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BIOFILM ERADICATION ACTIVITY OF PARTIALLY PURIFIED FRACTION OF ETHYLACETATE EXTRACT OF ACALYPHA WILKESIANA LEAVES ON *CANDIDA ALBICANS I, II* AND *PARAPSILOSIS*

[1]Maimuna Zubairu [2]Aliyu Salihu [2]Ismaila A. Umar [3]Karima Mohammed Rabiu [4]Elijah Ella

[1] Department of Biochemistry, Faculty of Science, Kaduna State University, Kaduna.
[2] Department of Biochemistry, Faculty of Life Sciences, Ahmadu Bello University, Zaria.
[3] Department of Biological Sciences, Yobe State University, Damaturu.
[4] Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria

Corresponding Author: muna270@yahoo.com

ABSTRACT
Candida spp produce highly structured biofilms composed of multiple cells encased in an extracellular matrix. Sessile cells are known to decrease drug diffusion toward biological targets. This research is aimed at evaluating the biofilm eradication activity of partially purified fraction of ethylacetate extract of *Acalypha wilkesiana* leaves on sessile cells of *C. albicans* I, II and *C. parapsilosis*. Column Chromatography-TLC was used to fractionate and partially purify the extract using a bioassay guided fractionation. Preparative TLC was used to purify the most potent fraction. Antifungal effects of the extract and percentage biofilm eradication was determined by microdilution method. Antibiofilm activity of the purified fraction on the morphology of the sessile cells of *Candida spp* was evaluated by scanning electron microscopy. The purified fraction had MIC of 1.25mg/ml on C. *albicans* I, II and *C. parapsilosis*, respectively. MFC of 2.5mg/ml on *C. albicans I* and *II* but there was no total death observed on *C. parapsilosis*. The fraction had IC_{50} significantly ($p \leq 0.05$) different from that of the reference drugs. There was significant ($p \leq 0.05$) increase in the biofilm eradicated by corilagin, amp B., voriconazole, caspafungin, compared to the purified fraction on *C. albicans* I, II and *C. parapsilosis*. There was decrease in biomass of sessile cells on the morphology of *C. albicans* I, II and C. *parapsilosis* in all treated plates compared to the control. Therefore, the purified fraction of ethylacetate extract of *A. wilkesiana* leaves has a potential to eradicate the biofilms formed by *Candida spp*.

Keywords: *Acalypha wilkesiana*, Microdilution, Scanning Electron Microscopy, Extracellular Matrix.

INTRODUCTION

One of the main factors complicating antifungal therapy in an increasing number of patients is the ability of fungal cells to form biofilms (Ramage *et al*., 2006). *Candida albicans* are known to produce structured biofilms composed of multiple cell types which include round, budding yeast-form cells, oval pseudo hyphal cells, and elongated cylindrical hyphal cells encased in an extracellular matrix (Ramage *et al*., 2005, Fox and Nobile, 2012). A biofilm is defined as a structured microbial community attached to a surface and encased within a self-produced extracellular matrix (Donlan, 2002). It is well established that biofilms are the predominant form of microbial growth in nature, and during infection (Ramage *et al*., 2009). It is a community of adherent cells with properties that are distinct from those of free-floating (planktonic) cells (Bachmann *et al*., 2002). Among pathogenic fungi, *Candida* spp. are the most frequently associated with biofilm formation (Ramage *et al*., 2005). Different types of biomaterials often used in clinics support colonization and biofilm formation by *Candida*, most notably intravascular catheters (Crump and Collignon, 2000: Kojic and Darouiche, 2004). Candidiasis associated with intravenous lines and bioprosthetic devices is problematic, since these devices can act as substrates for biofilm growth (Donlan, 2002). The presence of biofilms can result in serious problems due to their resistance to antimicrobial agents (Kruppa, 2009). This resistance is developed by the presence of quorum-sensing molecules that plays an important role in the biofilm formation and virulence, based on the local density of the fungal population present for the construction and/or dissolution of biofilm communities (Kruppa, 2009, Deveau and Hogan, 2011). Hyphae in biofilms contribute to the overall architectural stability, acting as a support scaffold for yeast cells and other hyphae. Thus, the ability to form hyphae and the ability of these hyphae to adhere to one another and to yeast cells are critical for biofilms development (Nobile and Mitchell 2005).

