Then, the slides were air dried. All the experiments were performed in triplicate. Acridine orange (0.01%) and crystal violet (0.1%) staining were separately developed. Then, acridine slides were observed by CLSM.

2.3.2. Bioguided fractionation of essential oils by silica gel column chromatography

A. cucullathus extract (9 g) was further submitted to a fractionation process by silica gel column chromatography (Zeuko´o et al., 2016).

The sample were dissolved in hexane (Hex)/Ethyl acetate (Ea) (70:30), then mixed with an equivalent quantity of silica powder then dried at room temperature. The column was mounted and saturated with hexane/Ethyl acetate/methylene chloride (Mc) (98:1:1). The dried mixture was poured in the column and eluted with 11 solvent-systems: Hex/Ea/Mc 98:1:1, Hex/Ea/Mc 97:2:1, Hex/Ea/Mc 93:5:2, Hex/Ea/Mc 88:7:5, Hex/Ea/Mc 85:10:5, Hex/Ea/Mc 80:15:5, Hex/Ea/Mc 75:15:10, Hex/Ea/Mc 65:20:15, Mc 100%, Ea 100%, Methanol (MeOH) 100%). The fractions obtained were collected. Then, the thin layer chromatography (TLC) followed by visualization successively with sulphuric acid, heat and Ultra Violet (UV) light was applied for the identification of spots for grouping collected fractions. Fractions with similar phytochemical profiles were pooled, grouped, and dried. The major fractions were analyzed by GC/MS to identify biomolecules. Finally, these fractions were screened for their anti-candidal activity using the protocol mentioned above.

2.4. Chemical analysis by GC-MS

The identification of extract components was performed by gas chromatography coupled with mass spectrometry (GC-MS) using Shimadzu equipment, MEGCs QP-2010 (Shimadzu Corporation, Japan). System control and data acquisition were achieved with an enhanced GC–MS Solutions 2.5 (Shimadzu Corporation, Japan). One microlitre of the derivatized sample extract was injected using auto injector (AOC-20i) and the separation was achieved by RTXs – PCB fused silica capillary column (60 m 0.25 mm i.d., 0.25 mm film thickness) (Restek, Pennsylvania, USA). The injector temperature was 220 °C, the temperature detector or the interface (GC-MS) was 240 °C. The initial column temperature was 60 °C, followed by an increase of 3 °C/min. up to 240 °C and maintained constant for 30 min. The mass spectrometer was programmed to perform readings in a range of 29–400 Da 0.5 s, intervals with ionization energy of 70 eV. It was injected 1 µL of each sample at a concentration of 10,000 ppm dissolved in hexane. The compounds were identified based on retention time (RT) and mass spectra obtained from full scan acquisition mode (m/z 50–500).

2.5. Statistical analysis

The results were statistically analyzed by ANOVA and Least Sensitive Difference (LSD) with Fisher test at 5% threshold probability factor. Excel was used for drawing curves, calculation of averages and standard deviations.

3. Results and discussions

3.1. Extraction yield of crude extracts

A total of four (04) extracts were obtained from plants and their extraction yields were presented in the following Table 1. The two aqueous extracts and two organic solvent extracts (hexane 100% and Hexane:Ethyl acetate 70:30) were used for the study. The aqueous extracts exhibited the best extraction yields ranging from 6.4% to 11%; compared to the organic solvent extracts where the yield interval ranged from 1.52% to 1.86%.

Table 1
Extraction yield.

Aqueous/solvent plant extracts (g/100 mL)	Yield (% W/W)
Aeollanthus heliotropioides (E)	6.4
Aeollanthus cucullathus (F)	11
Aeollanthus cucullathus (G)	1.86
Aeollanthus cucullathus (H)	1.52

E: Aqueous extract of *Aeollanthus heliotropioides*; F: Aqueous extract of *Aeollanthus cucullathus*; G: Hexane extract of *Aeollanthus cucullathus*; H: Hexane/Ethyl acetate (70/30) of *Aeollanthus cucullathus*; W/W: Weight/Weight.

Table 2
Minimum inhibitory concentration values.

Plant extracts	MIC(mg/mL)		
	C. albicans ATCC	*C. glabrata* (isolate)	*C. albicans* (isolate)
A. cucullathus (H)	0.625 ± 0.00	0.625 ± 0.00	0.625 ± 0.00
A. cucullathus (G)	5.00 ± 0.00	> 5.00	> 5.00
A. cucullathus (F)	> 5.00	5.00 ± 0.00	> 5.00
A. heliotropioides (E)	> 5.00	> 5.00	> 5.00

ATCC: American type collection culture; C.: *Candida*.

It was observed that polar solvents are more potent, because the extraction led to a large extraction yield up to 11%. The use of water as the extraction solvent exhibited higher values of the extraction yields for sample E (6%) and F (11%). In that particular case, some studies were performed. The extraction yields of polar solvents were greater than those with non-polar as hexane and ethyl acetate (Dhawan and Gupta, 2016). Particularly, water extraction could lead to even more secondary metabolites; as shown by the research results obtained for *Orthosiphon stamineus* leaves (Khan et al., 2014). The results also revealed that *Aeollanthus* plants are richer in polar secondary metabolites, while the activity is not always related to the extraction yield (Njume et al., 2011).

