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# Anti-candida activities of four bacterial endophytic extracts isolated from *Urena lobata*.

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## Abstract

**Purpose:** Most antifungals have drawbacks, including toxicity, efficacy, and cost, and their misuse has led to the emergence of resistant strains. Therefore, more is needed. This study aimed to evaluate the antifungal properties of bacterial endophytic extracts on Candida spp.

**Materials and Methods:** Biological samples were bacterial endophytes from *Urena lobata* and Candida species isolated from Stool. The Candida species were purified on chromogenic aga medium. The MIC was determined using broth microdilution starting from 200 µg/ml as the highest concentration. The bacterial endophytes were characterised based on morphology and motility. The inhibitory parameters, time kill kinetics assay and probable site of action of the extract were determined using the cell counting method and microscopic observation under methylene blue stain.

**Results:**The characterised Candida species were *Candida albicans*, *Candida glabrata*, *Candida dubliniensis*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei*. The selected extracts from screening were A23, A34, A52, A64, A80, and A96. The bacteria producing these extracts were all atypical nonmotile and were *Streptobacillus* spp. and *Bacillus* spp. The MIC ranged between 100 µg/ml and 200 µg/ml. The time kill kinetics assay revealed that the cidal effect of the extracts started after 3 to 9 hours of contact with the extracts. The shape of the cells posttreatment revealed that the probable site of action is on the cell membrane for A23 and the cell wall, nucleus, or mitochondria for A52, A64 and A80.

**Conclusion:**Bacterial endophytes have been proven through this study to be a suitable lead compound in antifungal resistance drug development.

**Keywords:** *Candida* spp, Antifungal, Bacterial Endophytes, *Urena lobata*, Gut

## 1. Introduction

Candidiasis is an infection caused by yeasts from the *Candida* genus. More than 17 different *Candida* species are known to be etiological agents of human infection; however, the key species of medical importance that affect the gastrointestinal track are *Candida albicans (C.a), Candida glabrata (C.g), Candida dubliniensis (C.d), Candida parapsilosis (C.p), Candida tropicalis (C.t), Candida lusitaniae and Candida krusei (C.k)*[1–5]. The neoplastic disease is a common condition in patients with Gastro-Intestinal Tract (GIT) candidiasis. The oesophagus,

followed by the stomach and small intestines, are the most common sites of infection. Frequently, the yeast colonises the ulceration that appears at these sites from another disease, such as malignant gastric or peptic ulcers. From there, candida species can colonise other deep organs. Although C. albicans is still the most common cause of GIT infection, in developing countries, an increasing number of papers state that non-C. albicans candida is the cause of GIT infection. This is correlated with the emergence in these countries with the resistance of the *nonalbicans Candida* to azoles. There is extensive use of fluconazole for chemoprophylaxis and the treatment of fungal infections due to its favourable oral bioavailability and safety[2,3,6,7]. This has led to Azole-resistant *C.albicans* frequently observed in candidiasis of the GIT of HIV patients [3,4,6] Apart from *albicans*, other species of the *Candida* genus, such as *C. glabrata*, *C. krusei*, and *C. lusitaniae,* have been reported to have reduced susceptibility to fluconazole [2–4,7]

The burden of this infection in terms of expense, morbidity, and mortality is non-negligible. In the US, the global management cost is at least 2 billion dollars per year. The majority of clinically used antifungals have various drawbacks in terms of toxicity, efficacy and cost [3,4,8]. Hence, there is a great demand for novel antifungals belonging to a wide range of structural classes that selectively act on new targets with fewer side effects. Natural products, either as pure compounds or as standardised plant extracts, provide unlimited opportunities for new hits and/or leads because of the matched lower availability of chemical diversity. As far as the environment is concerned, drug discovery from plants is limited and cannot focus on protected species. As an alternative, the discovery of novel bioactive metabolites from other natural sources, such as microorganisms inhabiting marine environments, desert soils, hot springs, and mangrove forests, has been of utmost importance in recent years[9–11].

