

Evaluation of the anti-candidal activity of methanolic leaf extract of *Cleistopholis patens* (fam. Annonaceae) on candida species isolated from stage II HIV patients.

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Abstract

Background: *Candida* species (sp) is implicated in causing opportunistic disseminated mycotic complications in stage II HIV patients. *Cleistopholis patens* is a West African medicinal tree reported to have significant antifungal activity against *C. albicans*.

Objectives: This study aimed to determine the anti-candidal activity of methanolic leaf extract of *Cleistopholis patens* against *Candida* species isolated from stage II HIV patients.

Methods: The minimum inhibitory concentration (MIC) of the extract and Nystatin® was determined by agar dilution method. The killing rate studies of the plant extract and Nystatin® were also determined.

Results: The extract had activity against all *Candida* isolates, with the MIC against the five isolates ranging from 6.0 - 9.8 mg/ml. Nystatin® also demonstrated plausible activity against the isolates with MICs ranging from 0.3125 – 25 mg/ml. *Candida albicans* strain 2 was the most sensitive to both extract and Nystatin® with MIC values of 6 and 0.3125 mg/ml respectively. *Candida krusei* was the least sensitive with MIC values of 9.8 and 25 mg/ml for the extract and Nystatin® respectively. The killing rate values for the extract ranged from -0.029 to -0.091 min⁻¹ and that of Nystatin® ranged from -0.076 to -0.112¹⁶ min⁻¹.

Conclusions: The results indicate that the methanolic extract of *Cleistopholis patens* is a promising clinical alternative besides Nystatin® in the treatment of infections caused by *Candida* species in stage II HIV patients.

Keywords: Anti-candidal activity, *Cleistopholis patens*, *Candida*, HIV Patients.

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Introduction

There is need for new effective and less toxic antifungal antibiotics for the treatment of disseminated mycotic infections in the light of the significant toxicities, failure rates of the currently available systemic antifungal agents and emergence of resistant strains of fungi for instance *Candida* sp implicated in opportunistic disseminated mycotic complications in stage II HIV patients¹. These events further heightened the search for new alternative drugs of plant origin in combating opportunistic mycotic infections associated with stage II

HIV infection². Discovery of new antimicrobials has relied primarily upon isolation of such agents from natural sources³. This search was extended to the leaves of the plant *Cleistopholis patens* (Benth.) Engl. & Diels found distributed in various parts of tropical Africa, in the rain forest region such as Burkina Faso, Cote d'ivoire, Ghana, Liberia, Sierra Leone, Togo and Nigeria⁴. It is a small to medium sized tree up to 27m high of secondary and swamp forest with distinctive airy crown of horizontal branches and branchlets with well dispersed leaves resembling compound leaves^{5,6}. A new alkaloid (3-methoxysampangine) which exhibited significant activity against *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans* was isolated from the root bark of *Cleistopholis patens*¹. The anti-plasmodial activity of nonvolatile and volatile extract from the stem bark of *Cleistopholis patens* had been reported^{7,8}. According to ethno medicinal report the stem bark of the plant is used in the treatment of jaundice⁹, infective hepatitis and stomach disorders¹⁰. The roots are used as a vermifuge¹⁰ and the leaves are said to remedy fever¹¹.

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This paper describes the anti-candidal activity of the leaves of *Cleistopholis patens* (Benth.) Engl.& Diels.

Materials and methods

Test microorganisms

Test microorganisms used were four strains of *Candida albicans* and a strain of *Candida krusei* previously isolated from stage II HIV patients visiting University of Nigeria of Teaching Hospital Enugu. The *Candida* spp were isolated using the method described by Jagdev and Arora¹². Two oral swabs were collected from each patient under sterile conditions. One swab was used for Gram staining and KOH wet mount and other swab was inoculated on Sabouraud's dextrose agar (SDA) with chloramphenicol and incubated at 25°C. All the cultures were observed every day for a week for the appearance of colonies suggestive of yeast like organism. The various isolates were identified and characterized at the mycology laboratory of the Department of Microbiology University of Nigeria, Nsukka using standard methods described by Larone¹³.

Plant material

The leaves of *Cleistopholis patens* were collected from Nsukka in Enugu state between the months of May and June. The leaves were authenticated by Mr. Alfred Ozioko the taxonomist at the International Centre for Ethnomedicine and Drug Development, Nsukka where a voucher specimen with voucher number INTERCEDD/505 was kept in the herbarium for future reference.