3.2. Minimum inhibitory concentration (MIC) values

It is observed that organic solvent extracts have inhibited the fungal growth at a range of concentrations ranging from 5 up to 0.625 mg/mL (Table 2). Hexane ethyl acetate extracts were more potent (with 0.625 mg/mL as MIC) than the other ones. Both *Candida albicans* and *Candida glabrata* expressed the same response when exposed to Hexane ethyl acetate extracts.

The activity of Hexane ethyl acetate extracts could be explained by the inhibition of some common *Candida* cell targets. *Candida albicans* and *Candida glabrata* share almost the same biological structures excepted at small differencies. They are constituted by similar basic cellular structures. The most usual *Candida* targets are sterols from membrane and β-glucans from the wall. In these cases the antimicrobial agents could bind ergosterol (polyenes), inhibit its biosynthesis (Azoles) or inhibit β-glucans synthesis (echinocandins). However, there are many other antifungal groups. But, these ones are the most commonly used among patients with candidiasis. However, these latter antifungals are tolerated by patients even at higher doses (Pea and Lewis, 2018). Then, this fact constitutes a leading cause to anticandidal resistance. In the best of our knowledge, there are no studies involving *A. cucullathus* extracts against pathogenic *Candida* strains, while some works were investigated on some other plant species within the same genus. They highlighted similar anticandical activity tested on *Cryptococcus neoformans* and *Candida* species. It is the case of *A. heliotropioides* (Ngo Mback et al., 2016). Antibacterial properties were also reported with MIC of 50 mg/mL and 100 mg/mL against *Escherichia coli* and *Salmonella* sp. (Martins et al., 2016). These last antibacterial results have revealed a better susceptibility of yeasts, than bacteria species. The observations suggest that the plant would be more specific to yeasts pathogenic cell targets than the common bacteria.

3.3. Determination of chemical composition of active extracts

The most active plant extracts were analyzed by gas chromatography coupled with mass spectrometry (GC/MS), to identify their qualitative and quantitative chemical composition. The GC/MS analysis revealed the presence 39 and 44 compounds respectively for H and G from *Aeollanthus cucullathus* (Table 3).

Table 3
Chemical composition of *Aeollanthus cucullathus* crude extracts.

N°	Compound names	Retention time	Relative quantity (area %)	
			A.cucullathus (G)	*A.cucullathus (H)*
1	(5-Decyl)benzene	9.15	0.92	–
2	1-Propylheptyl)benzene	9.3	0.35	–
3	Decane	9.62	2.47	–
4	Decane, 3-phenyl	9.62	–	0.62
5	Methyl (Z) – 5,11,14,17-Eicosatetraenoate	10.02	1.54	–
6	Trichloroacetic acid, tridec – 2-vynyl ester	10.02	–	0.49
7	5-Eicosene, (E)	10.12	–	1.9
8	Benzene, (1,3,3-trimethylnonyl)	10.26	–	0.86
9	Benzene, (1-butylheptyl)	10.88	–	3.33
10	Undecane	10.88	6.61	–
11	Benzene, (1-propyloctyl)	11.05	3.86	–
12	Undecane, 4-phenyl	11.05	–	1.78
13	Guaia – 1(5),11-diene	11.26	0.25	–
14	Benzene, (1-ethylnonyl)	11.41	3.314	–
15	Undecane, 3-phenyl	11.41	–	1.24
16	Undecane, 2-phenyl	12.05	–	2.16
17	Benzene, (1-methyldecyl)	12.06	4.87	–
18	10-Methylnonadecane	12.22	–	0.73
19	Octadecane, 1-chloro	12.23	0.17	–
20	Dodecane, 6-phenyl	12.53	10.49	3.01
21	Dodecane, 4-phenyl	12.81	4	1.26
22	Decane, 5-ethyl – 5-methyl	12.97	0.14	–
23	Dodecane, 3-phenyl	13.18	4.05	2.02
24	9-Octadecene, (E)	13.63	0.12	–
25	Benzene, (1-methylundecyl)	13.83	–	1.5
26	Dodecane, 2-phenyl	13.83	4.62	–
27	cis – 1-Chloro – 9-octadecene	14	0.17	–
28	Benzene, (1-pentyloctyl)	14.21	–	2.08
29	Tridecane, 6-phenyl	14.21	6.36	–
30	Tridecane, 5-phenyl	14.32	3.84	1.19
31	Tridecane, 4-phenyl	14.53	6.84	3.25
32	Tridecane, 3-phenyl	14.91	3.66	1.33
33	9-Eicosyne	15.13	1.27	–
34	Dodecane, 2-bromo	15.42	0.16	–
35	Tridecane, 2-phenyl	15.55	3.47	1.11
36	1-Oxa-spiro[4.5]deca6,9-diene – 2,8-dione,	15.66	0.39	–
37	14-Methyl – 8-hexadecenal Z	15.74	0.23	–
38	Disulfide, di-tert-dodecyl	15.82	–	1.11
39	Undecanoic acid, 10-methyl-, methyl ester	15.82	0.51	–
40	(1,2,2-Trimethylbutyl)cyclohexane	16.19	0.08	–
41	l-(+)-Ascorbic acid 2,6-dihexadecanoate	16.5	–	2.35
42	Crocetane	16.5	0.35	0.96
43	Ethyl docosanoate	16.92	–	1.39
44	Heptadecane, 2,6,10,15-tetramethyl	17.02	0.37	0.42
45	Tetradecanal	17.31	0.19	–
46	Cyclohexadecane	18.33	0.25	–
47	Heptadecane	18.56	1.48	–
48	Phytol	18.77	2.26	1.27
49	Cyclopentadecanone, 4-methyl	19.17	0.53	–
50	Linoleic acid ethyl ester	19.45	–	0.51
51	cis,cis,cis – 7,10,13-Hexadecatrienal	19.55	–	1.49
52	Hexadecane, 1-iodo	19.76	0.16	–
53	Tetratetracontane	20.05	17.02	28.71
54	alpha.-L-Galactopyranoside, methyl 6-deo	20.31	0.19	–
55	E – 10-Methyl – 11-tetradecen – 1-ol propionate	20.32	–	0.35
56	3-[(1S,2S) – 2-(2-Hydroxyethyl) – 1,3,3-trimet	20.53	–	2.87
57	Z – 7-Pentadecenol	20.86	–	4.4
58	5,19-Cyclo – 5.beta.-androst – 6-ene – 3,17-dione	20.95	0.1	–
59	2-(3-Hydroxy-propyl)-cyclohexanol	21.12	–	0.8
60	5,6,7,8-Tetrahydrofurazano[4,5-c]azepin – 4-	22.02	–	2.91
61	5-Methyl – 5-(4,8,12-trimethyltridecyl)dihydro	22.15	0.82	–
62	4,8,12,16-Tetramethylheptadecan – 4-olide	22.15	–	0.5
63	Azulene, 1,2,3,5,6,7,8,8a-octahydro – 1,4-dimethy	22.63	0.56	–
64	2H-Pyran, 2-(7 heptadecynyloxy)tetrahydro	23.22	0.25	–
65	1,1-Dimethyl – 4a-hydroxymethyldecalin – 2-o	23.38	–	0.4
66	Docosane, 7-hexyl	23.66	0.51	–
67	Tritetracontane	23.79	0.17	0.88
68	1,2-Benzenedicarboxylic acid, mono(2-ethyl)	24.72	–	7.36
69	Pimar – 15-ene – 8,11-diol	24.9	–	5.63
70	Spongestrin	27.12	–	5.24

