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Anti-Candidal Activities of Leaf Extracts of *Sansevieria aethiopica* (Thunb): A Medicinal Plant Use in the Treatment of Oral Candidiasis in Eastern Cape of South Africa

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Abstract

Candida albicans infections are on the increase in the recent time and its resistance to most fungicides has been documented. We studied the effect of extracts of *Sansevieria aethiopica* (Thunb) on *C. albicans* ATCC 10231 and their possible mechanisms of actions were proposed. The minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of the extracts were determined using macrobroth dilution method while the structural changes in the fungus after treatment with the extract was determined by electron microscope. Standard methods were used to determine the effects of the extracts on the proton pumping, internal pH and ergosterol synthesis. The MIC of the extracts ranged from 1.5625 to 3.125 mg/ml while extracts-treated cells showed alterations in the morphology; wrinkled surfaces, shrinkages, tears and holes. Proton pumping activity was lower in the treatment group compare to the control while the internal pH of test fungus ranged between 5.40 and 6.03. We observed a decreased in ergosterol content in the candidal cells treated with the plant extracts. At ½MIC of acetone, methanol and ethanol extracts of the plants the amount of 24(28)-dihydroergosterol to ergosterol were 0.0972/0.5128, 0.0939/0.3571 and 0.1032/0.3702 g/dry weight respectively. The extracts were able to inhibit the growth, affect the intracellular pH (by extension the membrane integrity) and interfere with the sterol metabolism in *C. albicans* ATCC 10231.

Keywords: *Candida albicans*, *Sansevieria aethiopica,* ergosterol, proton pumping, intracellular pH, fungicide

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Introduction

Candida albicans is a fungus usually reproduced by budding it is a normal flora of human gastrointestinal and vaginal tracts [1]. The occurrence of candidiasis is lower than the bacterial infections; however *Candida albicans* infections ranked fourth among nosocomial bloodstream infections leading to death [2-4]. *Candida* spp. are frequently colonizes mucous membrane of human where they act as opportunistic fungal pathogens which may develop to candidaemia [5,6]. The predisposing factors have been identified to include: high diabetes, pregnancy, usage oral contraceptives and antibiotics [7,8]. Candidal infections have increased significantly in the recent time with mortality rate of 40% [6-9]. *Candida* infection has been reported to be the one of the commonest causes of vaginitis among middle age women [10,11].

Medicinal plants are used for the treatment of contagious and physiological diseases globally. The applications of medicinal plants are prominent among rural dwellers in poor-resource nations [12,13]. The global report showed an increase in the resistance of disease causing microorganisms to different orthodox medicines [14-16]. This in no doubt called for alternative and effective therapy.

The use of medicinal plants has gained a wide recorgnition as a result of its safety, low cost and effectiveness [17,18]. This now shifted the focus to medicinal plants for the treatment of infections. These attributes make medicinal phytomedicine to be preferred to the conventional chemotherapeutics agents [17-20].

Sansevieria aethiopica (Thunb) is a perennial shrub with tough and erected leaves [21] used for the treatment of oral, ear and other

fungal infections [22-23]. Despite its popular uses of *S.* aethiopica its mechanisms of actions have not been reported hence the aim of this study. We proposed the possible mechanisms of actions of *S. aethiopica* against *C. albicans* in this study.

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. At the Giffen Herbarium of the Department of Botany, University of Fort Hare, Alice, South Africa, the plant was authenticated by Prof. D. Grierson and the voucher (DavMed 2012/2) was submitted to the same Herbarium. The plant was dried in the oven at 40 °C and pulverized to fine powder and a 50 g of ground plant sample was soaked in 500 ml of each of the solvents 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 Laborata 4000-efficient (Heldoph, Germany). Each of the dried extracts was dissolved in water + 2% dimethyl sulfoxide (DMSO). The reconstituted extracts were filter by 0.45 µl pore size membrane filter for sterility.

Source of the organism

Candida 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 x 10^7 cfu/ml) and diluted with sterile PDB to achieve the final concentration of 1.0 x 10^6 cfu/ml.

