Full Length Research Paper

Activity of Sansevieria aethiopica (Thunberg) leaf extracts on the planktonic and the biofilm of Candida albicans ATCC 10231

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Accepted 2 September, 2013

Increase in resistance of Candida species to conventional prescribed antifungal agents poses a challenge to health care. This calls for alternative therapies such as the use of antifungal plant extracts. Therefore, the objective of this study was to screen Sansevieria aethiopica (Thunberg) leaf extracts for anti-fungal activity against Candida albicans. A microbroth dilution method was used to determine the minimum inhibitory concentrations (MICs) and a biofilm enumeration assay was employed to determine the minimum biofilm inhibition concentrations (MBICs) and minimum biofilm eradication concentrations (MBECs) of the extracts. Electron microscopy was used to determine the effects of the extracts on the ultrastructure of the biofilm of the test fungus. Acetone extract had the least effect on C. albicans ATCC 10231 with MIC of 3.125 mg/ml. For the extracts, the MBICs and MBECs were higher than the corresponding MICs. The MBEC: MIC and MBIC: MIC were 8:1 and 2:1, 4:1 and 2:1, and 8:1 and 8:1 for acetone, ethanolic and methanolic extracts, respectively. Extract treated cells showed change in the morphology of the cells. Extracts of S. aethiopica were able to affect the proliferation of both planktonic and sessile cells of C. albicans. Isolation and elucidation of the bioactive ingredients and the effects of the active compound on C. albicans still warrants further investigation.

Key words: Candida albicans, Sansevieria aethiopica, planktonic, anti-candidal, scanning electron microscope, medicinal plants, extracts.

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen that can be exogenously or endogenously acquired. The pathogen is able to cause infections ranging from superficial to life threatening mycoses in immunocompromised hosts (Richardson, 2005; Dumitru et al., 2004; Grubb et al., 2008). Candida infections can be superficial, invasive or disseminating. Invasive infections have been reported with a 40% mortality rate (Bertagnolio et al., 2004; Pfaller and Diekema, 2007; Klevay et al., 2009). Predisposing factors to candidal infections include burns, cancer, catheterization, human immune deficiency virus (HIV), low-birth-weight, prolonged chemotherapy, prosthetic devices, and transplantation of organs (Pfaller and Diekema, 2007; Asciovglu et al., 2002).

Aside from increasing resistance to the available antifungal compounds, the toxicity of some compounds is high (Georgopapadakou and Walsh, 1994: Shreaz et al., 2011). Some major antifungals which are limited to a few chemical classes, have been implicated in hepatotoxicity and nephrotoxicity coupled with decreasing efficacy against C. albicans (Arthington-Skaggs et al., 1999; Dismukes, 2000; Pan et al., 2009). Hence the need for inexpensive and effective antifungals with less toxicity is imperative.

Medicinal plants have been the major health care...
measure of the resource-poor population in different parts of the globe (Tharkar et al., 2010; Duraipandiy and Ignacimuthu, 2011). Some of the extracts of plants have demonstrated positive responses during pharmacological investigations (Patel and Coogan, 2008; Suresh et al., 2010). Herbal preparations are used for the treatment of different types of infectious diseases and they have yielded lead compounds for the discovery of new drugs. *Sansevieria aethiopica* (Thunberg) is a member of the family Asparagaceae. It is a perennial shrub with tough, semi-succulent and erect leaves (Van Wyk et al., 2000). *S. aethiopica* is called “isikholokotho, isikwendle or isitokotoko” in South Africa where it has been long recognized for the treatment of different infectious diseases (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; Newton, 2001; Von Koenen, 2001). The objective of this study was to screen *S. aethiopica* leaf extracts for anti-candida properties with the specific objectives to determine the effects of the plant extracts on planktonic and sessile (biofilm) cells as well as their ultra-structure.