(*continued on next page*)

Table 3 (*continued*)

N°	Compound names	Retention time	Relative quantity (area %)	
			A.cucullathus (G)	*A.cucullathus (H)*
71	Spinacen	28.11	–	0.63

G and H: Hexane and Hexane ethyl acetate extract from *A. cucullathus* respectively..

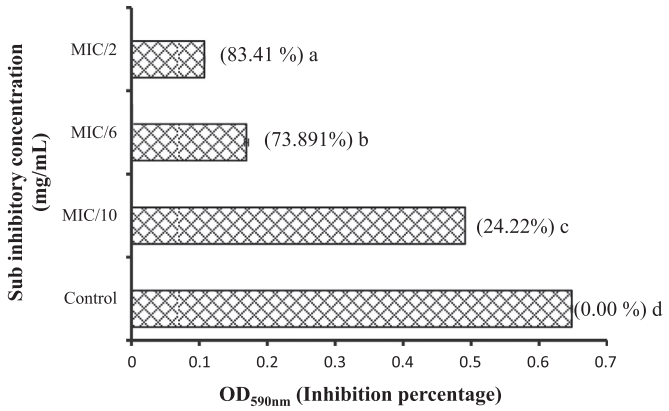

Fig. 1. Inhibition of *C. Albicans* biofilm formation by *A. cuculathus* Hexane-Ethyl acetate extracts. MIC: Minimum Inhibitory Concentration; [Number %]: Biofilm formation inhibition percentage. Bar charts bearing different alphabet are significantly different ($p = 0.05$) as referred to the Least Significance Difference (LSD) based multiple comparison test.

From the above table, it is shown that more than 60 volatile compounds were identified. The compounds are constituted by long alkane molecule chains as well as short ones. Hydrocarbons, cyclohexane, cyclopentane, alcohols, esters, sulfur-containing compounds and benzene derivatives were also present. Most of these compounds were not yet reported previously in the chemical content of *Aeollanthus cucullathus* extracts. The results revealed that four (04) compounds were found as major constituents. Tetratetracontane (17.02% and 28.72%), Dodecane, 6-phenyl (10.49%) and 1,2-Benzenedicarboxylic acid, mono (2-ethyl) (7.36%). Tetratetracontane were found in both extracts, G and H respectively with an amount of 17.02% and 28.71%. It is observed that the main two compounds of *A. cucullathus* Hexane extract were highlighted in *A. cucullathus* Hexane ethyl acetate extract. However, the second major compound of *A. cucullathus* Hexane ethyl acetate extract, 1,2-Benzenedicarboxylic acid is not present in *A. cucullathus* Hexane extract. In term of chemical composition similarities, the samples share almost the same compounds with different proportions. The chemical content differences could justify the best antimicrobial activity exhibited by these plant extracts. Moreover, the presence of acid ascorbic derivatives, l-(+)-Ascorbic acid 2,6-dihexadecanoate (2.35%) in *A. cucullathus* Hexane ethyl acetate extract, can suggest a possible antioxidant activity of *Aeollanthus cucullathus*.