Recently, there has been much concern on medicinal plants to reduce the adverse effects of various infections due to the resistance acquired by some microorganisms to some synthetic drugs and one of such plants is *Acalypha wilkesiana* (Copper Leaf). Plants have been a source of medicine in the past centuries and today scientists and the general public recognize their value as a source of new or complimentary medicinal products (Premanath and Lakshmideri, 2010).

The aim of this work is to evaluate the biofilm eradication activity of partially purified fraction of *Acalypha wilkisiana* leaves on *Candida albicans* I, *Candida albicans* II and *Candida parapsilosis.*

Materials and Methods

Chemicals and Reagents: Caspofungin, amphotericin B, voriconazole were purchased from Sigma Aldrich. Potato dextrose agar (PDA), sabaraud dextrose broth, chloramphenicol capsules, sample bottles, human serum, lactophenol cotton blue, API 20 C Aux Kit (Biomerieux SA France), 0.85% NaCl, McFarland standard scales 0.5 and 2, apiweb ™ Identification software (Ref. 40 011). All other chemicals used were of analytical grade except where otherwise stated.

Ethical Clearance: Ethical approval was granted by the Health Research Ethics Committee (HREC) of Ahmadu Bello University Teaching Hospital Shika with an assigned number, ABUTHZ/ HREC/AO1/2017.

Identification of Clinical Isolates of *Candida*

Three (3) Isolated *Candida* species were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital (ABUTH), Shika. Two of the samples were from high virginal swab while the remaining one was from intracervical swab. The *Candida* Isolates where characterized using API 20 C AUX strips.

Collection and Authentication of Plant Material: Fresh leaves of *Acalypha wilkesiana* were collected from the garden of National Ear Center, Independence Way, Kaduna which is located at Alt 603m, Latitude 10.52º and Longitude 7.45 º. Samples of *Acalypha wilkesiana* were authenticated in the herbarium section of the Department of Botany, Faculty of Life Sciences, Ahmadu Bello University, Zaria and the voucher specimen was deposited.

Extraction and Percentage Yield of Phytochemicals from Leaves of *Acalypha wilkesiana*

Leaves of *A. wilkesiana* were air dried and pulverized into uniform powder using mortar and pestle. Two Hundred grams (200g) of the pulverized leaves of *Acalypha wilkesiana* was soaked in 1.5 L of ethyl acetate for 48hours in a conical flask after which the mixture was filtered through Whatman No. 1 filter paper as described by Handa *et al*., (2008). The filtrate was concentrated at 40°C using rotary evaporator to get constant dry weight of the extract. The Percentage yield of the extract was calculated using the following formula:

$$\text{Yield (\%)} = W1 / W2 \times 100$$

Where: W1 = weight of extract after evaporation of solvent and W2 = dry weight of sample (powder).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of Ethylacetate Extract of A. *wilkesiana Leaves* on Planktonic cells of *C. albicans* I, II and *C. parapsilosis.*

Minimum Inhibitory Concentration (MIC) was determined by broth dilution method as described by Takahagi *et al*., 2009. Stock solutions were prepared in dimethyl sulfoxide for *ethylacetate, voriconazole, amphotericin B and caspofungin.* Sterile plastic microtiter plates containing flat-bottomed wells were utilized (Corning Costar). About 100µL sabaraud dextrose broth was added to each well. Drug-free medium wells were employed to provide sterility and growth controls. Mcfarland standard (0.5) of *C. albicans* (*I* and *II*) and *C. parapsilosis* were prepared. About 100µL aliquot of each was added to each well of the microdilution plate. Final concentrations of corilagin, voriconazole, amphotericin B, and caspofungin were serially diluted from 0.01mg/ml to 0.00125mg/ml and100µL were added to the corresponding wells. Purified fraction of ethylacetate extract of *Acalypha wilkesiana* leaves was also serially diluted from 100mg/ml to 12.56mg/ml and 100µL were added to the corresponding wells. The microtiter plates were incubated at ambient temperature for 24 hours and absorbance was taken after the incubation period at 630nm using microtiter plate reader. The MIC is defined as the lowest concentration of the test substance inhibiting microbial growth. (Rasooli and Abyaneh, 2004). The concentration of the antifungal in a microdilution well that had absorbance nearly that of the control well is taken as the MIC.

