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Evaluation of synergistic anticandidal activity of *Galenia africana* extract and fluconazole against *Candida albicans* and *Candida glabrata*

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Abstract: *Candida* infections have increased in recent years and are causing a serious public health concern. In addition, *Candida* species are becoming resistant to numerous antifungal drugs. It is for this reason that alternative treatment options are being explored, using medicinal plants, to curb this trend of drug resistance. The aim of this study was to evaluate the anticandidal activity of an ethanolic extract of *Galenia africana* (*G. africana*) alone and particularly in combination with fluconazole (FLC) against *Candida albicans* and *Candida glabrata*. The anticandidal activity was evaluated using various techniques. The minimum inhibitory concentration (MIC) of the extract was 6.25 mg/mL for both *Candida* strains. A synergistic effect was observed when *G. africana* was combined with FLC, against *C. albicans* and an indifferent effect against *C. glabrata* when interpreted by the fractional inhibitory concentration index (FICI) (0.36 and 1.002 for *C. albicans* and *C. glabrata*, respectively). Microscopic analysis revealed cell damage and decrease in cell size after *G. africana* treatment. Collectively, these results suggest that *G. africana* possessed antifungal activity against the *Candida* strains and a synergistic effect with FLC. Cell damage observed possibly contributed to this synergistic effect. This provides new information for the development of new antifungal agents.

Keywords: antifungal resistance; *Candida species*; *Galenia africana*; susceptibility testing; disk diffusion; Broth microdilution

Abbreviations: CLSI: Clinical and Laboratory Standards Institute; CFU: colony-forming unit; CPD: critical point dryer; DPBS: Dulbecco's phosphate-buffered saline; FIC: Fractional inhibitory concentrations; FICI: Fractional inhibitory concentration index; *G. africana*: *Galenia africana*; INT: p-iodonitrotetrazolium chloride; MIC: Minimum inhibitory concentration; MFC: Minimum fungicidal concentration; RPMI: Roswell Park Memorial Institute medium; SDA: Sabouraud Dextrose Agar; SDB: Sabouraud Dextrose Broth; SGA: Sabouraud Glucose Agar; SEM: Scanning electron microscopy; YNBG: Yeast Nitrogen Base Glucose

1. Introduction

The occurrence and frequency of fungal infections, which range from life threatening invasive and chronic infections (such as fungemia and meningitis) to recurrent superficial infections (such as oral and vaginal candidiasis), have been on the rise for a number of years now (Brown et al., 2012; Ksiezopolska and Gabaldon, 2018). In spite of the availability of treatment options, fungal infections significantly contribute to the high global morbidity and mortality rates (affecting millions of people, with an overall death toll of around 1 350 000 deaths per year) (Brown et al., 2012). Thus, creating a major public health concern due to the challenging aspect of managing these infections (Arendrup and Patterson, 2017; Fisher et al., 2018). Candidiasis is the major cause of fungal infections especially in immunocompromised and hospitalized individuals. It is particularly common in people suffering from cancer, diabetes, AIDS, as well as those with other severe diseases and transplant patients receiving immunosuppressive treatment following transplant surgeries (Al Ashaal et al., 2010; Arendrup, 2010). Candidiasis is caused by *Candida* species, commensal fungi found in the gastrointestinal tract, oral mucosa, skin, and vagina (Nobile and Johnson, 2015; Shao et al., 2007). *Candida* species are capable of causing both superficial and systemic infections due to their ability to adapt to different environments (Yan et al., 2012).