**MATERIALS AND METHODS**

**Source and extraction of plant sample**

Fresh leaves of *S. aethiopica* were collected from a single tuft in February, 2012, in Alice Township, Nkokobe Municipality of Eastern Cape of South Africa. The plant was authenticated by Prof. D. Grierson and the voucher (DavMed 2012/2) was submitted to the Giften Herbarium of the Department of Botany, University of Fort Hare, Alice, South Africa. The fresh plant sample was dried in an oven at 40°C and ground to fine powder. Fifty grams of the ground plant sample were soaked in 500 ml of each of the analytical grade solvents [acetone (99.5%), ethanol (99.0%), and methanol (99.8%)] for 12 h on Stuart Scientific Orbital Shaker (Manchester, UK). The sample was then suction-filtered through Whatman number 1 filter paper and washed with another 200 ml solvent. The filtrate was concentrated with Laborota 4000 efficient (Heidolph, Instruments, Schwabach, Germany). The dried extract was dissolved in the respective extracting solvent (1 ml) and then topped up to the required volume. The doubling dilution was done to achieve different concentration ranging from 100.00 to 0.75125 mg/ml. The reconstituted extracts were filtered through a 0.45 μm pore size membrane (Millipore Inc.) filter for sterility.

**Source of the organism**

*C. albicans* ATCC 10231 was collected from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The organism was grown in potato dextrose broth (PDB) for 18 h and was standardized to 0.5 McFarland scale (=1.0 × 10^7 cfu/ml) and diluted with sterile PDB to achieve the final concentration of 1.0 × 10^6 cfu/ml.

**Anti-candida assay**

The minimum concentrations of the extracts that inhibited the test organism (*C. albicans* ATCC 10231) were determined using the method of Eloff (1998). An 18 h broth culture of *C. albicans* ATCC10231 in PDB was standardized to 0.5 McFarland scale (1.0 × 10^7 cfu/ml). This was further diluted to a concentration of 1.0 × 10^5 cfu/ml. A 100 μl of each of the extracts (100.00 to 0.75125 mg/ml) filter by 0.45 μm pore size membrane filter for sterility was inoculated with 100 μl of the standardized inoculum of *C. albicans* ATCC 10231 into 96 well microtitre plates with amphotericin B (Sigma-Aldrich, Germany) serving as control. The experiment was conducted in duplicates and repeated twice. The plates were covered with parafilm and incubated at 37°C for 24 h. The growth of the test organism was detected by adding 50 μl of 0.2 mg/ml p-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, Germany) and re-incubated for 2 h at 37°C. The reduction in the colour of INT to red which indicates the biological activity was used as indicator for microbial growth. The minimum inhibitory concentration (MIC) was defined as the least concentration with no colour change. Each experiment was duplicated and repeated two times.

**Time kill assay**

The broth macrodilution technique of National Committee for Clinical Laboratory Standards (2005) was used to determine the rate of killing of the test fungus by the extracts. The assay was performed with a final inoculum of approximately 1.0 × 10^6 cfu/ml in a final volume of 30 ml. The final inoculum was verified with plating 1.0 ml of 10^1 dilution on yeast nitrogen broth (YNB) supplemented with 2% glucose and incubated for 24 h at 37°C. Different concentrations of sterile extract were incorporated into 50 ml of glucose supplemented YNB to give a final concentration of 1/2 MIC, 1 MIC, and 2 MIC. The tubes were incubated at 37°C and shaken every 30 min. At each sample time of 0, 2, 4, 6, 8, 10 and 12 h, aliquots (0.2 ml) of the samples were withdrawn, diluted in ten-fold series in sterile normal saline and plated onto PDA and incubated at 37°C for 24 h. Controls were set up consisting of a negative control without plant extract but the diluents, while the positive control plate was supplemented with 0.5 mg/ml amphotericin B. The experiment was conducted in duplicates and repeated twice. The candidal colonies developed were counted and compared with the control.