The Minimum Fungicidal Concentration (MFC) of the extract with the best inhibitory activity was determined using the method of Russell and Furr (1977). This method involves sub culturing portions of the agar from petridishes that showed no growth in the tests for determination of MICs. These agar portions were transferred into petridishes containing freshly prepared Sabouraud Dextrose Agar (SDA). These plates were incubated at 25-27°C for 2 days and observed daily for growth. The absence of growth at the end of incubation period signifies total cell death. The minimum concentration of the plant extracts that produces total cell death is taken as the MFC (Siddiqui *et al*., 2013).

The IC_{50} of the purified fraction of ethylacetate extracts of *Acalypha wilkesiana* leaves was carried out using micro dilution method as described by Jessica *et al*., 2013. The IC_{50} is defined as the concentration where the response is reduced by half. The following formular was used for calculations of cell inhibition:

$$\text{\% cell inhibition} = 100 - \{(At- Ab)\}/ (Ac- Ab) \times 100$$

Where,

At=Absorbance value of test compound
Ab= Absorbance value of blank
Ac= Absorbance value of control.

Purification of Antifungal Agent Present in Ethylacetate Extract of *Acalypha wilkesiana* Leaves

The ethylacetate extract of *Acalypha wilkesiana* leaves was fractionated using column chromatography thin layer chromatography as described by Gaurav and Nissreen,2013. Two solvent systems were used for the fractionation. Silica gel was used as the adsorbent. About 50g of silica was made into slurry with ethylacetate and placed in a cylindrical tube that is plugged at the bottom by a piece of glass wool using a funnel. The extract was dissolved in ethylacetate. A pipette was used to add the sample to the top of the column. The stopcock was opened and the solvent was allowed to drain. A very small amount of solvent was used to wash down any sample that may have clung to the sides of the column. This additional solvent was also drained. To elute the Sample through the Column: a pipette was used to add 4ml of solvent. A funnel was placed at the top of the column and the remainder of the column was filled with the solvent. The stopcock was opened and the solvent was allowed to drain through the column. The mobile phase was collected as it drained from the column into test tubes. Additional solvent with increase polarity starting from absolute n-hexane (100%), n-hexane: ethylacetate (4:1, 3:2, 2:3, 1:4), and absolute ethylacetate (100%) were added to the top of the column as needed until all the desired compounds have eluted from the column.

Fractions that showed the same retention factor (RF) were pooled together. The most potent fraction was selected in a bioassay guided fractionation. Preparative thin layer chromatography was carried out to purify the most potent fraction. Formula for calculating retention factors is given as:
Rf= distance moved by the spot / distance moved by solvent

Percentage Biofilm Eradication

Percentage Biofilm Eradication was determined by the microdilution method as described by (Djordjevic *et al*., 2002). A 100 µl aliquot of SDA broth was added to each well, and 100 µl cell suspension (0.5 Mcfarland standard) of each of the three strains of *Candida* was added to corresponding wells and incubated for 24 hours at 37^{o}C for biofilm growth. After incubation, supernatant was discarded and wells washed twice with 200 µl phosphate buffer (PB) and incubated for 2 hours at 37^{o}C followed by addition of the ethylacetate extract of *Acalypha wilkesiana* leaves at concentration corresponding to the MIC, 2 MIC and 4 MIC. The plates were, allowed for initial yeast adherence. Subsequently, the wells were washed with phosphate-buffered saline (PBS) to remove loosely bound cells, fresh medium was added, and the plates were incubated for 48 hours at 37^{o}C. Absorbance values were read at 600 nm using a microplate reader. Corillagin, amphotericin B, voriconazole and caspafungin were used as positive controls and untreated biofilm served as the growth control.

Effects of Purified Fraction of *Acalypha wilkesiana* Leaves on the Morphology of Sessile Cells of *C. abicans I, II* and *parapsilosis*

Biofilm eradication activities of the purified fraction of *Acalypha wilkesiana* leaves on the morphology of the sessile cells was observed by scanning electron microscopy as described by Shafreen *et al*., 2014. Biofilms were formed on 24 well microtiter plates as earlier described and then the discs were fixed with 4.0% formaldehyde and 2.0% glutaraldehyde. The sessile cells were then transferred to corresponding glass slides. The samples were dehydrated with a series of ethanol washes and allowed to dry for 24 hours, then sputter-coated with palladium- gold thin film and examined on a JOEL-JSM 5300 scanning electron microscope.