Candida albicans is the leading cause of persistent and severe fungal infections (Horn et al., 2009). However, the prevalence of infections caused by non-*albicans* species is on the rise (Sardi et al., 2013), which include *Candida glabrata, Candida krusei*, *Candida tropicalis*, and *Candida parapsilosis* (Krcmery and Barnes, 2002). Despite *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* being the most common *Candida* species, accounting for over 90% of the cases, more than 30 other *Candida* species have been associated with candidemia (Gabaldon et al., 2016; Pfaller et al., 2010; Pfaller et al., 2011). However, *C. albicans* and *C. glabrata* have been reported to be the most frequently isolated *Candida* species. For instance, a North American study conducted in major medical centers showed that *C. albicans* was the most commonly isolated species followed by *C. glabrata*. Moreover, the increase and spread of non-*albicans* infections could be attributed to severe immunosuppression, increased broad-spectrum antibiotic use and old age (Horn et al., 2009; Panghal et al., 2011). Over the years, it has been observed that a large number of fungal species, including *Candidia* species, are resistant to a variety of antifungal drugs worldwide (Ingham et al., 2012). Furthermore, it has been shown that prior fluconazole exposure can influence the incidence of fluconazole- resistant *Candida* bloodstream infections (Oxman et al., 2010; Tumbarello et al., 2008) in patients with underlying medical conditions such as HIV/AIDS and cancer, patients in intensive care, and those undergoing treatment with other therapeutic drugs (Garnacho-Montero et al., 2010; Ruhnke et al., 1994; Slavin et al., 2010). Fluconazole resistance is also seen in candidemia resulting from *C. glabrata* infection (Lee et al., 2009; Tumbarello et al., 2008). This is attributed to the ability of *Candida* species to form drug-resistant biofilms which significantly contributes to their disease causing characteristic in humans (Rajendran et al., 2010). The advent of drug resistant species and the need for treatment options with fewer side effects have paved the way for the evaluation of alternative antifungal agents, using medicinal plants, in the hope of developing novel therapeutic agents (Bonifacio et al., 2019; Hofling et al., 2010; Soliman et al., 2017).

For centuries, herbal medicines have been traditionally used in Africa, and around the world, to treat various diseases. This is mainly due to the belief that medicinal plants are widely available, extremely effective, and cheaper than conventional drugs (Awuchi, 2019). *Galenia africana* (*G. africana*) is a medicinal plant commonly referred to as “kraalbos” or “geelbos” (Van Der Lugt et al., 1992). It is predominantly found in the Namaqualand region of South Africa as well as the western and southern Karoo (Kellerman et al., 1988; van Wyk et al., 1997). It is documented that the indigenous Khoi-San people chewed the plant to alleviate toothaches and it has also been used to treat venereal sores, asthma, wounds on both animals and humans, eye infections, and skin diseases (Watt and Breyer-Brandwijk, 1962). Other therapeutic uses include the treatment of venereal disease, ringworms, relieving of eye inflammation and using it in lotions for the treatment of skin diseases. The aerial parts of *G. africana* have also been used to treat coughs, including tuberculosis (Mativandlela et al., 2009; Watt and Breyer-Brandwijk, 1962). *G. africana* extracts have also demonstrated antifungal characteristics against *Botrytis cinerea* (Fielding et al., 2015). The current study was a preliminary study mainly aimed at investigating the effect of *G. africana* alone and

particularly in combination with fluconazole (FLC) against *Candida albicans* and *Candida glabrata* using different methods.

2. Materials and Methods

2.1. Preparation of the G. africana plant extracts

The plant material was collected from Komaggas farmers in Namaqua District, Northern Cape Province. The formal identification of the plant material used in this study was done by Mr. F. Weitz (Herbarium, Department of Botany, University of the Western Cape, Bellville). Voucher specimens are kept in the UWC herbarium (Vries 1; herb no 6595). The dried leaves and shoots were dried for several weeks to maintain the bioactivity and then passed through a hammer mill to produce a powdery material (approximately 2-3 mm). To standardize the material substance within the plant, the dry weight was measured against the total volume of liquids used in the maceration process. The 20% (w/v) yellow-green to brown-green plant tincture was macerated by mixing the milled plant material, using 60% ethanol as a solvent. This was expressed as a ratio with the generally accepted standard strength of a plant tincture being 1:5 which is 1-part dry weight of plant material to 5 parts of macerating liquid. The expiry date applied was four years from the date of maceration. The extraction took place in cold ethanol at room temperature in a shaker for 12 h maximum. The extract had a pH of 6.9 and was then used as a stock solution. Two types of *G. africana* extracts were used in this study. The first was a 200 mg/mL extract which was commercially prepared, by Brenn-O-Kem (Pty) Ltd (Wolseley, South Africa), with 60% ethanol and then oven dried under negative pressure to produce crystals. The dried *G. africana* crystals were then crushed and re-suspended in RPMI 1640 media buffered with 0.165 M MOPS (both purchased from Sigma-Aldrich, USA) to make a stock solution that was utilized in the subsequent experiments. However, a 500 mg/mL extract was prepared and used for the disk diffusion assay. The second was a 200 mg/mL extract in 60% ethanol that was not dried and was supplied in liquid form. The working concentrations of both *G. africana* extracts were made up in RPMI 1640 media buffered with 0.165 M MOPS.