**Determination of minimum biofilm eradication concentration (MBEC)**

The method of Antunes et al. (2011) was used to determine the concentrations of the extracts that will clear the formed biofilm. A 20 μl of standardized inoculum of *C. albicans* ATCC 10231 was added into each of the wells of 96-well microtitre plate and incubated at 37°C for 18 h. Sterile normal saline was used to wash the plates to remove the planktonic cells. 100 μl of YNB (Oxoid) was added into the wells followed by 100 μl of the sterile *S. aethiopica* extracts. The plate was incubated at 37°C for 24 h after which it was rinsed with sterile normal saline and allowed to air dry. The wells were stained with 1.0% crystal violet for 5 min and excess stain rinsed off. The plates were air dried again and the dye bound to the adherent cells was extracted with 100 μl of 33% (v/v) glacial acetic acid per well. The optical density was measured at 545 nm by DAR800 Microplate Reader (Calabasas, Canada) and the reading was taken and compared with negative control. MBEC was the minimum concentration of extract that gave an absorbance similar to the negative control.

**Anti-candidal adherence assay (biofilm inhibition assay)**

The method of Wasfi et al. (2012) was used with modification to determine the effect of sub-MICs of the plant extracts on *C. albicans* ATCC 10231 adherence. 100 μl of standardized cell
The activity of the extracts of *S. aethiopica* on planktonic cells and biofilms of *C. albicans* ATCC 10231.

<table>
<thead>
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<th>Plants</th>
<th>MIC</th>
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<th>MBIC</th>
<th>MBEC/MIC</th>
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<tbody>
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<td>25.00</td>
<td>6.250</td>
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<td>4</td>
</tr>
<tr>
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<td>6.250</td>
<td>3.125</td>
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<td>2</td>
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<tr>
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<td>12.500</td>
<td>12.500</td>
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<tr>
<td>Amphotericin B</td>
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<td>0.032</td>
<td>0.0160</td>
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<td>2</td>
<td>2</td>
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<table>
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<tr>
<th>MBIC</th>
<th>MBEC</th>
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<tr>
<td><em>S. aethiopica</em></td>
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% Reduction in biofilm = 100 × [(Absorbance of control - Absorbance of test) / Absorbance of control]

Where Absorbance of control

**In situ electron microscopy**

The method of Kamilla et al. (2009) was adapted to determine the effect of the extracts of *S. aethiopica* on the ultra-structure of the *C. albicans* ATCC 10231 biofilm after exposure. The standardized inoculum of *C. albicans* ATCC 10231 was seeded on a sterile plate (100 mm by 15 mm) of PDA with sterile cotton wool swab and incubated for 2 h at 37°C. After incubation, the plate was re-incubated for another 12 h at 37°C. A 5 mm diameter sterile borer was used to remove plugs from the petri dish and each of the plugs was placed on a double-stick adhesive tab on a stub. The sample was vapour fixed with 2% osmium tetroxide for 1 h, dried with liquid nitrogen and later transferred to freeze dryer (Vistis Benchtop K) for 5 h. The dried samples were gold coated before viewing with scanning electron microscope (JEOL JSM-6390LV, Japan).

**RESULTS AND DISCUSSION**

*Candida* species are important opportunistic fungal pathogens due to the increasing frequency of infections especially in patients with cancer, diabetes, and HIV (Hamza et al., 2006). The summary of the activity of extracts of *S. aethiopica* leaf extracts on the planktonic and the biofilm of *C. albicans* ATCC 10231 is shown in Table 1. The activities of the extracts were compared with amphotericin B. *C. albicans* ATCC 10231 was relatively resistant to aceton extracts which had MIC of 3.125 mg/ml. Considering the result of the MICs, extracts of *S. aethiopica* have fungicidal effects on *C. albicans* ATCC 10231 though at varying degrees. This result is similar to that obtained by Torey and Sasidharan (2011) who reported standardized methanolic extract of *Cassia spectabilis* to have a pronounced activity against three local isolates of *C. albicans* with MIC of 6.25 mg/ml. In like manner Mai et al. (2013) reported that methanolic extracts of *Sansevieria trifasciata* was effective against *C. albicans*. The activities of the extracts and the concentrations of their phytochemicals (phenolic, alkaloid, tannin, and other compounds) (Lattanzio et al., 2001; Brul and Coote, 1999).