The antimicrobial results revealed *A. cucullathus* Hexane Ethyl acetate extract as the best extract, with the best anticandidal activity (MIC = 0.625 mg/mL). However, *A. cucullathus* Hexane extract remained less active, despite the presence of similar compounds as the ones found in *A. cucullathus* Hexane ethyl acetate extract. The high amount of tetratetracontane (28.71%) in *A. cucullathus* Hexane ethyl acetate extract could be the reason of a better anticandidal activity than *A. cucullathus* Hexane extract. Tetratetracontane is a long chain alkane molecule ($C_{44}H_{90}$). It was already proved that tetratetracontane could exhibit antimicrobial activity and cytoprotective activity (Ertas et al., 2015; Kanimozhi and Ratha bai, 2012). Tetratetracontane was also found in lipid extract from brown and red algae. It constitutes a substitute to polyunsaturated fatty acids (PUFAs) as food additives in replacement of toxic PUFAs (Kumar et al., 2017). Moreover, from active extracts of *Citrus limonum* against cholesterase activity, tetratetracontane was found as one of the major compounds in a percentage of 18.41% (Castro et al., 2016). On the same way, some scientists showed that *Lycopsis orientalis* essential oils exhibited an activity against acetyl- and butyryl-cholinesterase enzymes. They obtained from these extracts a moderate inhibitory activity up to 0.2 mg/mL with a tetratetracontane content of 9.4% (Ertaş et al., 2014). These results may suggest the possible involvement of tetratetracontane in anti-alzheimer activity. More, it is known that lipase and phospholipase extracellular enzymes from *Candida* play an important role as virulence factors in the pathogenicity development. The eventual activity of tetratetracontane against some enzymes could be used as a starting point for the research of new antifungals targeting *Candida* pathogenic hydrolytic enzymes.

3.4. Antibiofilm activity against Candida species

The antibiofilm activity of the most active sample (*A. cucullathus* Hexane Ethyl acetate extract), was expressed in percentage of inhibition (%). The following histogram figures represent the inhibition potential of *Aeollanthus cucullathus* extracts at different sub inhibitory concentrations ½ MIC (0.312 mg/mL), 1/6 MIC (0.104 mg/mL), and 1/10 MIC (0.0625 mg/mL). In the case of *Candida albicans*, all the tested sub inhibitory concentrations led to the biofilm formation reduction. Even up to the lowest sub inhibitory concentration MIC/10 (0.062 mg/mL), an inhibition of the biofilm formation was recorded. ½ MIC and 1/6 MIC allow respectively a six times and three times *C. albicans* biofilm formation reduction. The results showed the potent antibiofilm activity of *Aeollanthus cucullathus* extracts at the tested concentrations.

From the previous results (Fig. 1.), the antifungal response of *C. albicans* planktonic cells is ten times less than the antibiofilm response of *Candida* sessile cells. These observations suggested that an antifungal agent could fail to inhibit the pathogen in its growth, but could stop or decrease its resistance, by increasing its susceptibility towards antifungals. Also, the ten times reduced MIC from 0.625 mg/mL up to 0.0625 mg/mL respectively against single cells and biofilm development, suggests that *Aeollanthus cucullathus* may not only act on common cell targets which are membrane, wall or physiological cell structures of the planktonic yeast. But, the interest extract could also inhibit other cell features involved during biofilm formation. For example, the genes expression in the case of quorum sensing as a key role in biofilm formation. *Candida albicans* biofilm formation is genetically managed by more than 50 transcriptional regulators. The proteins responsible are a 'core' set of nine regulators (Ndt80, Bcr1, Rfx2, Flo8, Rob1, Brg1, Gal4, Tec1 and Efg1) that is required for biofilm formation (Fox et al., 2015; Nobile and Johnson, 2016). Some researchers have shown that active compounds could inhibit biofilm Efg1 gene expression by interfering at the level of morphogenesis switching through the Ras1-cAMP-Efg1 pathway (Morici et al., 2016).