2.2. Candida strains and culture conditions

The *Candida* type strains, *C. glabrata* (ATCC 26512) and *C. albicans* (ATCC 90028), were a kind gift from Prof Charlene WJ Africa. *C. glabrata* and *C. albicans* were sub-cultured on Sabouraud Dextrose Agar (SDA) and incubated for 24 h at 37°C. These type strains were used in this study due to their high prevalence (as previously outlined in the introduction section). Sterile glass test tubes, containing 7 mL of saline, were inoculated with colonies picked from the SDA. The inoculums were then adjusted to 0.5 McFarland's standard with a known concentration of approximately 3×10^8 microorganisms per mL using a nephelometer. This technique uses known densities of microorganism suspensions for standardization (McFarland, 1907). In the broth microdilution method, cell suspensions were standardized using Sabouraud Dextrose Broth (SDB) to the desired concentration. Confirmation of the two strains was carried out by aseptically streaking the fungal strains on Oxoid chromogenic differential medium. On this medium, *C. albicans* grow as green colonies, whereas *C. glabrata* produce beige/brown or purple/mauve colonies.

2.3. Antifungal susceptibility testing using the disk diffusion assay

The disk diffusion assay was conducted according to the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2009) with minor modifications, and used to evaluate the antifungal effect of *G. africana* extract against *C. albicans* and *C. glabrata*. Briefly, a 500 mg/mL stock solution of the extract was used to make serial dilutions of 3.91, 7.81, 15.63, 31.25, 62.5, 125 and 250 mg/mL. Sterile filter disks (9 mm) were then loaded with 40 µL of

the different extract concentrations. Control disks impregnated with 25 µg/mL fluconazole and 50% ethanol (solvent used to dissolve the *G. africana* crystals) were also included. The disks were then allowed to dry at 37°C overnight. Inoculums of *C. albicans* and *C. glabrata* (adjusted to 0.5 McFarland standard) were then swabbed onto the Yeast Nitrogen Base Glucose (YNBG) agar plates and allowed to stand for about 30 min. The disks were then placed onto the cultured plates and incubated at 37°C for 24 h. All experiments were performed in triplicate and results are expressed as arithmetic means of these replicates. Determination of antifungal susceptibility and resistance was done after 24 h of incubation by measuring zones of inhibition.

2.4. Determination of minimum inhibitory concentration (MIC) using the broth microdilution method

The MIC of *G. africana* was determined by the broth microdilution method and performed according to CLSI reference methods for yeasts (CLSI, 2008). Briefly, twofold dilutions of the dried and ethanolic *G. africana* extracts were prepared by diluting them in RPMI 1640 medium (Sigma-Aldrich, USA) to yield the following concentrations: 0.015, 0.031, 0.061, 0.122, 0.244, 0.489, 0.977, 1.954, 3.91, 7.815 and 15.63 mg/mL of the dried extract; 0.024, 0.049, 0.098, 0.195, 0.391, 0.781, 1.562, 3.125, 6.25, 12.5 and 25 mg/mL of the ethanolic extract. A 1:100 cell suspension (of both *Candida* strains) was prepared containing a final concentration of 1-5 x 10^3 CFU/ml and 100 µL of this standardized suspension was then added to wells containing 100 µL of the extract to yield the desired concentrations. The tests were performed in triplicate. Control wells consisted of RPMI media alone, cell suspensions alone, fluconazole (2 µg/mL and 32 µg/mL for *C. albicans* and *C. glabrata* respectively) alone and sterile water alone. Each well contained a final volume of 200 µL and plates were incubated at 37°C for 24 h. After the incubation period, 40 µL (0.2 mg/mL) of *p*-iodonitrotetrazolium chloride (INT) was then added to each well and incubated for another 30 min at 37°C. The MIC was determined as the lowest concentration of the extract showing no colour change and exhibiting complete inhibition of fungal growth as seen by the lack of colour change from yellow to pink.