Endophytes are microorganisms that colonise asymptomatically inside the tissue of a host plant and maintain mutualistic association for the whole or part of their lifecycle[12–17] There are many reports stating their biological activities. These include antiparasitic[13,18], antiviral[13,16] antioxidant[13,19], anti-inflammatory[13], anticancer[13,18], antibacterial[13,15,16,18–20] and antifungal activities[13,16,18,21,22]. The potential of endophytes is correlated with the host plant. In that regard, two bacterial endophytes isolated from two different plants might express different secondary metabolite synthesis[9,12,14,16]

*Urena lobata* Linn, otherwise known as Caesar weed, is a shrub that grows between 0.6- 3 m tall and up to 7 cm in basal diameter. It is a member of the Malvaceae family and is known for its numerous medicinal properties. Various extracts of leaves and roots are used in herbal

medicine to treat diverse ailments, such as colic, malaria, gonorrhoea, fever, toothache, and rheumatism [23–25]. To the best of our knowledge, we did not find a report on endophytes from Urena lobata. The aim of this study, therefore, was to evaluate the antifungal activity of bacterial endophytes of *Urena lobata*.

## 2. Materials and Methods

### 2.1. Biological Samples

Bacterial endophytes isolated from the leaves, fruits, and roots of *Urena lobata* were used. Six Candida isolates were isolated from the stools at the Gynaeco-obstetric and Pediatric Hospital of Douala, Cameroon, and freely provided to us.

### 2.2. Plant identification and harvesting

*Urena lobata* in good phytosanitary health were harvested at the following geographical position 4°05’22″N 9°48’04″E, elevation: 530 m, Douala, Littoral, Cameroon, within the PK17 campus of the University of Douala. The plant parts were harvested and transported to the Microbial Bioproducts Research Unit of the Laboratory for Pharmacology, Faculty of Medicine and Pharmaceutical Sciences, the University of Douala. A voucher specimen was identified at the National Herbarium with the identification number HNC 2576**.**

### 2.3. Endophyte isolation.

Endophyte isolations were performed as we previously described[19,22]. Briefly, roots, stems, stem bark, branches, flowers, flower buds, fruits and leaves were brought to the laboratory immediately after collection. After washing with tap water, the organs were sliced into 1 cm x 1 cm pieces using a sterile blade. After that, the slices were washed again in tap water. Then, the samples were sterilized by immersion in ethanol at 70°, alternated by a passage in 1% sodium hypochlorite and rinsed in sterile distilled water.

The sterile slices were then dried under aseptic conditions and seeded on a plate containing nutrient agar + fluconazole. The plates were sealed and incubated at room temperature. The plates were observed each day for bacterial isolation.

The strains were then purified by successive subculturing of the bacteria isolated on nutrient agar until pure strains characterised by uniform morphotypes were obtained in the Petri dish. The strains were named after the Microbial Product Research Unit (MPRU) of the Laboratory

for Pharmacology at the FMSP. In preparation for fermentation, these pure strains were transplanted into Petri dishes containing Mueller-Hinton agar.

### 2.4. Extract preparation

The extracts were prepared as previously described with slight modifications[26]. The modifications used eight colonies of bacteria rather than 0.5 Mc Farland and the extraction solvent was dichloromethane methanol rather than ethyl acetate.

Briefly, four batches of each bacterium were prepared by inoculating eight colonies of each bacterium in 50 ml of Mueller Hinton broth and incubating for 1, 2, 3, or 4 weeks. The culture was agitated daily. At the corresponding end of the fermentation, 50 nl of methanol was added to each bottle, allowed to stand for 24 hours and then washed three times with pure dichloromethane. The organic layer was dried at room temperature, and the dried residues were weighed, dissolved in pure DMSO and stored in a refrigerator before pharmacological testing.

### 2.5. Characterisation of Endophytic Bacteria

The most efficient endophytic bacteria were characterised microscopically following the protocol demonstrated by Begum [27] with slight modification. The bacterial isolates were characterised morphologically under the microscope to determine whether they were cocci, bacillus, Vibrio, Spirillum or Spirochetes.