Statistical Analysis

The results were expressed as mean ± Standard deviation (SD). All tests were performed in triplicate. Significant differences were analyzed between groups using one-way analysis of variance (ANOVA). Test for differences between two means was performed using Turkey-Kramer multiple test. The values of $p \leq 0.05$ was considered as significant.

RESULTS AND DISCUSSION

Table 1 shows that purified fraction of *Acalypha wilkesiana* leaves has MIC of 1.25mg/ml each on C. *albicans I, II* and *C. parapsilosis*. MFC of 2.5mg/ml on *C. albicans* I and II but there was no total cell death observed on *C. parapsilosis at* 2.5mg/ml. This result conforms with the findings of Muyideen *et al.*,2013 which stated that the ethylacetate extract of *Acalypha wilkesiana* leaves had a broad-spectrum activity on fungi species. The reference drugs showed significant ($p \leq 0.05$) decrease in MIC and MFC compared to the purified fraction of *Acalypha wilkesiana* leaves. Voriconazole showed no total cell death on all the *Candida* strains used for this experiment, this is in conformity with the previous studies where voriconazole produced partial growth inhibition on *Candida* species and complete inhibition of growth on other fungal species (Javier *et al*., 2006).

Corilagin showed MIC of 0.04mg/ml each on *C. albicans* I, II and C. *parapsilosis* but, there was no total cell death observed on *C. parapsilosis* which was contrary to a previous study where corilagin showed fungicidal activities on *Candida parapsilosis* and non on *Candida albicans* (Guilherme *et al*., 2015). Purified fraction of *Acalypha wilkesiana* leaves had IC_{50} of 6.89 0.52, 6.95 0.38 and 8.72 1.51 on *C. albicans* I, II and *C. Parapsilosis*, respectively which was significantly different from the IC_{50} of voriconazole, amphotericin B and caspafungin.

Figure 1 showed the percentage biofilm eradicated by the purified fraction of *Acalypha wilkesiana* leaves and reference drugs on *C. albicans* I, II and *C. parapsilosis*. There was

significant ($p \leq 0.05$) reduction in biomass of sessile cells treated with the purified fraction of *Acalypha wilkesiana* leaves compared to the negative control which agrees with the report of Douglas *et al*., 2015 on significant effects of purified compounds from *Acalypha wilkesiana* leaves in reducing total biomass. There was no significant difference between the biofilms eradicated by voriconazole, amphotericin B and caspafungin on *C.albicans I* , *II* and *C. parapsilosis*. **Plates 1, 2** and **3** showed scanning electron micrograph of control sessile cells of 48 hours cultured *C.albicans* I , II and *parapsilosis*, respectively. Examined by SEM 3000. There was loss of biofilm biomass in plates treated with corilagin,voriconazole, amphotericin B, caspofungin and purified fraction of *Acalypha wilkesiana* leaves. This conforms with the findings of Silva *et al.*, 2011 who reported that antifungal agents reduced germ tube formation, fungal metabolism and proliferation, it is possible that purified fraction of *Acalypha wilkesiana* leaves interfered with the synthesis or expression of filament-associated adhesins on the surface of the cells, thereby reducing induced adhesion and total biomass.

Table 1: Minimum Inhibitory Concentration and Minimum Fungicidal Concentration (MIC / MFC) of Purified Fraction of Ethylacetate Extract of *Acalypha wilkesiana* Leaves, Corilagin, Voriconazole, Amphotericin B. and Caspofungin on *C. albicans* I, II and *C. parapsilosis*.

Fraction / Drugs	*C. albicans* I mg/ml MIC / MFC	*C. albicans* II mg/ml MIC / MFC	*C. parapsilosis* mg/ml MIC / MFC
Purified Fraction	1.25 / 2.5	1.25 / 2.5	1.25 / -
Corilagin	4×10^{-2} / 4×10^{-2}	4×10^{-2} / 4×10^{-2}	4×10^{-2}/-
Voriconazole	12.5×10^{-6} / -	12.5×10^{-6} / -	12.5×10^{-6}/ -
Amphotericin B	12.5×10^{-6} / 25×10^{-4}	12.5×10^{-6} / 25×10^{-4}	12.5×10^{-6} / 25×10^{-4}
Caspofungin	12.5×10^{-6} / $25 \text{ x} \times 10^{-4}$	12.5×10^{-6} / 25×10^{-4}	$\times 10^{-6}$ / 25×10^{-4}