2.5. Determination of minimum fungicidal concentration (MFC)

The minimum fungicidal concentration of the dried and ethanolic extracts was evaluated in order to establish whether *G. africana* was fungicidal (able to kill the yeast cells). The MFC was determined by sub-culturing a 10 µL aliquot from each negative well (well that did not produce any colour change) and from a positive well (cell suspension control well) onto drug-free Sabouraud glucose agar (SGA) plates. The plates were then incubated for 18-24 h at 37°C. The concentration that yielded no visible growth on SDA was taken as the MFC (Bagiu et al., 2012; Costa et al., 2015).

2.6. Evaluation of the activity of G. africana in combination with fluconazole using the checkerboard assay

The checkerboard assay was performed to assess the interaction between the *G. africana* ethanolic extract and fluconazole as previously described (Hsieh et al., 1993; Petersen et al., 2006). The ethanolic extract was chosen for the checkerboard assay as it exhibited greater antifungal activity against the two *Candida* strains as determined by the MIC and MFC values. Briefly, the MIC of the extract was determined after a 24 h incubation period. The concentrations tested ranged between 0.78 and 50 mg/mL for *G. africana* and between 0.12 and 64 µg/mL for fluconazole. The fractional inhibitory concentration index (FICI) was used to estimate the interaction between *G. africana* and fluconazole in this assay. It was calculated by adding the MIC of the extract and fluconazole in combination divided by the MIC of extract and fluconazole when used alone. The results were interpreted as follows:

FICI ≤ 0.5 was defined as synergy, 0.5 < FICI < 1 as additive, 1 < FICI < 4 as indifference and antagonism as FICI > 4 (Olajuyigbe and Afolayan, 2013).

2.7. Sensititre YeastOne colorimetric MIC procedure

The Sensititre YeastOne antifungal susceptibility test is a broth microdilution method utilized to evaluate the interaction between *G. africana* and various antifungal drugs. This colorimetric microdilution assay relies on a colorimetric indicator to indicate yeast cell growth (Posteraro and Sanguinetti, 2014; Sanguinetti and Posteraro, 2018) and was performed as previously described (Pfaller et al., 2012). Briefly, before adding the cell suspension to the YeastOne inoculation broth, *G. africana* was added to yield final concentrations of 6.25 (the MIC) and 12.5 (2 x MIC) mg/mL. A working suspension of approximately 1.5-8 x 10^3 CFU/mL (of both *Candida* species) was then prepared in the same broth (TREK Diagnostics Systems). Broths containing cell suspensions but without the extract were also included as controls. The YeastOne plates were then rehydrated by adding 100 µL of the working solution (containing the extract and cell suspension) into each well. The plates were sealed with the adhesive seal and incubated for 24 h at 37°C. The plates were read manually by visually reading them under normal laboratory lighting. Yeast growth in the wells containing the antifungal solutions was represented by a change in the colorimetric growth indicator from blue (negative, no growth) to red (positive, growth). The MIC was recorded as the lowest concentration of antifungal agent inhibiting yeast growth as evident by the lack of development of a red colour in the growth well (first blue colour). The intensity of colour produced was compared to that of the positive control wells (Pfaller et al., 2012).