The colonies were then transferred to EMB agar and mannitol salt agar and incubated for 24 to 48 hours.

### 2.6. Differentiation of Candida species

Candida species were isolated and differentiated using Chromatic™ Candida[28]. Suspected Candida species collected from the GIT were streaked on plates containing chromogenic agar. It was later incubated aerobically at 37°C for 48 hours. After incubation, the colour and morphology of the colonies were observed and interpreted according to the provider's prescription.

### 2.7. Antifungal Screening of Endophytic Extracts for Antifungal Activity

The broth microdilution technique was used as previously described [7] with a fixed concentration of each extract.

Briefly, to 40 µl of Sabouraud dextrose broth, 10 µl of endophyte extracts (10 mg/ml in 10% DMSO) were added to each well of the 96-well plate, and 50 µl of the inoculum ($5x10^3$ CFU/ml in Sabouraud dextrose broth) of Candida species was added. The negative and positive controls were prepared under the same conditions. Ketoconazole and nystatin were used as positive controls, and the negative control was prepared under the same conditions by replacing the volume of the antifungal agent with the culture medium. The plates were incubated for 48 hours at 37°C. The plate was incubated for 48 hours and observed. The plates were observed for turbidity or a clot of cells at the bottom of the well. Only extract that showed inhibition of all 3 species and was chosen to determine the minimum inhibitory concentration. The test was performed in duplicate.

### 2.8. Determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The antifungal activity of the extracts obtained from the bacterial endophytes was evaluated by determining the minimum inhibitory concentration and minimum fungicidal concentration using the broth microdilution method given that it is the standard method recommended internationally for antibiotic susceptibility testing[29].

### 2.9. Determination of the minimum inhibitory concentration (MIC)

The MIC of the extract of bacterial endophytes is needed to inhibit the visible *in vitro* growth of a microorganism (fungi)[30]. The MIC was determined using the broth microdilution method given that it is considered the standard method recommended internationally for antibiotic susceptibility testing[29]. Briefly, a 2-fold serial dilution was performed of the extract and the reference drug (ketoconazole and nystatin), creating concentrations from 200 µg/ml to 3.125 µg/ml. Fifty microliters of freshly prepared $5\times10^3$ CFU/ml from 0.5 McFarland Standard Inoculum dilution, the plates were incubated for 48 hours at $^{37°C}$ and scored for growth. Selected wells were filled with SDB only and SDB with microorganisms to serve as negative and blank controls, respectively. This process was performed for each microorganism (*C. albicans, C. tropicalis, C. krusei, C. glabrata, C. dubliniensis* and *C. parapsilosis)* per extract.

### 2.10. Determination of the minimum fungicidal concentration (MFC)

The minimum fungicidal concentration (MFC), also known as the minimum lethal concentration (MLC), is the most common estimation of fungicidal activity. The MFC is

defined as the lowest concentration of antimicrobial agent (extract of bacterial endophytes) needed to kill 99.9% of the final inoculum after incubation for 72 hours. [31,32]

The MFC was performed using a modified procedure from Maria [32]. The MFC was performed from the MIC. No serial dilution was involved. A sterile 96-well plate was used. Samples were taken from a well with no visible growth in the MIC assay and subcultured onto freshly prepared SDB plates containing 150 µl of Sabouraud Dextrose broth. The subcultured plates for fungi were incubated at 37°C for up to 72 hours. The MFC was taken as the concentration of the extract that did not show any fungal growth on the fresh broth medium. Wells with no observed growth indicate that the extract is fungicidal, while wells with any visible growth indicate that the extract is fungistatic.

### *2.11.* Determination of the time-kill kinetics of the endophytic extracts

The time-kills kinetic study is the establishment of the rate at which a fungus is killed by the endophytic bacterial extract as a function of survival data recorded at enough exposure time points[31].