For all the extracts, the MBICs and MBECs were higher than the corresponding MICs. The MBEC/MIC values to the three extracts were 8:1, 4:1 and 8:1, respectively. The MBIC: MIC values for both acetone and ethanolic was 2:1 while higher value (8: 1) was recorded for methanolic extract. *C. albicans* is a biofilm producer, the cell exists predominantly and enclosed and shielded away from the antimicrobials and host defense mechanisms (Branchini et al., 1994; Samaranayake et al., 2002). This reason may account for the higher concentrations of the extracts that were needed to inhibit and eradicate the biofilm compared to the MIC in planktonic broth culture.

At the 2 MIC, which is the highest concentration tested for the time-kill response, the rate of reduction of the organism was higher than at the lower concentrations. The fungus was completely inhibited by both acetone and methanolic extracts at 8 h of incubation. Similar observations were reported by Knoblock et al. (1989), Abe et al. (2003), Ahmad et al. (2005) and Khan and Ahmad (2012a, b). A time-kill response of *C. albicans* ATCC 10231 on exposure to the extracts is represented in Figures 1 to 3. Compared with the control at 12h there was reduction in the viable counts of the fungus. At concentration twice the MIC, all the extracts showed fungicidal activity on the organism and at the concentration, the total population of the fungus was brought to zero earlier than at the MIC. The extracts exhibited a concentration-dependent growth inhibition on *C. albicans* ATCC 10231.

Time-kill assays showed that the extracts reduced the
load of *C. albicans* significantly. These results are in agreement with previous findings that extracts of other plants had fungicidal properties against *Candida* species and other pathogens (Birdsall et al., 1997; Coulburn et al., 2007; Iauk et al., 2007; Iwazaki et al., 2010; Ncube et al., 2012). The crude extracts were very effective against the pathogen at MIC and 2 MICs. Acetone and methanolic extracts inhibited the organism completely at 6th and 8th hour of exposure to the 2 MICs and MICs of the extracts.

The untreated cell (Figure 1A) showed a pronounced biofilm formation with both primary and secondary layers while there was inhibition in the test. This observation indicates the effects of the extracts on the pathogen and supported previous findings of Torey and Sasidharan (2011) that extracts of *C. spectabilis* have antibiofilm property against the *C. albicans*. Extract from plant has been documented to inhibit the formation of biofilm. They...
do this by inhibiting the biochemical activity of the cells (Agarwal et al., 2008). Acetone treated (B) showed change in the morphology of the cells, the initial oval shape changed to elongated cells and it also showed shrinkages of some cells. Ethanolic extract treated cell (C) showed no sign of budding. The cells were elongated and cemented together but there was no emergence of cells to form the second layer of biofilm. The methanolic extract treated cells (D) showed few daughter cells budding out of the parent cells. This extract showed least effect on the biofilm of the organism as shown in Figure 4.

Figure 3. Time-kill assay of methanol extracts of *S. aethiopica* on *C. albicans* ATCC 10231.

Figure 4. Anti-biofilm properties of the extracts of *S. aethiopica* against *C. albicans* ATCC 10231. A, B, C and D represent control, acetone extract-treated, ethanol extract-treated and methanolic extract-treated cells, respectively.
Conclusion

The extracts of *S. aethiopica* were able to affect the proliferation of both planktonic and sessile cells of *C. albicans* ATCC 10231. Isolation and elucidation of the bioactive ingredients and the effects of the active compound on *C. albicans* still require further investigation.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the financial supports of the National Research Foundation, South Africa and the University of Fort Hare, Alice 5700, Eastern Cape, South Africa.

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