Extraction solvents and other materials

Analytical grade of methanol (BDH, England) was used. Other materials used include Nystatin®, Sabouraud's dextrose agar (SDA) (Oxoid, England), Dimethylsulphoxide (DMSO) (BDH, England) and chloramphenicol (Fidson, Nigeria).

Extraction of *Cleistopholis patens*

The collected leaves were shade dried and milled. The powder was cold macerated in methanol and allowed to stand overnight. It was then clarified with the aid of cotton wool. Subsequently the methanol extract was evaporated to dryness and stored in a refrigerator.

Collection of test microorganisms

Slant cultures of test fungi were obtained from the Mycology laboratory of the Department of Microbiology University of Nigeria. The inoculated slants were incu-

bated at 25°C for three days, and subsequently stored in a refrigerator.

Standardization of test microorganisms

A loopful of the SDA slant cultures above was obtained and introduced into a sterile test tube containing 5ml sterile distilled water. The volume was adjusted by dilution to obtain cell population of 1.0×10^6 cfu/ml equivalent to 1.0 McFarland standard.

Preliminary sensitivity test of extract and Nystatin®

A modification of the agar dilution method described by Esimone et al¹⁴ was used. Exactly 1.5 g of the dried methanolic extract was dissolved in 15ml of Dimethylsulphoxide (DMSO) to obtain a stock concentration of 100 mg/ml. Subsequently 0.5, 1 and 2 ml of the stock concentration were diluted with 9.5, 9 and 8ml of sterile molten SDA in three sterile petri dishes respectively. The following concentrations were obtained namely 5, 10 and 20mg/ml. The setup was thoroughly mixed by swirling and allowed to set, after which the SDA plates were dried in the dryer at 37°C for 1hr. Thereafter the SDA plates were inoculated with the test organisms and incubated at 25°C for 24 hrs after which the results were recorded. The method was repeated for Nystatin® against the test isolates. However 0.5, 1.5, and 2.5ml of stock concentration of Nystatin® of 100 mg/ml were diluted with 9.5, 8.5 and 7.5 ml of sterile molten SDA in three sterile petri dishes to obtain concentrations of 5, 15, and 25mg/ml of Nystatin®.

Minimum Inhibitory Concentration (MIC) of extract

A modification of the agar dilution method described by Esimone et al¹⁴ was used. Arithmetic serial dilutions of 100 mg/ml stock concentration of extract were made using sterile molten SDA to obtain final agar concentrations ranging from 5 to 20 mg/ml. The plates were allowed to set after which they were dried at 37°C for 1hr. Thereafter the plates were streaked with the test microorganisms and incubated at 25°C for 24 hrs. The MIC of the extract was determined.

Minimum Inhibitory Concentration (MIC) of Nystatin®

The same method described above was similarly employed to determine the MIC of Nystatin®.

Determination of the killing rate of extract and Nystatin®

A modification of the killing rate method described by Iroha et al¹⁵ was used. Exactly 1 ml of standardized culture of the fungi (1×10^6 cfu/ml equivalent to 1.0 McFarland's standard) was inoculated into test tubes containing 9 ml of the antimicrobial agents (extract or Nystatin® in concentration thrice their MIC values) in double strength SDA. Prior to viable cell count determination, the antimicrobial effect of the extract or Nystatin® in the samples withdrawn at various time intervals was terminated by diluting the withdrawn samples 1000 folds. Thereafter the viable count was determined using the surface viable count method.

Statistical analysis

The statistical analysis of the MICs and killing rate values for extract and Nystatin® was done with SPSS (version 20). Data was tested for statistical significance using Student's independent samples T-test and differences were considered significance at $P < 0.05$.

Results

Preliminary sensitivity test results

Result of the preliminary sensitivity test shown in Table 1 indicated that the plant extract had activities against test organisms down to the concentration of 10mg/ml. No growth inhibition was observed for all the test organisms at the concentration of 5mg/ml.

Table 1: Sensitivity results of *Candida* isolates to Extract and Nystatin®

S/N	Organism	Concentration (mg/ml)	
		Extract	Nystatin®
1	<i>Candida albicans</i>	10	5
2	<i>Candida albicans</i>	10	5
3	<i>Candida albicans</i>	10	5
4	<i>Candida albicans</i>	10	5
5	<i>Candida krusei</i>	10	25

Key: - = Growth inhibition: + = No growth inhibition

Furthermore all isolates except *Candida krusei* were sensitive to Nystatin® down to 5mg/ml. *Candida krusei* was sensitive at the concentration of 25mg/ml but resistant at 15 and 5mg/ml respectively.