Determination of minimum inhibitory concentration (MIC)

Macrobroth dilution method of Clinical and Laboratory Standards Institute (CLSI) [24] was used to determine the *in vitro* antifungal activity of the extracts of *S. aethiopica*. Extract of the plant sterilized by membrane filtered was incorporated into Potato Dextraose Broth in test tubes to give a final concentrations ranging from 0.078125 to 10.00 mg/ml. The tubes were inoculated with 100 µl of standardized inoculum of *C. anlbicans* ATCC 10231. A tube Amphotericin B and with only broth were used as a positive and negative controls respectively. The tubes were incubated aerobically at 37 °C for 24 h. The first tube in the series with no sign of visible growth was taken as the MIC.

Determination of minimum fungicidal concentration (MFC)

A loopful of culture from the first three broth tubes that showed no growth in the MIC tubes were inoculated on sterile PDA plates and observed for growth after incubation at 37 °C for 24 h. The MFC was taking as the least concentration of the extracts that showed no growth. MIC index (MICI) was calculated as the ratio of MFC and MIC. The result was interpreted as follow: MFC/MIC ≤2.0 was considered bactericidal, if >2 but <16 it was considered bacteriostatic and lastly if the ratio is ≥16.0, the extract was considered ineffective as reported by Shanmughapriya et al. [25].

In situ electron microscopy

The effects of the extracts of *S. aethiopica* on the ultra-structure of the *C. albicans* ATCC 10231 was determined by the method of Kamilla et al. [26] with minor modifications. Standardized inoculum of *C. albicans* ATCC 10231 (with optical density of 0.1 at the wavelength of 650nm) was seeded on a sterile plate of Potato Dextrose Agar and incubated for 2 h at 37 °C. The plant extracts at different minimum fungicidal concentrations was applied on the culture and the plate was further incubated for another 6 h at 37 °C. Five mm sterile borer was used to remove plugs from the culture 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).

Proton pumping activity

The method of Shreaz et al [27]. was used to determine the effect of the extracts on the proton pumping in *Candida albicans* ATCC 10231. The test fungus was culture in Yeast Nitrogen Broth supplemented with 20 % glucose and grown for 18 h at 30 °C with periodic shaking. After incubation the cells were harvested by centrifuging at 3500 × g for 10 min at 4 °C and washed twice with sterile distilled water. Harvested cells (0.1 g) were re-suspended in 5 ml solution containing 0.1 M KCl and 0.1 mM $CaCl_2$. The ¼ MIC and ½ MIC of extracts of *S. aethiopica* were added separately into the suspension after which the pH was adjusted to 7.0 using 0.01 M HCl/NaOH. Dimethyl sulfoxide (2%) and Amphotericin B (6 µg/ml) were used as negative and positive control respectively in the assay. The pH of the suspension was adjusted to 7.0 using 0.01 M HCl/NaOH. Test extracts were added to achieve the desired concentrations (¼ MIC and ½ MIC) in 5 ml solution. A 100 ml of glucose was added to the suspension to achieve a final concentration of 5 mM and the pH of the suspension was monitored, over a period of 60 min, using Crison Basic 20 pH meter (Barcelona, Spain).

Measurement of intracellular pH (pHi)

Modified method of Bhatia et al [28]. was used to determine the intracellular pH of the test fungus. As described above, *C. albicans* ATCC 10231 cells were grown, harvested and washed twice with sterile distilled water as described earlier and 0.1 g of the cells was suspended in 5 ml 50 mM KCl. The extract of *S. aethiopica* was added at sub MICs in each case and Amphotericin B was also added to the control group. The pH of the medium was adjusted to 7.0 using Crison Basic 20 pH meter (Barcelona, Spain) and incubated at 37 °C for 30 min with constant shaking. The pH of the medium was monitor and the point of no further change in the pH was taking as the internal pH.