The time kill study of extracts of the bacterial endophytes was conducted as described by Adusei [33] with some modifications using the method of counting viable cells. The process was performed on *C. albicans* with the most active extracts. Briefly, the extract with the fungicidal effect was tested on 48-hour-old *C. albicans*. Using the previously stated protocol. The concentrations correspond to MIC and 2xMIC. Aliquots (10 µl) of the mixture from each well were taken at time intervals of 0, 3, 6, 9, 12, and 24 h and aseptically inoculated into SDA in sterile Petri dishes and incubated at 37°C for 24 h. A control test was performed alongside the untreated organisms. The colonies were counted, and the number of colonies forming units was evaluated and plotted against time.

### 2.12. Tentative mechanism of action

The plates were prepared according to the time kill kinetics, and the observation of fungal shape after 24 hours of contact with the MIC and 2x MIC of the most efficient extracts was performed after staining the content of the well with methylene blue and observation under a binocular microscope.

### 2.13. Data Analysis

The data obtained from this study were entered into Microsoft Excel 2016 to create tables and graphs. Further analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test from GraphPad Prism Version 8.0.1 for Windows.

## 3. Results and discussion

### 3.1. Results

Among the 105 extracts obtained from 12 endophytes, six extracts were selected for the study after showing no fungal growth at 100 µg/ml.

#### 3.1.1. Presentation of the most efficient bacterial endophytes and their extract characteristics

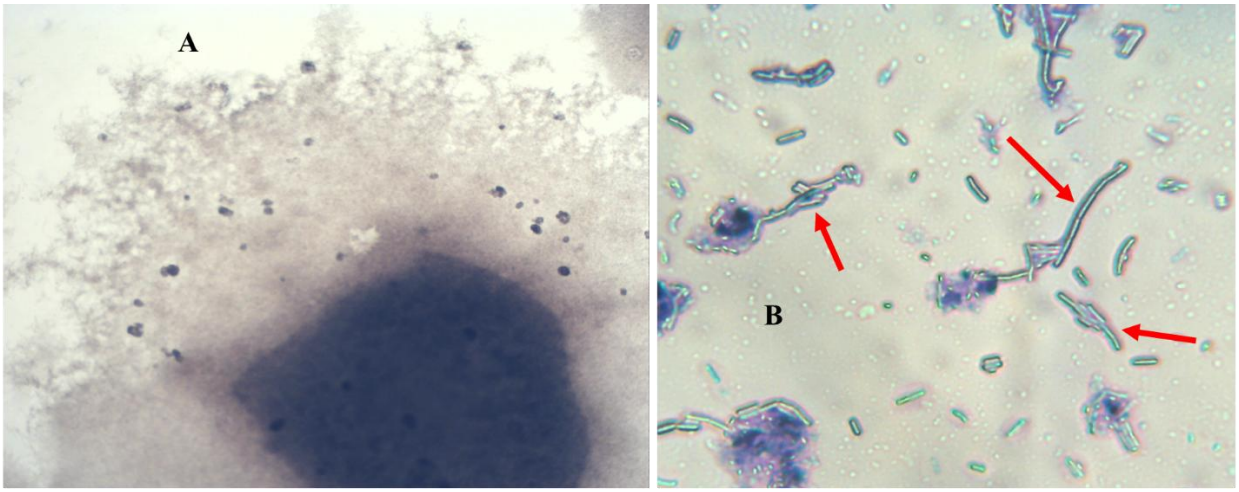
The bacterial endophytes were given code names after the Microbial Bioproducts Research Unit (MBRU) followed by a number corresponding to the code for the plants given at the entry of the laboratory. The table shows the origin of the extracts that showed complete inhibition of the fungal strains Table 1. These extracts were obtained after fermentation for 2 weeks each except for A64, which was obtained after 4 weeks of fermentation. They were obtained from different plant organs, notably fruits, leaves, and roots.

**Table 1. The extract's origins and characteristics.**

| Bacterial endophyte | Extract obtained | Plant organ | Fermentation Week |
|---|---|---|---|
| MPRU5771 | A23 | Leaf | 2 weeks |
| MPRU5433 | A34 | Fruit | 2 weeks |
| MPRU5711 | A52 | Leaf | 2 weeks |
| MPRU5431 | A64 | Fruit | 4 weeks |
| MPRU5031 | A80 | Root | 2 weeks |
| | A96 | | 3 weeks |

MBRU=Microbial Product Research Unit.