MIC Determination of Plant Extract and Nystatin®

The MIC values (Table 2) of the plant extract ranged from 6 - 9.8mg/ml. The highest MIC value was recorded against *Candida krusei* whereas *Candida albicans*

strain 2 had the lowest MIC value for the plant extract. The MIC values of Nystatin® against these isolates ranged from 0.3125 -25 mg/ml. The highest MIC value for Nystatin® was obtained against *Candida krusei* and the lowest value was recorded against *Candida albicans* strain 2 as indicated in the table. Strains 1, 2, and 4 of *Candida albicans* were more sensitive to Nystatin® at lower concentrations than they were to the plant extract. Furthermore *Candida albicans* strain 3 was more sensitive to the plant extract than to Nystatin®.

Table 2: Minimum inhibitory concentration of plant extract and Nystatin® against *Candida* isolates

		Concentration (mg/ml)	
S/N	Organism	Extract	Nystatin®
1	<i>Candida albicans</i>	7.0 ± 1.00	2.5 ± 0.00
2	<i>Candida albicans</i>	6.0 ± 1.00	0.3125 ± 22.0
3	<i>Candida albicans</i>	9.0 ± 2.00	22.0 ± 2.00
4	<i>Candida albicans</i>	9.4 ± 0.40	0.625 ± 0.00
5	<i>Candida krusei</i>	9.8 ± 0.10	25.0 ± 2.65

Also *Candida albicans* strains 3 and 5 were more susceptible to Nystatin® at higher concentrations of 22 and 25 mg/ml than they were to the plant extract. The statistical analysis showed that there was significant dif-

ference at $P < 0.05$ between the MICs of the plant extract and Nystatin® against *Candida* isolates.

Killing rate study of plant extract and Nystatin®

The results of the killing rate study of the plant extract and Nystatin® are shown in Figures 1-3.

Figure 1. Plot of natural log number of survivor cells of *C. albicans* strains 1-4, and *C. krusei* against time for extract.