The same experiment has also been performed for the inhibition of *C. glabrata* biofilm formation (Fig. 2.). A less antibiofilm activity was observed with *C. glabrata* compare to *C. albicans*. *Aeollanthus cucullathus* extracts exhibited an inhibition rate of 1.89 up to 30% respectively at 1/10 MIC (0.0625 mg/mL) and At ½ MIC (0.312 mg/mL). *C. glabrata* on the opposite of *C. albicans* is a non-dimorphic yeast. It means that it cannot produce filaments. Its biofilm is constituted by an assemblage of planktonic cells gathering inside a matrix, with the absence of hyphae. This difference is important in the way to try to find an explanation

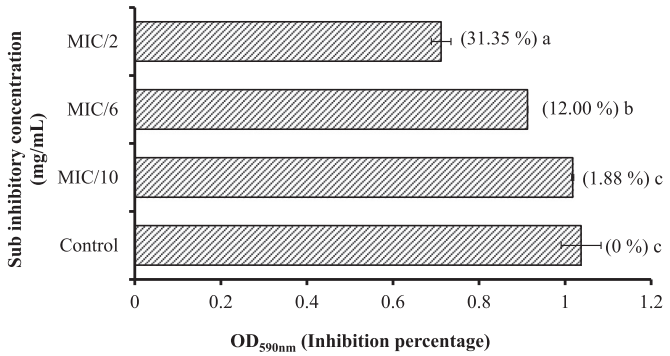

Fig. 2. Inhibition of *C. glabrata* biofilm formation by *A. cuculathus* Hexane-Ethyl acetate extracts. MIC: Minimum Inhibitory Concentration; [Number %]: Biofilm formation inhibition percentage. Bar charts bearing different alphabet are significantly different (p = 0.05) as referred to the Least Significance Difference (LSD) based multiple comparison test.

over the antibiofilm susceptibility of one pathogen to another one.

Many researchers have shown that, *C. glabrata* is the second opportunistic and pathogenic yeast after *C. albicans* (Fidel and Vazquez, 1999; Pfaller et al., 2014). It is also worth to mention that antifungal drugs tolerance was obtained towards *C. glabrata* when it is treated with azoles. This tolerance is due to the altered sterols within the cell membrane. This membrane change allows the fungi evasion from antifungal treatments specifically azoles. Moreover, *C. glabrata* has the ability to take up exogenous sterols, both when the ergosterol biosynthesis pathway is blocked and when under normal conditions (Nakayama et al., 2007). This escape ability expressed by *C. glabrata* in some cases could make it more resistant to the common antimicrobials than the other *Candida* species. The particularity in the antifungal resistance mechanism commonly adopted by *C. glabrata* could be explained by its less antibiofilm susceptibility towards the tested *Aeollanthus* extracts compared to *C. albicans*.

From the microscopic pictures below (Fig. 3), the biofilm formations were inhibited, as confirmed with the above antibiofilm activity highlighted by optical densities results. Fig. 3 depicted the first four images of *C. albicans* biofilm. By comparison to the control, it is observed some changes in the biofilm morphology. Image A represents the assay without any antifungals. At that level, the biofilm is well structured (long filaments and sessile cells). Pictures B, C, D, are those treated with *A. cucullathus* Hexane Ethyl acetate extract respectively at MIC/10 (0.0625 mg/mL), MIC/6 (0.10 mg/mL) and MIC/2 (0.312 mg/mL).

All these sub inhibitory concentrations inhibited hyphae formation. Even at MIC/10, no filaments were observed, but at this concentration some biofilm formation stages were completed. Here at MIC/10 of *A. cucullathus* Hexane-Ethyl acetate extract, cells are well packed, adhered together inside the matrix but without any forms of pseudo hyphae or filaments. At MIC/6 and MIC/2 it clearly observed an inhibition of the first stage of biofilm formation. So, the cell adherence was inhibited by the treatment. Here, no well-structured biofilm was identified. Almost all the biofilm features are inhibited. *A. cucullathus* could inhibit filamentation up to 0.062 mg/mL and could also inhibit the adherence which is the first biofilm formation stage, at 0.10 mg/mL. Further, one important feature in the biofilm structure, is its thickness. From the CLSM analysis thickness changes were highlighted. In the control assay the thickness and the intensity (transmitted from the integrated acridine orange dye by cells) were respectively $T_A = 5.14$ µm and $I_A = 51.4$. When treated with 0.0625 mg/mL, *C. albicans* biofilm thickness and intensity have decreased up to $T_B = 4$ µm and $I_B = 36.7$. The interest extract has also modified the thickness of the biofilm as well as its development.

Concerning *C. glabrata*, the biofilm is less diffuse compared to *C. albicans* biofilm. But, according the strains used in the present study, CLSM analysis revealed that *C. glabrata* biofilm possesses a greater thickness ($T_B = 4$ µm) than *C. albicans*. Also, its biofilm cells stained with acridine orange would be able to transmit a higher intensity signal ($I_B = 36.7$). From the treatments at respective sub inhibitory concentrations, there is no significant difference between control assay (A′) and the treated assay (B′) at MIC/10. However, a small reduction of the biofilm formation is observed when treated with the extract at MIC/6 (C′) and MIC/2 (D′). Here, clumped cells were decreased compare to the control (A′) and the assay at MIC/10 (B′).