### 3.1.2. Characterization of the most efficient bacterial endophytes

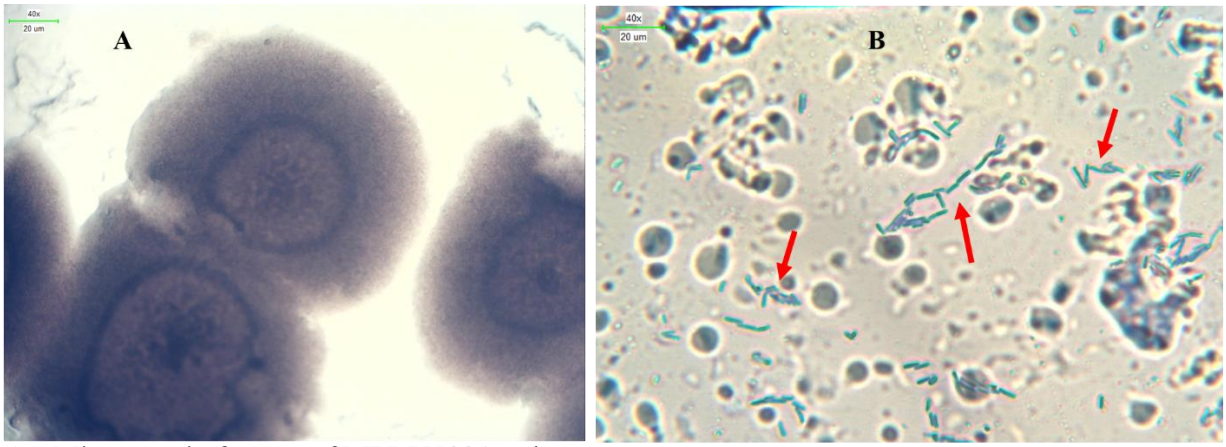



A= Microscopic feature of MPRU5433 colony with filamentous margin

B = Microscopic feature of MPRU5433 with red arrows showing cells with bacilli like shape in chains.

**Figure 1: Characteristics of MPRU5433 under a microscope at a magnification of 40x.**

From Figure 1, the shape of the colony margin and the alignment of the bacterial cells suggest that MPRU5433 is a Streptobacillus sp. The bacteria failed to grow on EMB agar and mannitol salt agar. They might be classified as atypical streptobacillus spp.




A= Microscopic feature of MPRU5031 colony with the entire margin

B = Microscopic feature MPRU5031 with red arrows showing bacilli cells

**Figure 2: Microscopic characteristics of MPRU5031 at 40x magnification.**

From Figure 2, the shape of the colony margin and the features of the bacterial cells suggest that MPRU5031 is a *Bacillus* sp. The bacteria failed to grow on EMB agar and mannitol salt agar. They might be classified as atypical Bacillus spp.

The overall shape and cells are presented in these figures. The global results of all the most active bacteria are presented in table 2.

**Table 2.** Characterisation of bacterial endophytes

| Bacterial endophyte | Morphology | Motility |
|---|---|---|
| MPRU5771 | ND | ND |
| MPRU5433 | *Streptobacillus sp* | Non motile |
| MPRU5711 | *Bacillus sp* | Non motile |
| MPRU5431 | ND | ND |
| MPRU5031 | *Bacillus sp* | Non motile |

ND = Not determined; sp = species

The results showed that MPRU5433 was found to be a *Streptobacillus spp*., MBRU5711 and MBRU5031 were both found to be *Bacillus spp.,* and they were all nonmotile.