Determination of ergosterol content in the plasma membrane

Total intracellular sterols were extracted as reported by Arthington-Skaggs et al [29]. with slight modification. Standardized inoculum of *C. albicans* ATCC 10231 was plated in Potato Dextrose Broth (PDB) medium containing sub-MICs of extracts of *S. aethiopica* except, the control, and incubated for 24 h at 37 °C. The cells were harvested by centrifugation at 2,700 rpm for 5 min washed twice with distilled water and cell pallets weighed. A 5 ml of 25% alcoholic potassium hydroxide (25 g of KOH and 36 ml of sterile

distilled water, brought to 100 ml with 100% ethanol) solution was added to each sample and vortex mixed for 2 min and incubated in an 80 °C water bath for 1 h. After incubation, tubes were allowed to cool to room temperature and 2 ml of sterile distilled water and 5 ml of *n*-heptane, followed by vigorous vortex mixing for 3 min. The samples were stored at -20 °C for 24 h. The *n*-heptane layer was scan spectrophotometrically by UV-VIS 3000PC over a wavelength range of 220 and 300 nm. Ergosterol and late sterol intermediate 24(28) dehydroergosterol (DHE) in the extracted sample were quantified from the result as followed Ergosterol content was calculated as a percentage of the wet weight of the cell by the following equations:

% Ergosterol + %24(28) DHE = (A_{282}/290)/pellet weight

%24(28) DHE = (A_{230}/518)/pellet weight

% Ergosterol = % Ergosterol + %24(28) DHE - %24(28) DHE

Where 290 and 518 are E value (in percentage per cm) determined for crystalline ergosterol and 24(28)dehydroergosterol respectively.

Statistical Analyses

All the experiments were conducted in triplicates and repeated twice. Data are shown as mean ± standard deviation (SD) for quantitative variables and as absolute qualitative variables. Analyses were conducted by Dunnett Multiple Comparisons Test to compare both treatment groups with the control using SPSS version 17.0 (SPSS, Chicago, IL, USA).

Results and Discussion

The search for new antifungal drugs is very necessary in the face of current resistance of pathogens to the present chemotherapy [30]. The MIC of the extracts ranged between 1.5625 and 3.125 mg/ml. Acetone extract had the highest MIC of 3.125 mg/ml and both Acetone and ethanolic extracts had MFC of 6.250 mg/ml as shown in **Table 1**. Acetone and methanolic extracts had fungicidal effects on the test yeast. Plant extracts or plant-derived compounds have been very effective against *Candida* spp. This results support the reports of other researchers that plant extracts are very effective in the treatment of candidiasis [16,18,20].

The cells in the control plate are with a normal, round and smooth-surfaced while those in the control in the treated plate showed alterations in the cell morphology (**Plate 1**). In the plate B there were burst cells that have released out the cytoplasmic contents. Treated cells showed changes in their morphology, a number of damaged cells features were shown. Wrinkled surfaces, shrinkages, tears and holes in the cells were observed in the treated cells. The effects of the extracts of *S. aethiopica* with cell membrane integrity as evidenced by shrinkage of cell surface and lysis of sessile cells indicate their effectiveness in the eradication of *C. albicans.* This is similar to reports of other researchers that have indicated the compounds from plants exert disruption of cell membrane of the yeast cells [31,32]. The changes in membrane morphology of *Candida albicans* ATCC 10231 treated with extracts of *S. aethiopica* we observed is similar to the findings of Pan et al [33]. who reported extracellular material and membrane perturbations as a result of membranes destruction in *C. albicans.*

Glucose-dependent medium acidification provides a relative measure of proton pumping by the plasma membrane. The effect of extracts of *S. aethiopica* on glucose-dependent proton pumping by *C. albicans* ATCC 10231 was evaluated. Compared with the control, there was a significant difference in the data obtained in the treatment groups: ethanolic and methanolic extracts of *S. aethiopica* at ½ MIC and ¼ MIC as shown in Table 2. Glucose dependent proton pumping showed a dose-and time-dependent inhibition of proton pumping. The proton-pumping ability of fungi is crucial for the regulation of the internal pH of a fungal cell. When fungal cells depleted of their carbon sources are exposed to glucose, the sugar is rapidly taken up by the cells

Table 1 Antifungal activity of extracts of *S. aethiopica* (mg/ml) on *C. albicans* ATCC 10231.