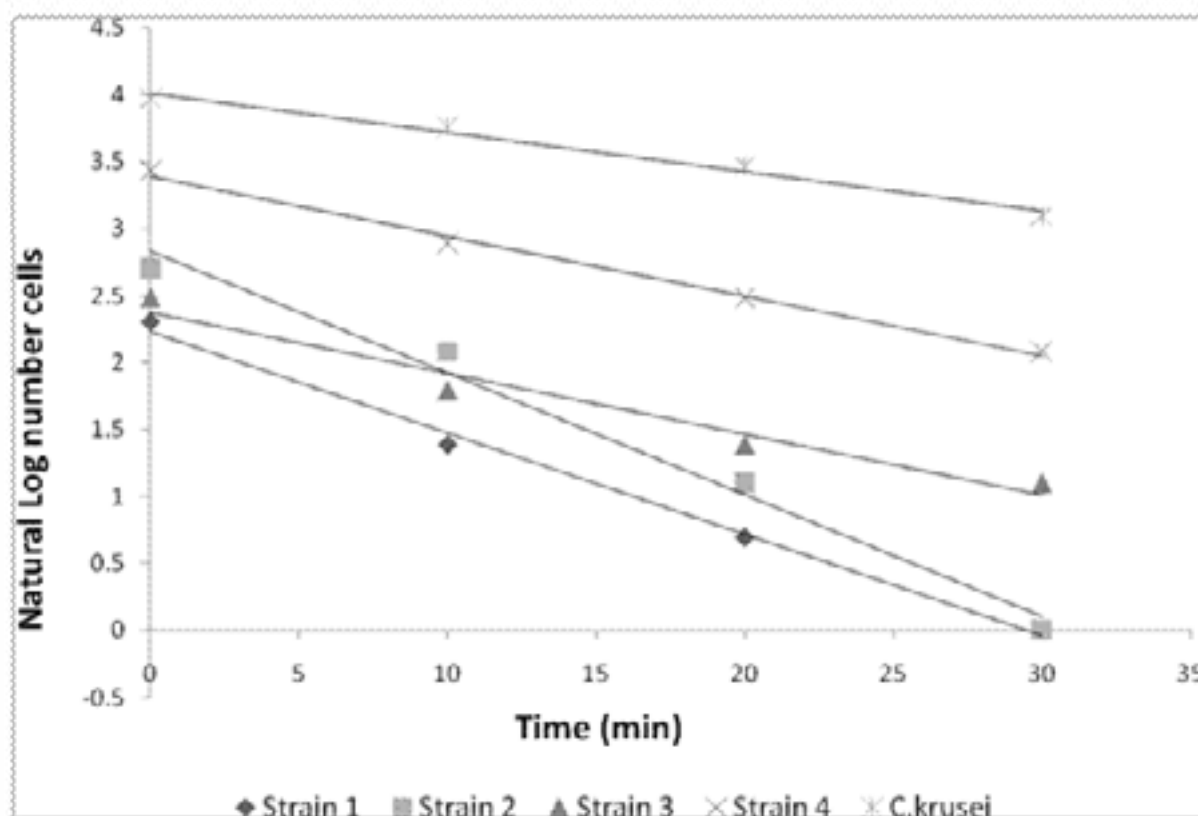


Figure 2. . Plot of Log number of survivor cells of *C. albicans* strains 1,2,and *C. krusei* against time for Nystatin®.

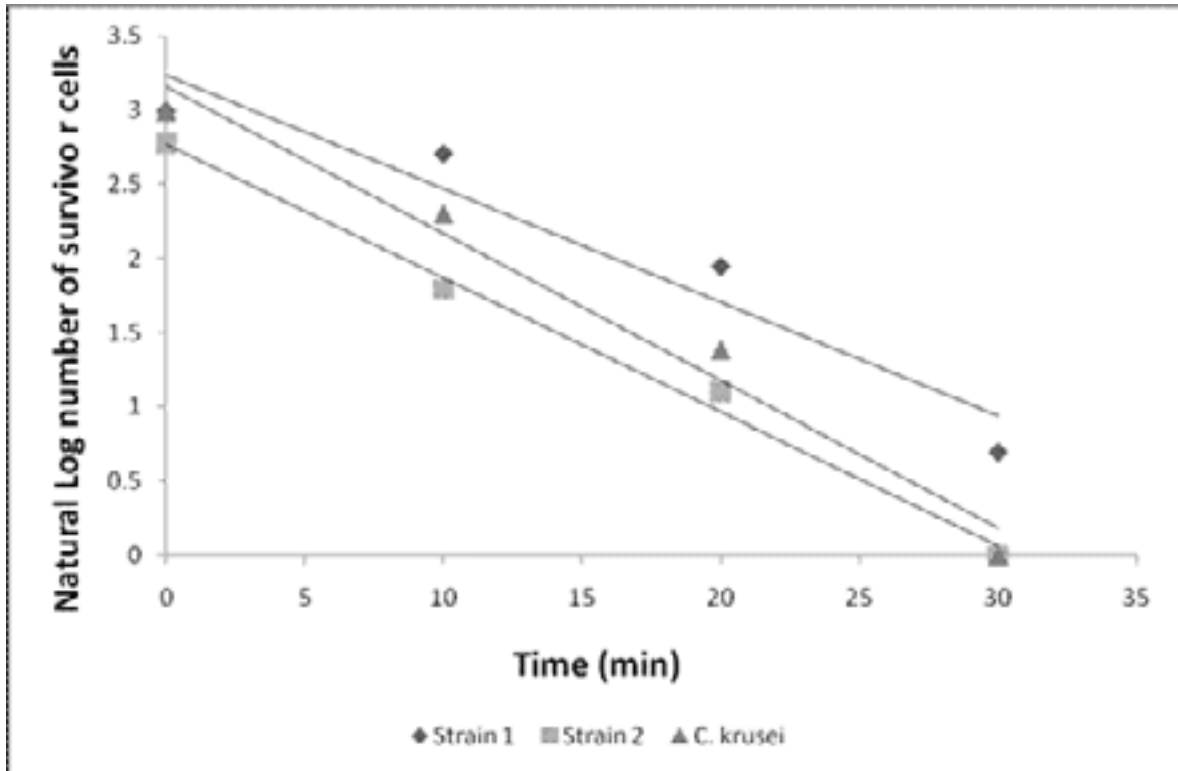
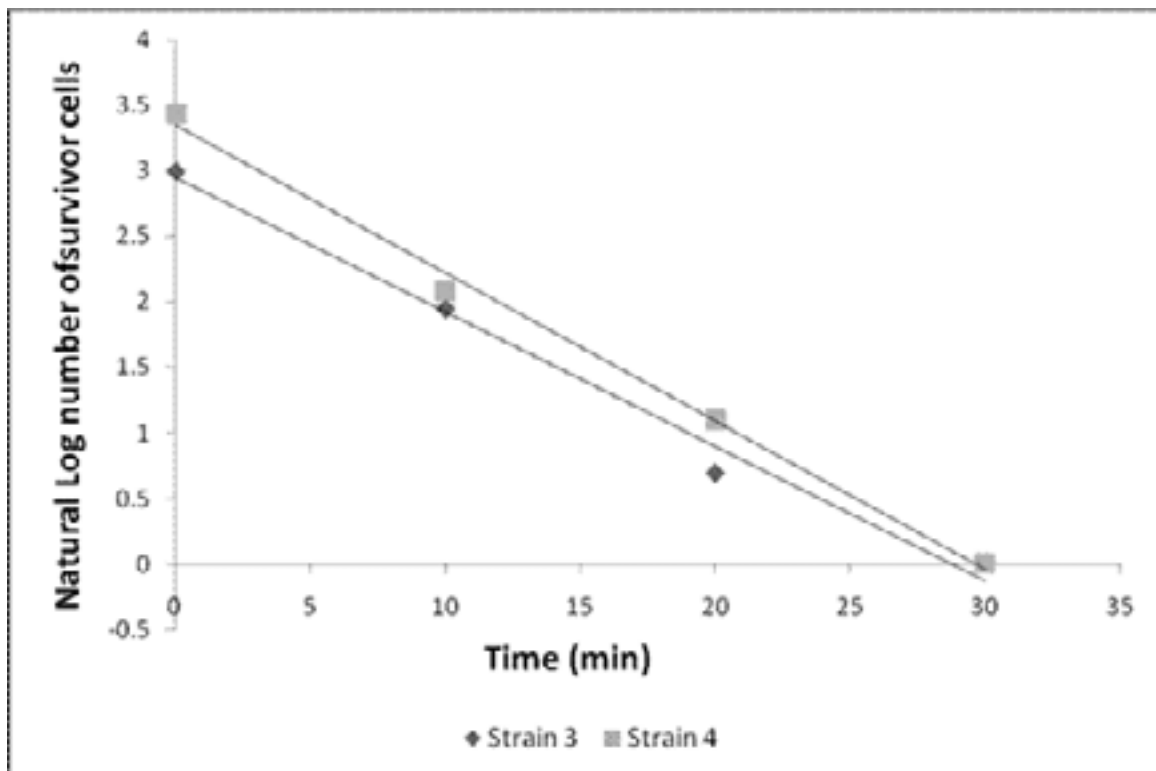