2.8. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to evaluate the effect of the extract on the *Candida* cells. SEM was performed on yeast cells treated with the extract in the disk diffusion assay. The aim was to evaluate the effect of the extract on the yeast cell structure despite the extract only showing minimal activity in this method. The yeast cells within the inhibition zones (microcolonies) were carefully collected using fine spatulas, to avoid damaging the cell structures, and fixed onto glass cover slips using 2.5% glutaraldehyde in Dulbecco's phosphate buffer saline (PBS) for 1 h. The cover slip was then washed twice with PBS and twice with sterile distilled water in 5 min cycles. Samples were then dehydrated in graded concentrations of ethanol (50, 70, 90 and 100%) in 10 min cycles. After the dehydration process, samples were then removed from the 100% ethanol and placed into the critical point dryer (CPD) or air dried in order to remove any remnants of ethanol. Once all samples were dried, they were then sputter-coated with gold-palladium alloy and analyzed using the scanning electron microscope.

3. Results

3.1. Antifungal susceptibility testing using the disk diffusion assay

The disk diffusion assay was utilized in the analysis of the antifungal activity of *G. africana* against *C. albicans* and *C. glabrata*. The dried *G. africana* extract was dissolved in 50% ethanol to make up a 500 mg/mL stock solution. Treatment of *C. albicans* with *G. africana* produced small zones of inhibition, with the presence of microcolonies, at concentrations of 15.6 to 250 mg/mL. *G. africana* concentrations of 3.91 and 7.8 mg/mL did not produce any visible zones of inhibition. Zones of inhibition produced after the treatment of *C. glabrata* with *G. africana* were small and contained microcolonies at concentrations of 31.25 to 250 mg/mL. Those produced after treatment with 15.62 mg/mL of *G. africana* were not well defined but contained microcolonies. Concentrations of 3.91 and 7.8 mg/mL did not produce any inhibition zones. Control plates were also included which contained 50% ethanol

and fluconazole (25 µg/mL). There were no zones of inhibition present after treatment of both *Candida* strains with 50% ethanol. Treatment of the *Candida* species with fluconazole yielded an inhibition zone of 18 mm against *C. albicans*, with the presence of microcolonies. However, there was only a small zone of inhibition produced when used against *C. glabrata*.

3.2. Determination of MIC using the broth microdilution method

The broth microdilution assay was used to evaluate the effect of both the dried and ethanolic *G. africana* extracts. The MIC of the dried *G. africana* extract against *C. albicans* was 3.91 mg/mL while the MIC of the dried extract against *C. glabrata* was 1.95 mg/mL. The ethanolic extract produced an MIC of 6.25 mg/mL against both *C. albicans* and *C. glabrata*.

3.3. Determination of MFC

Figure 1 shows the results obtained after sub-culturing the aliquots from the negative wells (wells with no growth) in the microdilution assay onto SGA plates. The results revealed that the dried *G. africana* extract was fungistatic at all the concentrations (even the highest one used) as demonstrated by the presence of growth on the SGA plates. The results obtained after sub-culturing the aliquot from the wells treated with the ethanolic extract are shown in Figure 2. The MFC of the ethanolic extract against *C. albicans* was 6.25 mg/mL and that of *C. glabrata* was 12.5 mg/mL. This showed that the MIC=MFC=6.25 mg/mL (the MIC and MFC values were similar) for *C. albiacans* whereas *C. glabrata* had an MFC of 12.5 mg/mL which was different from its MIC of 6.25 mg/mL (MFC > MIC). The MFC results demonstrated that the ethanolic extract exhibited greater antifungal activity compared to the dried extract. The ethanolic extract was fungicidal at concentrations of 6.25 and 12.5 mg/mL for *C. albicans* and *C. glabrata*, respectively. Given that the dried extract was fungistatic, and not fungicidal, even at a high concentration, the ethanolic extract was selected for the checkerboard and Sensititre susceptibility testing methods to assess the combination effects.