### 3.1.3. Minimum inhibitory concentration (MIC)

The MIC in µg/ml of the 6 extracts was determined for each *Candida spp.* and is represented in the figures below:

**Table 3.** MIC (µg/ml) of different extracts

| Specie | A23 | A34 | A52 | A64 | A80 | A96 | Keto | Nyst |
|---|---|---|---|---|---|---|---|---|
| *Candida glabrata* | 100 | 100 | 100 | 100 | 100 | 200 | 100 | 100 |
| *Candida krusei* | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| *Candida parapsilosis* | 100 | 200 | 100 | 100 | 100 | 100 | 100 | 100 |
| *Candida albicans* | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| *Candida tropicalis* | 100 | 200 | 100 | 100 | 100 | 200 | 100 | 100 |
| *Candida dubliniensis* | 100 | 200 | 100 | 100 | 100 | 100 | 100 | 100 |

*Keto. = ketoconazole; Nyst. = Nystatin*

Table 3 shows the activity of the extracts on the Candida species. A23, A52, A64 and A80 all showed the best activity at a concentration of 100 µg/ml. A34 was active at 100 µg/ml against *Candida glabrata, Candida krusei,* and *Candida albicans* and at 200 µg/ml against *Candida parapsilosis, Candida tropicalis* and *Candida dubliniensis.* On the other hand, A96 was active at 100 µg/ml against *Candida krusei, Candida parapsilosis, Candida albicans* and *Candida dubliniensis* and at 200 µg/ml against *Candida tropicalis* and *Candida glabrata*. All six

species had no activity with the standard drug of reference – ketoconazole and nystatin using the same concentration as the extract were active at 100 µg/ml.

### 3.1.4. Minimum fungicidal concentration (MFC)

The results of the minimum fungicidal concentration in µg/ml of the extracts on the six candida species are shown in the table below:

**Table 4.** MFC of different extracts

| Specie | CG | CK | CP | CA | CT | CD | Conclusion |
|---|---|---|---|---|---|---|---|
| **A23** | 200 | 200 | 200 | 200 | 200 | 200 | **Fungicidal** |
| A34 | 100 | >200 | >200 | >200 | >200 | >200 | Fungistatic |
| A52 | 100 | 100 | 100 | 100 | 100 | 100 | **Fungicidal** |
| A64 | 100 | 200 | 200 | 100 | 100 | 100 | **Fungicidal** |
| A80 | 100 | 100 | 100 | 100 | 100 | 100 | **Fungicidal** |
| *A96* | >200 | 200 | 200 | 200 | >200 | 200 | Fungistatic |
| Keto | 100 | 100 | 100 | 100 | 100 | 100 | **Fungicidal** |
| Next | 100 | 100 | 100 | 100 | 100 | 100 | **Fungicidal** |

*Keto. = ketoconazole; Nyst. = nystatin; CG = Candida glabrata; CK = Candida krusei; CP = Candida parapsilosis; CA= Candida albicans; CT = Candida tropicalis; CD = Candida dubliniensis*

Table 4 above shows the MFC of the various extracts as well as the reference compounds. Extract A23 produced an MFC concentration of 200 µg/ml after subcultivation. Extract A34 had an initial reaction at 100 µg/ml on *C. glabrata,* but in the subsequent species, its MFC concentration was undetermined, implying that it could be a fungistatic agent. The A52 and A80 extracts all presented excellent activity at 100 µg/ml against all Candida spp. and are thus considered to be the most effective extracts. They are fungicidal. The A64 extract produced varied concentrations of inhibition, but it is still considered fungicidal. A96 inhibited *C. krusei, C. parapsilosis, C. albicans and C. dubliniensis at 200 µg/ml*. However, it was unable to inhibit the growth of *C. glabrate* and *C tropicalis* and was thus considered fungistatic.

### 3.1.5. Time kills kinetics

Time-kill kinetics were performed for the most active extracts, and it was performed on *C. albicans* due to its epidemiological importance.