Figure 3. Plot of Log number of survivor cells of *Candida albicans* strains 3 and 4 against time for Nystatin®.



The general formula used to express the rate at which a specified concentration or amount of an antimicrobial

agent kills microbial cells in a population is given by $\ln N_t = \ln N_0 - Kt$ where K represents the death rate constant or rate of kill of the antimicrobial agent.

Table 3: The values of the slopes (rate of kill) and R² for the extract and Nystatin® on the isolates

S/N	Isolates	Slope values		R ² Values	
		Extract(min ⁻¹)	Nystatin®(min ⁻¹)	Extract	Nystatin®
1	<i>Candida albicans</i>	-0.076 ± 0.002	-0.076 ± 0.001	0.994	0.926
2	<i>Candida albicans</i>	-0.091 ± 0.001	-0.090 ± 0.001	0.986	0.993
3	<i>Candida albicans</i>	-0.045 ± 0.002	-0.102 ± 0.002	0.960	0.988
4	<i>Candida albicans</i>	-0.044 ± 0.003	-0.112 ± 0.001	0.994	0.995
5	<i>Candida krusei</i>	-0.029 ± 0.002	-0.099 ± 0.00153	0.984	0.975

It is obtained as the slope when Log number of survivors is plotted against time (t). The values of the R² and slopes (rate of kill) for the extract and Nystatin® deduced from Figures 1-3 are shown in Table 3.