Extracts	MIC	MFC	MFC/ MIC	Interpretation
Acetone	3.125	6.250	2	Fungicidal
Ethanol	1.5625	6.250	4	Fungistatic
Methanol	1.5625	1.5625	1	Fungicidal
Amphotericin B (mg/ml)	0.0080	0.0160	2	Fungicidal

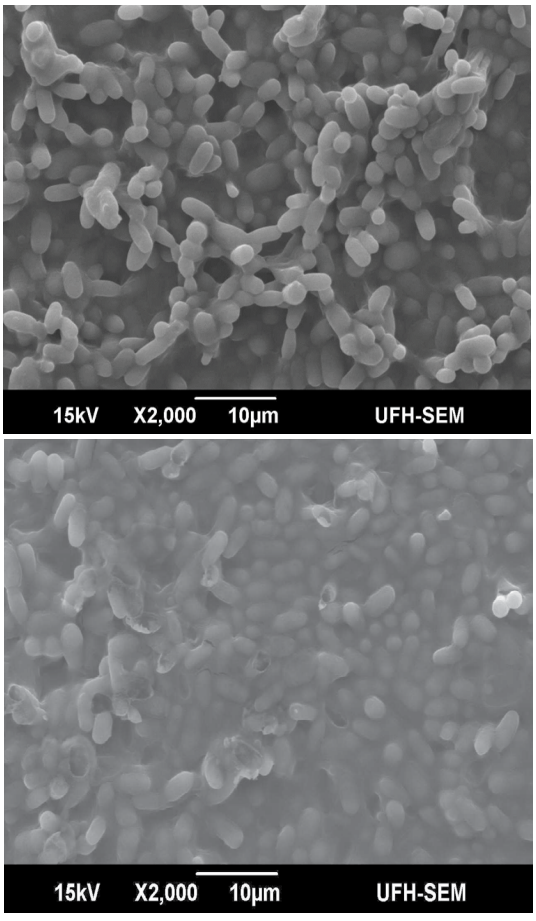

Plate 1 Scanning electron micrographs of effects of extracts of *S. aethiopica* on *C. albicans* ATCC 10231. Label (A) represents the control while (B) is a representative of the treated group.

Table 2 Effect of extracts of *S. aethiopica* on proton pumping of *C. albicans* ATCC 10231.

Extracts	Conc.	Time in minutes						
		0	10	20	30	40	50	60
Ethanolic	½ MIC[a,e,f]	7.00	6.25±0.13	6.21±0.23	6.17±0.19	6.02±0±0.73	5.98±0.67	5.51±0.49
	¼ MIC	7.00	6.23±0.46	6.11±0.45	6.10±0.82	5.82±0.93	5.77±0.43	5.62±0.92
Methanol	½ MIC[b,f]	7.00	6.34±0.34	5.99±0.64	5.73±0.73	5.44±0.46	5.32±0.82	4.70±0.84
	¼ MIC[c,f]	7.00	6.33±0.47	5.83±0.69	5.65±0.46	5.4±0.48	5.26±0.73	4.89±0.45
Acetone	½ MIC[d,f]	7.00	6.32±0.55	5.74±0.93	5.48±0.37	5.21±0.29	4.93±0.48	4.39±0.45
	¼ MIC[e,a,f]	7.00	6.12±0.11	5.53±0.28	5.37±0.12	5.02±0.46	4.32±0.25	4.15±0.45
Control[f,a,b,c,d]		7.00	5.82±0.92	5.25±0.72	5.00±0.84	4.73±0.99	4.23±0.37	4.01±0.57

[a,b,c,d]Group showing significant difference at 0.05 probability level

Table 3 Intracellular pH (pHi) in presence of the extracts of *S. aethiopica*in *C. albicans* ATCC 10231.

Extracts	Concentrations		
		¼ MIC	½ MIC
Acetone	Mean	5.91	5.40
	SEM	0.34	1.24
Ethanol	Mean	6.03	5.52
	SEM	1.01	0.72
Methanolic	Mean	5.87	5.24
	SEM	0.32	0.02
Control (Amphotericin B)	Mean	7.38	
	SEM	0.34	

Table 4 Inhibition of ergosterol biosynthesis in *C. albicans* ATCC 10231.