(-) indicates no fungicidal activity

Table 2 : Inhibitory Concentration (IC_{50}) of Purified Fraction and the Reference Drugs on *C.albicans I, II* and *parapsilosis*

Subfractions and Drugs	*C. albicans* I mg/ml	*C.albicans* II mg/ml	*C. parapsilosis*
Purified Fraction	6.89 ± 0.52[a]	6.95 ± 0.38[a]	8.72 ± 1.51[a]
Corilagin	0.038 ± 1.2× 10^{-5}[b]	0.037± 4.2 × 10^{-5}[b]	0.044 ± 4 × 10^{-5}[b]
Voriconazole	0.000700 ± 1.8 × 10^{-6}[c]	0.000704 ± 6.3 × 10^{-6}[c]	0.000750 ± 4.0 × 10^{-6}[c]
Amphotericin B	0.00069 ± 7.8 × 10^{-6}[c]	0.00071 ± 8.93× 10^{-6}[c]	0.00072 ± 9.1 ×10^{-6}[c]
Caspafungin	0.00072 ± 2.04 × 10^{-6}[c]	0.00072 ± 3.31 × 10^{-6}[c]	0.00072 ± 9.1 × 10^{-6}[c]

Values having different superscripts across a column (a- c) and row (1-6) are significantly ($p \leq 0.05$) different.

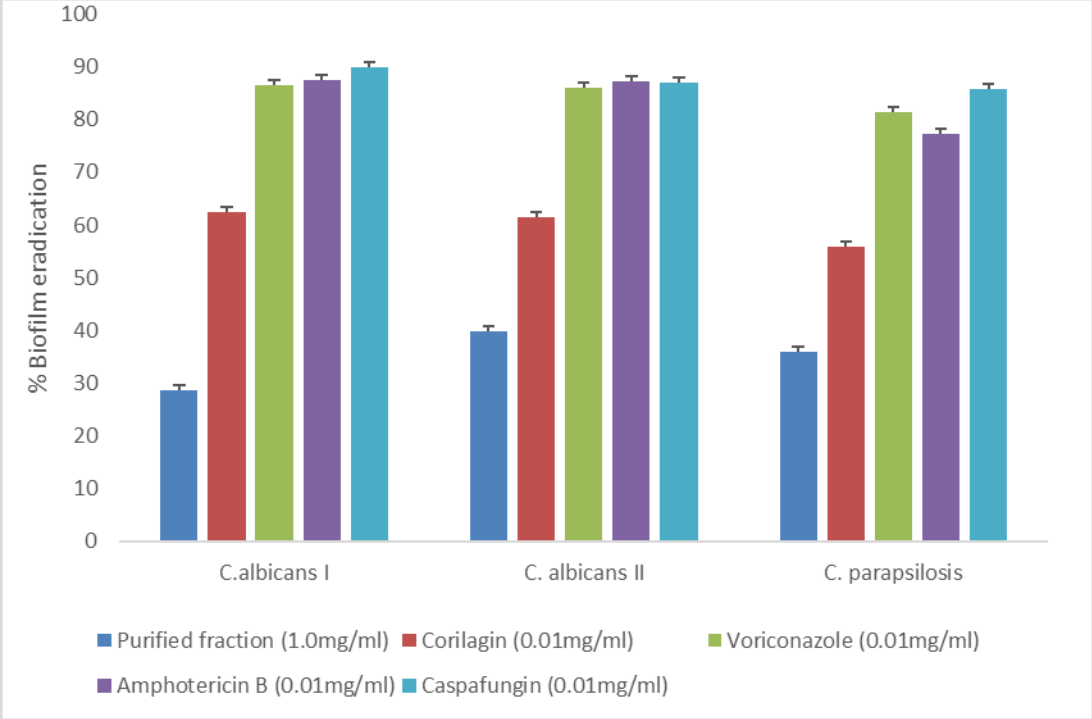

Fig. 1: Percentage Biofilm Eradicated by Purified Fraction of *Acalypha wilkesiana* Leaves and Reference Drugs on *C. albicans* I, II and *parapsilosis*.