In the best of our knowledge, it is the first time that *A. cucullathus* organic solvent extracts were analyzed for its chemical content and antibiofilm activity determination. The plant extract is mostly constituted by n-alkanes, mainly tetratracontane ($C_{44}H_{90}$) with a percentage of 28.71%. However, no reports have been yet published about the anti-candidal biofilm of tetratetracontane. Further, alkane compounds are also present in essential oils (Fafal et al., 2016) and they are sharing the same apolar properties as tetratetracontane. Essential oils were also extracted using solvents as hexane, suggesting that essential oils could be present in sample H. Many research works were focused on the antibiofilm activity of essential oils against *Candida* species. These statements could also justify the antibiofilm activity of the interest *A. cucullathus* Hexane-Ethyl acetate extract. The essential oils of *Ocimum*, *Mentha* and *Rosmarinus* belonging the same *Lamiaceae* family as *Aeollanthus*, were widely reported for their antibiofilm activities against fungi (Nazzaro et al., 2017). The antibiofilm mechanism of action of compounds from essential oils is diverse. They can act on different biofilm targets, by downregulating the expression levels of the adhesin genes HWP1 and ALS3; and can suppress the expression of CYR1 and CPH1 which encode components of the cAMP-PKA and MAPK hyphal formation regulatory pathways (Hsu et al., 2012).

3.5. Bio-guided fractionation of *A. cucullathus* hexane ethyl acetate extracts and chemical analysis

The most active sample against *Candida* species were submitted to a chemical process of separation by column chromatography. The Table 4 below highlights the fractionation yields of *A. cucullathus* Hexane Ethyl acetate extract.

From the separation of *A. cucullathus* Hexane Ethyl acetate extract, 210 fractions were obtained. They were grouped based on their Thin Layer Chromatography (TLC) profile similarities in a total of 32 fractions from A up to Z6. Three major fractions respectively C (12.82%), D (27.32%) and F (7.22%) (C: 4; D: 5–7; F: 9–12) were obtained. Each fraction yield was recorded (Table 4). Five (05) fractions were obtained in traces, leading to a total of 27 remaining fractions, which could be really exploited. The three major fractions C, D and F were analyzed by GC-MS to determine their chemical profile.

The chemical analysis was performed for the major fractions C, D and F and the results were collected, integrated and presented in the following Table 5.

According the above results 71 compounds were isolated from the three major fractions C, D and F. Five (05) major compounds were identified; tetratetracontane (31.28%) in fraction C, tetratetracontane (26.63%), 1,2-benzendicarboxylic acid,mono (2-ethyl) (14.06%) in fraction D and l-(+)-Ascorbic acid 2,6-dihexadecanoate (50.76%), L-Ascorbic acid (17.44%) in fraction F. The presence of Longiverbenone (0.28%) found in a small amount only in F fraction as uncommon compound was noticed. All the main components identified were from alkanes, carboxylic acids, acids and ester natures. The chemical composition of fractions C and D is related to its crude *A. cucullathus* Hexane Ethyl acetate extract, and with some similarities observed in *A. cucullathus* Hexane extract; through the presence of tetratetracontane which is also present as a major compound in both crude extracts. However, some particularities can be raised. The presence of Vitamine C and its derivatives - l-(+)-Ascorbic acid 2,6-dihexadecanoate (50.76%) and L-Ascorbic acid (17.44%) in fraction F. Also, l-(+)-Ascorbic acid 2,6-dihexadecanoate was identified at high