The rate of kill of the extract against the *Candida* isolates ranged from -0.029 to -0.091 min⁻¹ and that of Nystatin® ranged from -0.076 to -0.112 min⁻¹. The killing rate value for the extract against strain 2 is slightly higher than that of Nystatin®. Nystatin® recorded higher killing rate values against the rest of the isolates than the extract except for *Candida albicans* strain 1 in which the values were the same. Also the killing rate values of Nystatin® against strains 3 and 4 of *Candida albicans* were more than twice the values of the killing rate of the extract against the same organisms. In the case of *Candida krusei*, the killing rate value for Nystatin® was more than thrice the value obtained for the extract against the same organism. However the killing rate values of the extract against *Candida albicans* strain 4 (-0.044 min⁻¹) and strain 3 (-0.045 min⁻¹) were very close to each other. There was a significant difference between the killing rates values of the plant extract and Nystatin® against the *Candida* isolates at P < 0.05.

Discussion

Observations made from the preliminary sensitivity tests results (Table 1) of both the extract and Nystatin®

respectively indicated that all *Candida* species used in the study were sensitive to the extract even at 10mg/ml concentration. At 5mg/ml all showed resistance to the extract but were all sensitive to Nystatin® at this concentration except *Candida krusei*. Furthermore it was observed from the sensitivity result of Nystatin® that all the *Candida* species used except *Candida krusei* were sensitive to Nystatin® at 5mg/ml concentration. The later was sensitive to Nystatin® only at 25 mg/ml but manifested resistance at 5 and 15mg/ml. The activity demonstrated by the methanolic leaf extract of *Cleistopholis patens* in this study was because of the presence of copious amount of terpenoids, saponins, glycosides, steroids, and alkaloids¹⁶ which are secondary metabolites with known antimicrobial activity as demonstrated in previous studies by other workers^{17,18,19,20}. In addition, the MIC values of Nystatin® against the *Candida* species except *Candida albicans* strain 3 and *Candida krusei* were lower than the MIC values of the extract against these organisms. From our study, infections caused by these *Candida* species in those HIV patients with the exception of *Candida albicans* strain 3 and *Candida krusei*, would be better treated with higher doses of Nystatin® than the extract because of the high MIC values of Nystatin® against the organisms. In a similar study by Hufford et al²¹ three anti-candidal constituents were isolated from the root bark of *Cleistopholis pat-*

ens. However the MIC of Eupolauridine (one of the constituents isolated) was found to be 1.56µg/ml which was greater than 0.39µg/ml the MIC value for amphotericin B against the three strains of *Candida albicans* used. Consequently they considered eupolauridine as a promising potential new antifungal drug. The methanolic extract in this present study has a promising potential as a clinical alternative to Nystatin in treatment of infections caused by *Candida albicans* and *Candida krusei*. In contrast, it is interesting to note that the MIC value of the extract against *Candida albicans* strain 3 and *Candida krusei* was significantly lower than that of Nystatin® (which was more than twice the MICs for the extract). This was deviation from the findings of Hufford et al¹. In another study, Liu et al²¹ isolated a new alkaloid, 3 – methoxysampangine in association with three already known alkaloids from the root bark of *Cleistopholis patens* which had activity against *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*.

The results from Figure 1 showed that the killing potency of the extract is highest especially against *Candida albicans* strain 2 whose population was killed within 30 min, followed by strain 1 whose population was killed within less than 30 min. For the rest of the strains, the extract could not kill their entire population within 30 min. It is an established fact that MIC is a measure of the potency of an antimicrobial drug²² hence the varying potencies of the extract against the strains of *Candida* spp used in this study reflected the MIC values of the extract against the test *Candida* strains. Thus small values of MIC of a drug against a microbe signify higher potency²². In contrast, Nystatin® exhibited the highest potency against strain 2 killing the entire population within 30 min (Fig. 2). The next in potency was against strain 4 where the entire population was killed within more than 29 min but less than 30 min (Fig. 3). Nystatin® was less potent against *Candida albicans* strain 3 and *Candida krusei* as reflected in the MIC recorded against these organisms.

Conclusion

The methanolic extract of *Cleistopholis patens* had significant anti-candidal activity as indicated in its killing potency and MIC values against *Candida* spp used in this study. Consequently the extract is considered a potential clinical alternative besides Nystatin® in the treatment of infections caused by *Candida* species in Stage II HIV patients if formulated into a dosage form.

This study highlights among other things the need for continued search for new prototype antimicrobial drugs primarily from higher plant sources, for therein lies the probability of discovery of new drugs relatively free from the challenges of toxicity and cross-resistance associated with present drug therapies. Such discovery will help in addressing opportunistic disseminated mycoses which are common complications of acquired immunodeficiency syndrome.

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