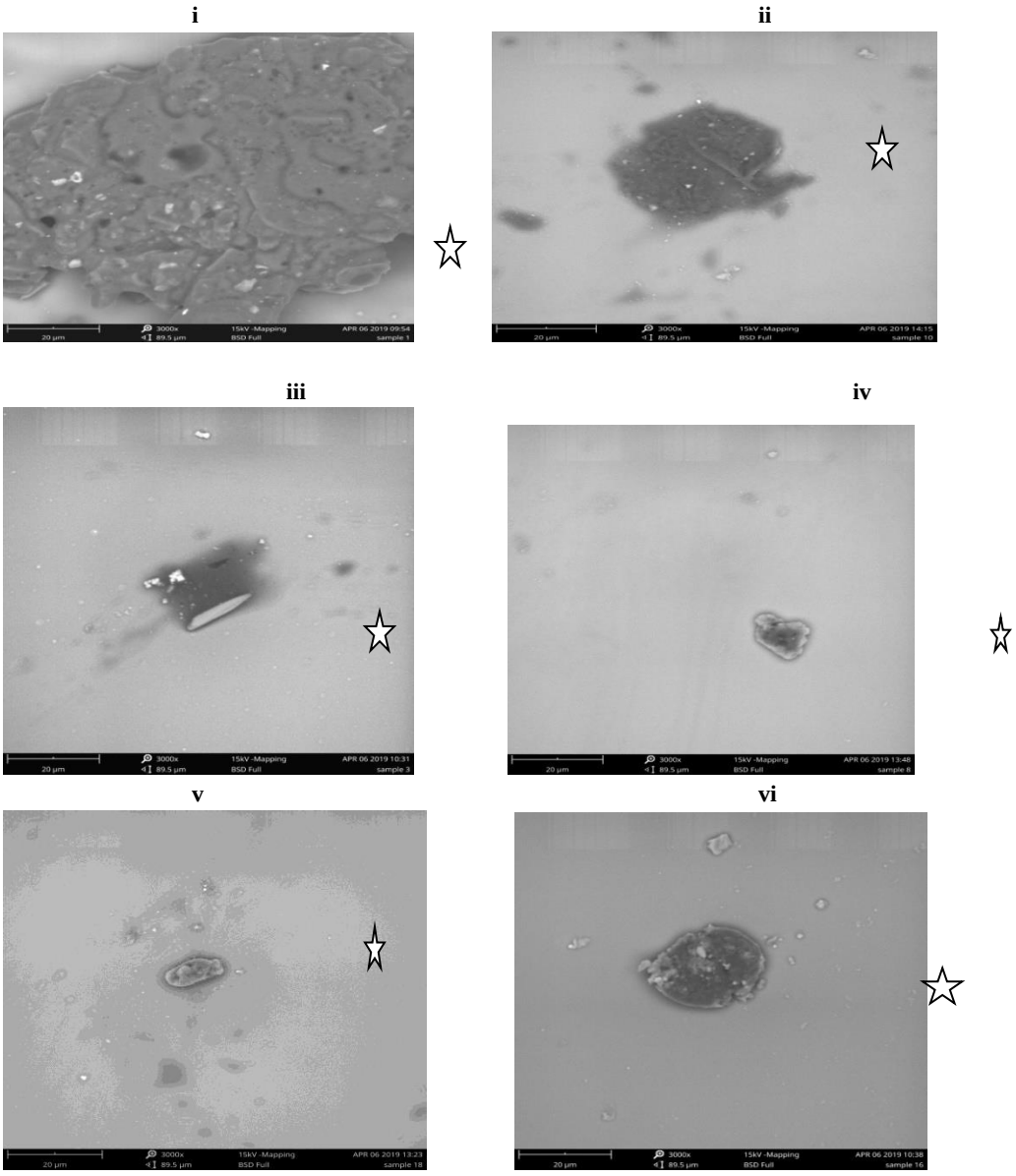

Plate 1: Scanning Electron Micrograph of 48hours cultured C. albicans* I *as control plate (i) Loss of Biofilm Biomass ( ) in treated cells with Corilagin plate (ii), Voriconazole plate (iii), Amphotericin B. plate (iv), Caspofungin plate (v) and Purified fraction of Acalypha wilkesiana Leaves (vi) .