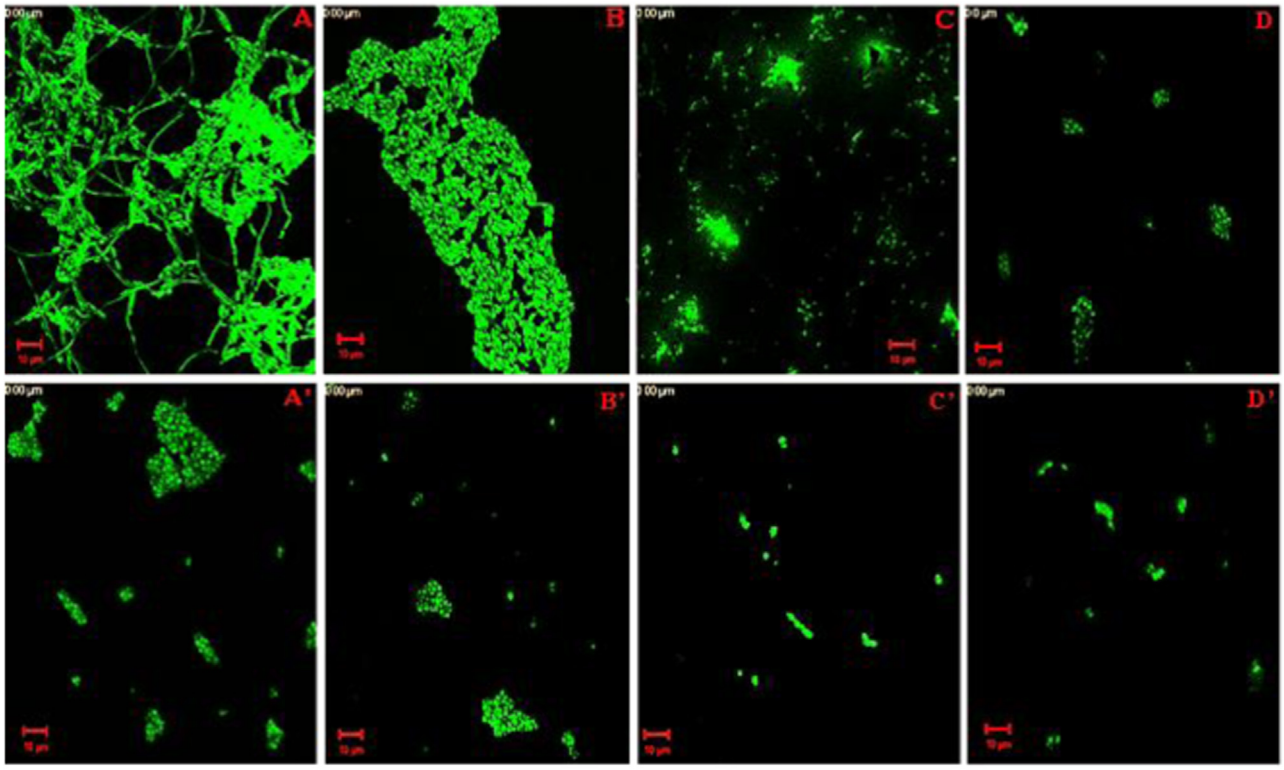

Fig. 3. CLSM imaging of treated and untreated *C. albicans* and *C. glabrata* biofilms. A: *C. albicans* Control; B: assay at MIC/10; C: assay at MIC/6; D:assayt at MIC/2; T: Thickness; I: Intensity; T_A = 5.14 µm/I_A = 51.4; T_B = 4 µm/I_B = 36.7 A′: *C. glabrata* Control; B′: assay at MIC/10; C′: assay at MIC/6; D′: assay at MIC/2; T: Thickness; I: Intensity; $T_{A'}$ = 6.77 µm/$I_{A'}$ = 10.63;$T_{B'}$ = 2.83 µm/$I_{B'}$ = 2.

Table 4
Yield of fractionation.

A. cucullathus Hexane Ethyl acetate extract	Extraction yield (%)
Fraction A	0.22
Fraction B	1.78
Fraction C	12.82
Fraction D	27.32
Fraction E	3.00
Fraction F	7.22
Fraction G	1.78
Fraction H	1.33
Fraction I	0.89
Fraction J	0.78
Fraction K	0.67
Fraction L	1.89
Fraction M	0.22
Fraction N	0.44
Fraction O	0.22
Fraction P	Traces
Fraction Q	Traces
Fraction R	0.78
Fraction S	0.11
Fraction T	0.22
Fraction U	Traces
Fraction V	Traces
Fraction W	Traces
Fraction X	0.22
Fraction Y	1.33
Fraction Z	1.11
Fraction Z1	5.44
Fraction Z2	1.56
Fraction Z3	0.78
Fraction Z4	0.44
Fraction Z5	1.11
Fraction Z6	1.00

proportions. They were also identified in the initial crude extracts but in a small amount.

Probably, some isomers from the crude extract would have been being converted into ascorbic acid and its analogues during the fractionation process. This kind of chemical composition changes observed could be the result of many molecular interactions and re-arranging chemical processes within a mixture. In fact, temperatures, solvents and others chemicals involved during the fractionation process could allow interactions between compounds and extrinsic factors, leading to chemical structure changes (Gokhale et al., 1994). These changes could lead to the production of artefacts; which can be in this case ascorbic acid and derivatives. Ascorbic acid possesses many applications in the field of pharmacy, medicine, food industry and cosmetics (Gorkom et al., 2018; Soto et al., 2018; Taira et al., 2018; Varvara et al., 2016). The presence of ascorbic acid in *A. cucullathus* fractions could suggest its application as antioxidant in the field of food protective agent or in the field anti-aging substances. Ascorbic acid could exhibit antibiofilm activity against multiresistant microbial strains by reducing the biofilm formation at sub inhibitory concentrations up to 4 µg/mL (Mirani et al., 2018). The chemical content of *Aeollanthus cucullathus* could be further exploited in the field of drugs discovery, specifically for anti-infective, antibiofilm and antioxidant researches.

4. Conclusion

The above research works highlighted the antibiofilm potential of *A. cucullathus* extracts and its chemical content. The results suggested that the plant inhibits *C. albicans* biofilm by blocking the filamentation process and by reducing the biofilm thickness. The major compounds detected in *A. cucullathus* were mainly constituted of alkanes, carboxylic acids, acids and esters. Among them, the presence of tetratetracontane, ascorbic acid and their derivatives as major compounds were identified. Further antimicrobial studies should be considered in

Table 5
Chemical composition of *A. cucullathus* fractions.