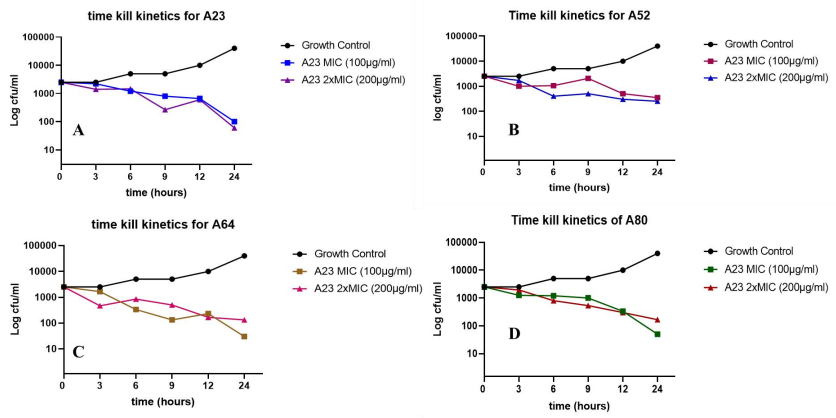
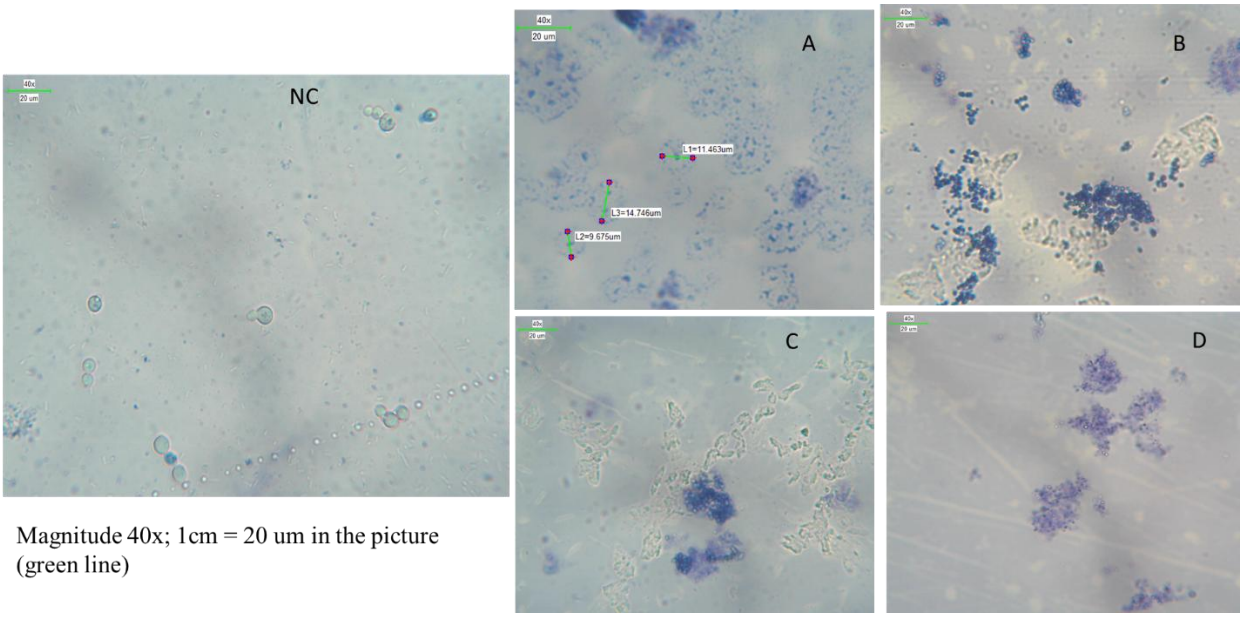



Figure 1. Time kill kinetics of bacterial endophytic extracts A23, A52, A64, and A64 on *Candida albicans*

Figure 1 represents the time of action of the extracts at different concentrations (MIC and 2xMIC). It is observed that with the concentration equal to the MIC, there is a steady decline in the population of microorganisms that reach a number less than Log 100 CFU/ml after 24 hours. and a rapid decrease after 12 hours in all extracts except for extract A52.