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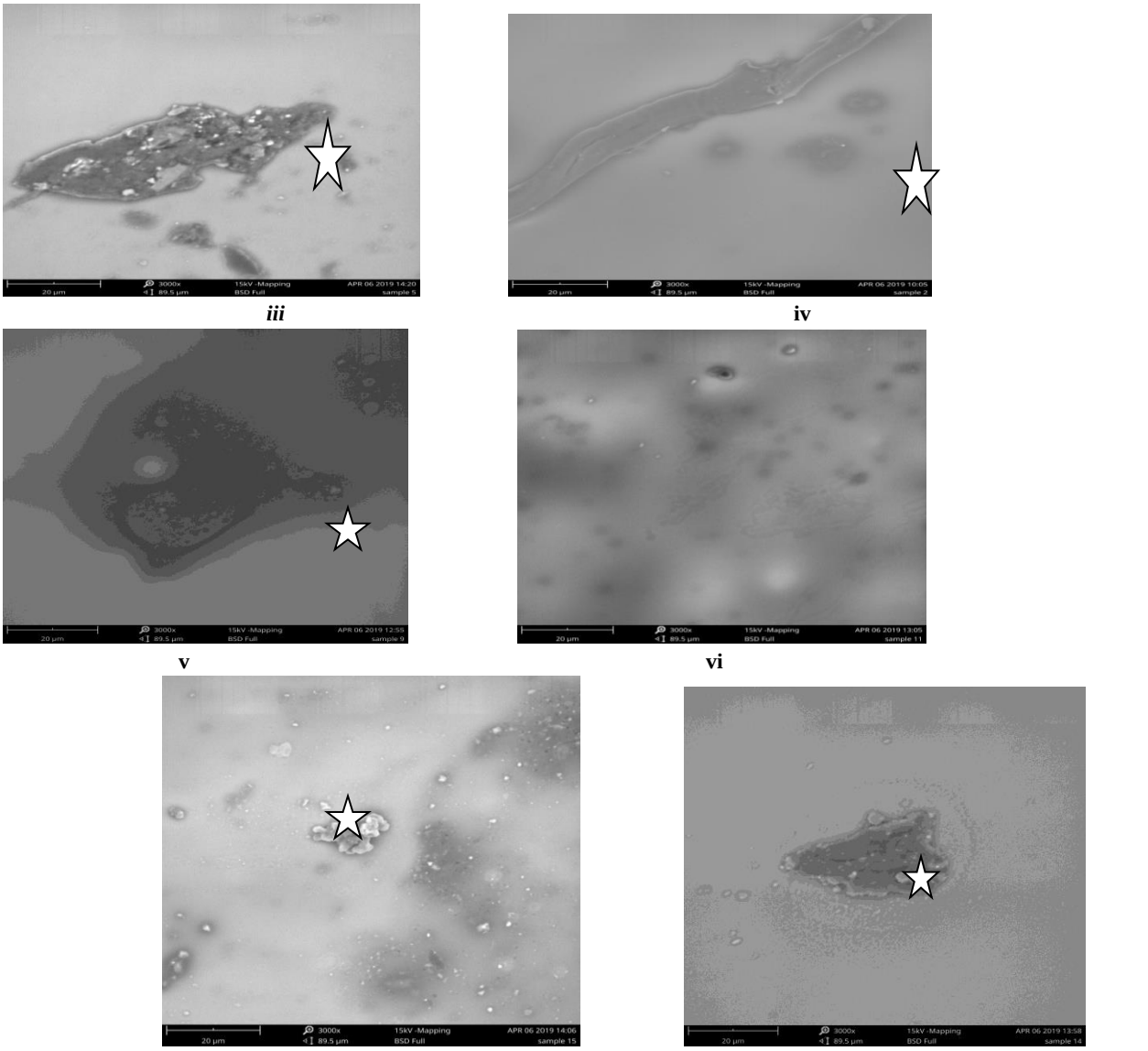

Plate 2 : Scanning Electron Micrograph of 48hours cultured C. albicans* II *in Control plate (i) Loss of Biofilm Biomass( ) in treated cells with Corilagin plate(ii), Voriconazole plate (iii), Amphotericin B. plate (iv), Caspofungin plate (v) and Purified fraction of Acalypha wilkesiana leaves