Extract		Pallet weight (g)	Percentage of the wet weight		
			Ergosterol + 24(28)DHE	24(28)DHE	Ergosterol
Acetone	½MIC[a]	1.23±0.13	0.4619±0.0302	0.0798±0.0016	0.3822±0.0240
	¼MIC	2.49±0.36	0.6100±0.0023	0.0972±0.0035	0.5128±0.0722
Methanol	½MIC[b]	1.28±0.11	0.2883±0.0104	0.0843±0.0204	0.2040±0.0492
	¼MIC[c]	2.01±0.09	0.4510±0.0033	0.0939±0.0310	0.3571±0.0240
Ethanol	½MIC[d]	1.39±0.14	0.2655±0.0308	0.0776±0.0100	0.1879±0.0085
	¼MIC	2.87±0.40	0.4734±0.0200	0.1032±0.0378	0.3702±0.0449
Control[a,b,c,d]		4.81±1.73	1.4786±0.0382	0.8203±0.2391	0.6583±0.0231

by the proton motive force generated by the proton gradient due to the pumping out of intracellular protons [27,34].

The effect of extracts on the proton-pumping ability of *C. albicans* was determined by glucose-induced acidification of external medium. The extracts inhibited the glucose-induced acidification of the external medium by *C. albicans* in a concentration-dependent manner. As shown in **Table 2**, at ½MIC the rate of inhibition of glucose-induced acidification of the external medium by *C. albicans,* measured in term of the pH of the medium, within 30 min of the experiment ranged between 6.25-5.51, 6.34-4.70 and 6.32-4.39 for ethanolic, methanolic and acetone extracts respectively. Paired Samples Statistics (Paired T-test) at probability level of 0.05 showed a significance difference between the control and the three test groups treated at the ½MIC. The activity of the lower concentration ¼MIC was expectedly lower that the ½MIC in all the treatment groups.

Regulation of intracellular pH is a fundamental to the growth of *Candida* and activation of plasma membrane ATPase as it is involved in maintenance of pHi [35,36]. From the **Table 3**, the internal pH of test fungus ranged between 5.87 and 6.03 for the cells treated with ¼MIC. The value was lower in the cells treated with ½MIC than those treated with ¼MIC of the plant extracts. For the cells treated with ½MIC the highest pHi was recorded in ethanolic extract treated cells while the least was observed in the methanolic extracts. The decrease in pHi was more in cells exposed to the extracts compared with the control. Prolonged intracellular acidification has been reported to have deleterious effect on cell viability [37,38]. This also affects the efflux of potassium ions [39] and cellular ion homeostasis [37,40].

Sterol quantification has been reported by Arthington-Skaggs et al [30]. To be more predictive of *in vivo* outcome than the broth microdilution procedure. We noticed a decrease in the level of the ergosterol and 24(28)DHE in the treated cell compared to the control. The amounts of the sterols were least in the cell treated with ½MIC of the extract of *S. aethiopica.* This showed a concentration dependent effect of the plant extracts. At the ½MIC of acetone, methanol and ethanol the amount of 24(28)DHE/ergosterol were 0.0972/0.5128, 0.0939/0.3571 and 0.1032/0.3702 respectively **(Table 4)**. This finding has been corroborated by other researchers [41-43]. Ergosterol is a significant component of *Candida* spp. and its content is affected by chemical and physical factors [44].

We observed a decreased in ergosterol content Candida cells treated with the plant extracts. The difference in the activity of the extract may be due to their difference in terms of interactions

with cholesterol as reported by Henriksen et al [45]. This is as a result of interference with the sterol metabolism which is the primary cellular process. This shows that the extracts target the cell membranes of *Candida albicans*. In conclusion, the extracts of *S. aethiopica* have been able to inhibit the growth of *C. albicans* ATCC 10231 and also we observed that the extracts were able to affect the intracellular pH and interfere with the sterol metabolism. This plant has multiple actions on the cell wall of the test fungus.

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