N°	Compound names	R. Time	Relative quantity (Area %)		
			Fraction C	Fraction D	Fraction F
1	2,4-di-*t*-Butylphenol	8.66	–	–	0.87
2	Decane, 5-phenyl	9.15	–	0.58	–
3	Decane, 4-phenyl	9.3	–	0.37	–
4	Decane, 3-phenyl	9.62	–	0.6	–
5	Methyl (Z) − 5,11,14,17-eicosatetraenoate	10	–	–	0.45
6	5-Eicosene, (E)	10.13	5.9	1.76	–
7	Benzene, (1,3,3-trimethylnonyl)	10.26	1.26	–	–
8	Decane, 2-phenyl	10.27	–	0.88	–
9	Undecane	10.86	–	–	0.99
10	Undecane, 5-phenyl	10.88	5.4	3.92	–
11	Naphthalene	11.01	–	–	1.02
12	Benzene, (1-propyloctyl)	11.06	2.43	–	–
13	Undecane, 4-phenyl	11.06	–	1.89	–
14	Undecane, 3-phenyl	11.41	2.42	1.79	–
15	Undecane, 2-phenyl	12.06	4.22	2.74	–
16	Crocetane	12.23	7.98	1.09	–
17	Tridecane	12.51	–	–	1.13
18	Dodecane, 6-phenyl	12.54	2.89	–	–
19	Dodecane, 5-phenyl	12.62	3.11	4.87	–
20	Dodecane	12.8	–	–	0.49
21	Dodecane, 4-phenyl	12.82	3.01	1.89	–
22	Longiverbenone	12.887	–	–	0.28
23	Dodecane, 3-phenyl	13.18	2.58	1.74	–
24	Myristic acid	13.38	–	–	7.04
25	Eicosane	13.76	0.54	0.5	–
26	Dodecane, 2-phenyl	13.84	2.85	1.9	–
27	Myristaldehyde	13.99	–	–	0.15
28	Tridecane, 6-phenyl	14.22	4.16	2.91	–
29	Tridecane, 5-phenyl	14.33	2.6	1.56	–
30	9-Eicosyne	14.43	1.98	0.54	–
31	Methyl heptadecyl ketone	14.49	–	–	0.52
32	Tridecane, 4-phenyl	14.54	1.94	4.31	–
33	Tetradecanoic acid	14.64	–	–	0.45
34	Diisobutyl phthalate	14.84	–	–	0.79
35	Tridecane, 3-phenyl	14.91	1.7	1.74	–
36	Tetratetracontane	15.42	31.28	26.63	–
37	Heptadecane, 2,6,10,15-tetramethyl	15.43	–	0.54	–
38	Octadecane	15.54	–	–	0.14
39	Tridecane, 2-phenyl	15.55	2.64	1.52	–
40	(R)-(-)-(Z) − 14-Methyl − 8-hexadecen − 1-ol	15.68	–	–	0.13
41	Palmitic acid	15.82	–	–	0.24
42	Disulfide,di-tert-dodecyl	15.84	–	1.73	–
43	Isophytol	16.18	–	–	0.1
44	Dibutyl phthalate	16.36	–	–	1
45	Pentadecane, 8-hexyl	16.52	–	1.76	–
46	Ethyl docosanoate	16.94	–	3.15	–
47	l-(+)-Ascorbic acid 2,6 -dihexadecanoate	17.11	–	–	50.76
48	9,12-Octadecadienoic acid, methyl Ester	18.46	–	0.35	–
49	Tritetracontane	18.57	–	2.57	–
50	Tetrapentacontane	18.57	–	–	0.23
51	Phytol	18.81	–	–	0.51
52	Linoleic acid ethyl ester	19.47	–	1.08	–
53	cis,cis,cis − 7,10,13-Hexadecatrienal	19.58	–	2.77	–
54	7-Tetradecenal	19.58	–	–	10.44
55	L-Ascorbic acid	20.02	–	–	17.44
56	Pentadecanal-	20.33	–	0.32	0.19
57	Tributyl acetylcitrate	21.02	–	–	1
58	Heneicosane	21.49	–	–	1.13
59	Phen − 1,3-diol, 2-[3-oxohexadecanoyl]	22.02	–	0.44	–
60	Tetratriacontane	22.14	–	0.94	–
61	2(3H)-furanone	22.19	–	–	0.96
62	Azulene	22.68		–	0.96
63	1-Decanol, 2-hexyl	22.76	1.02	–	–
64	Cyclodocosane, ethyl-	22.77	–	0.4	–
65	Heptadecane	24.16	–	–	0.6
66	1,2-Benzenedicarboxylic acid, Mono (2-ethyl)	24.79	–	14.06	–
67	Bis-(3,5,5-trimethylhexyl) phthalate	26.02	–	0.52	–
68	Phthalic acid, ditridecyl ester	27.17	–	0.54	–
69	Phthalic acid, dinonyl ester	28.04	–	2.05	–

(*continued on next page*)

Table 5 (*continued*)

N°	Compound names	R. Time	Relative quantity (Area %)		
			Fraction C	Fraction D	Fraction F
70	Spinacen	28.13	8.16	0.43	–
71	Tetradecanal	28.21	–	0.61	–

R.Time: retention time; C, D, F: Major fractions of Hexane-Ethyl acetate extracts from *A. cucullathus*.

the aim to better appreciate and understand the plant antibiofilm mechanism of action against the pathogenic and resistant *Candida* yeast.

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Conflict of interest

Authors declare no conflict of interest.

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