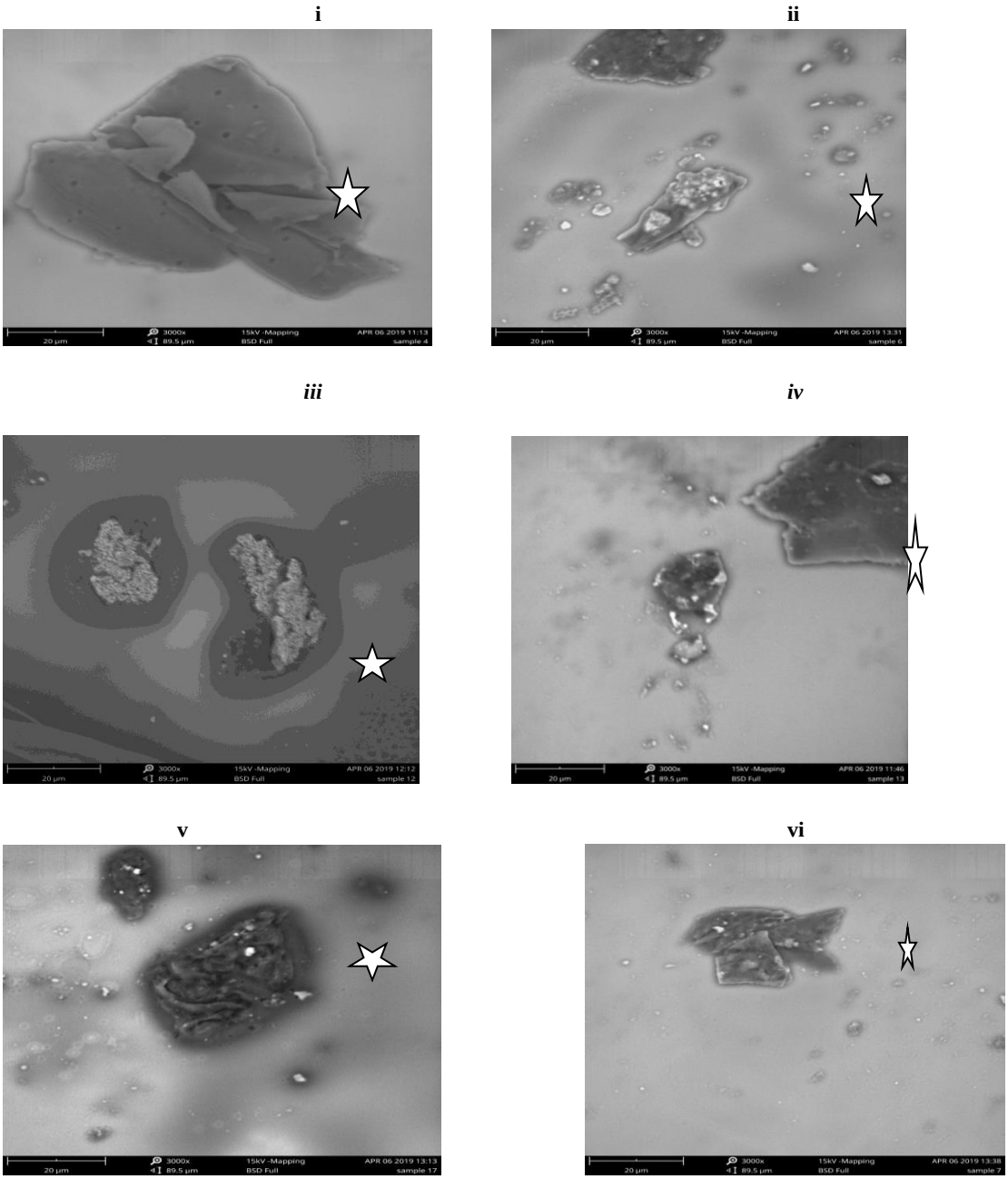

Plate 3: Scanning Electron Micrograph of 48hours cultured C. parapsilosis as control plate (i) Loss of Biofilm Biomass( ) in treated cells with Corilagin plate (ii), Voriconazole plate (iii), Amp B. plate (iv), Caspofungin plate (v) and Purified Fraction of Ethylacetate Extract of Acalypha wilkisiana Leaves plates (vi).

CONCLUSION

Purified fraction of *Acalypha wilkesiana* leaves reduced the biomass of *C. albicans* I, II and *parapsilosis* sessile cells and therefore has the ability to diffuse through the biofilms.

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