### 3.1.6. Posttreatment behavior of *Candida albicans*

This determination was done microscopically after comparing the effects of the extract on the yeast




Magnitude 40x; 1cm = 20 um in the picture (green line)

NC= negative control (no treatment); A= *C. albicans* treated with A23 for 24 h; B= *C. albicans* treated with A52 for 24 h; C= *C. albicans* treated with A64 for 24 h; D= *C. albicans* treated with A80 for 24 h

**Figure 2.** Twenty-four-hour posttreatment behaviors of *C. albicans*.

From the figure above, there is a clear difference in the morphology of the cells before and after the use of the extract. The diameter of the cells before the addition of the extract was

approximately 6.1 µm. After A23 was added, the cells increased in size to an average diameter of 9 µm, followed by cellular lysis. The possible site of action of this extract could be in the cell membrane of *C. albicans* by creating pores on the cytoplasmic membrane.

From Figure 2 above, there is a clear difference in the morphology of the cells before and after the use of extract A52. After A52 was added, there was cellular shrinkage of the cells. The cell is stained because it is dead. The possible site of action of this extract could be in the cell wall of *C. albicans,* affecting the osmotic fragility of the cell. After the extract A64 was added, there was complete lysis of the cell, they try to form biofilm as a means of protection, but their cell wall is affected so they lyse. After extract A80 was added, the cells were completely lysed. Such cellular lysis could be attributed to disruption of the cell membrane.

### 3.2 Discussion

Endophytes, which can be either endophytic fungi or bacteria, have been said to be present in almost all both aquatic and terrestrial plants[9,12,14,16–18]. This huge diversity of endophytic microorganisms hinders the establishment of a uniform protocol for the characterisation and identification of endophytes. Due to the limitations of this study, endophytes were not characterised at the genetic level but through their microscopic and macroscopic characteristics. Even if this method is not a formal identification of the microorganism, it can help to cluster them prior to identification. This has been reported elsewhere[34].

#### 3.2.1 Screening for antifungal activity among numerous extracts

A total of 99 extracts were screened for antifungal activity at 100 µg/ml. The screening was performed for all the extracts on 3 Candida species isolated from stools named *C. krusei*, *C. dubliniensis*, *and C. albicans*. To the best of our understanding, we did not find a report on the antifungal activities of the bacterial endophytic extract from *Urena lobata*. However, these results are in line with those reported in the literature. Bacterial endophytic extracts from Panax ginseng [10,35–38] and organic volatile compounds from bacterial endophytes [21] have been reported to have antifungal effects. In addition, several reviews have stated the potential of endophytes for their antifungal effects on phytopathogen mould. The strains used here were all resistant to both nystatin and ketoconazole (MIC=100 µg/ml). This resistance to antifungal agents has been reported in a number of publications[5,7,8,39,40]. The MIC obtained for extracts A23, A52, A64 and A80 on *Candida species* was the same as that of the

reference compounds. The study revealed that extracts A23, A52, A64 and A80 all showed fungicidal activity between 100 µg/ml and 200 µg/ml. A study carried out on evaluating the anticandidal potential of endophytic bacteria isolated from *Dryopteris uniformis* (Makino) showed that the extracts from the bacteria had MFC values between 250 µg/ml and 500 µg/ml[38].

In the best of our reading, we did not find a report on the time-kill kinetics of the endophytic extracts on candida strains. Extract A23 started killing cells before 3 hours, while extracts A52, A64, and A80 started acting on *C. albicans* cells after 3 hours at 2x CMI, but the effect was significant for CMI after 12 hours of contact.

Regarding the posttreatment *Candida albicans* cell behavior, we did not find authors who present the effect of the endophyte from *Urena lobata* on the yeast cells. However, this work is in line with that from Das et al.,[38] showing that endophytic bacteria produce extracts with destructive effects on candida albicans cells, as revealed by electron microscopy.

## 4. Conclusion

This work highlights the antifungal effect of extracts from four endophytic bacteria and suggests that these extracts start having an effect after 3 to 9 hours of treatment and that the site of action of this endophytic extract might be the cell membrane or any other component that leads to membrane stability.

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