3.4. Evaluation of the activity of G. africana in combination with fluconazole using the checkerboard assay

Results of the checkerboard assay revealed that the MIC of fluconazole alone was 0.5 µg/mL whereas that in combination was 0.12 µg/mL against *C. albicans*. The MIC of *G. africana* alone was 6.25 mg/mL and the MIC in combination was 0.78 mg/mL. The FICI obtained was 0.36 which indicated that the interaction between fluconazole and *G. africana* against *C. albicans* was synergistic since the value was ≤ 0.5. Treatment of *C. glabrata* with the extract-fluconazole combinations revealed that the MIC of fluconazole alone was 64 µg/mL whereas that in combination was 0.12 µg/mL. The MIC of *G. africana* alone and in combination was 6.25 mg/mL. The FICI obtained was 1.002 which classified the interaction as being indifferent. This meant that the interaction observed was as a result of the *G. africana* extract that had the greatest antifungal activity compared to fluconazole.

3.5. Sensititre YeastOne colorimetric MIC procedure

The Sensititre YeastOne colorimetric MIC procedure was used to evaluate the interaction between combinations of antifungal agents and *G. africana* against *C. albicans* and *C. glabrata*. The MIC interpretive criteria shown in Table 1 are included for easy comparison to the results obtained using this procedure (Table 2). Table 2 outlines the results obtained after the treatment of *C. albicans* and *C. glabrata* with different antifungal drugs alone as well as in combination with *G. africana*.

Table 1. MIC interpretive criteria (clinical breakpoints in µg/mL) for antifungal drugs and *Candida* species using CLSI broth microdilution methods[a].

	C. albicans			*C. glabrata*		
Antifungal drug	**S**	**I**	**R**	**S**	**I**	**R**
Anidulafungin	≤0.25	0.5	≥1	≤0.12	0.25	≥0.5
Caspofungin	≤0.25	0.5	≥1	≤0.12	0.25	≥0.5
Micafungin	≤0.25	0.5	≥1	≤0.06	0.12	≥0.25
Itraconazole	≤0.12	0.25-0.5	≥1	≤2	–	>2
Fluconazole	≤2	4	≥8	–	≤32	≥64
Posaconazole	≤0.06	–	>0.06	≤2	–	>2
Voriconazole	≤0.12	0.25-0.5	≥1	≤0.5	–	>0.5
5-Flucytosine	≤4	8-16	≥32	≤4	8-16	≥32
Amphotericin B	≤2	–	>2	≤2	–	>2

[a] Data compiled from (Pfaller and Diekema, 2012). S- sensitive; I- intermediate; R- resistant.

Table 2. Sensitire susceptibility test results of *C. albicans* and *C. glabrata* treated with antifungal drugs alone as well as in combination with *G. africana* (6.25 and 12.5 mg/mL).

	MIC of antifungal drug (µg/mL)					
	C. albicans			*C. glabrata*		
Antifungal drug	**Drugs alone**	**Drugs + GA[a]**	**Drugs + GA[b]**	**Drugs alone**	**Drugs + GA[a]**	**Drugs + GA[b]**
5-Flucytosine	= 0.5	**≤ 0.06**	**≤ 0.06**	≤ 0.06	≤ 0.06	≤ 0.06
Amphotericin B	= 0.5	**≤ 0.12**	**≤ 0.12**	= 2	**≤ 0.12**	**≤ 0.12**
Anidulafungin	≤ 0.015	≤ 0.015	≤ 0.015	= 0.06	**≤ 0.015**	**≤ 0.015**
Caspofungin	= 0.03	**≤ 0.008**	**≤ 0.008**	= 0.12	**≤ 0.008**	**≤ 0.008**
Fluconazole	= 0.25	**≤ 0.12**	**≤ 0.12**	= 16	**≤ 0.12**	**≤ 0.12**
Itraconazole	≤ 0.015	≤ 0.015	≤ 0.015	= 0.5	**≤ 0.015**	**≤ 0.015**
Micafungin	≤ 0.008	≤ 0.008	≤ 0.008	= 0.015	**≤ 0.008**	**≤ 0.008**
Posaconazole	≤ 0.008	≤ 0.008	≤ 0.008	= 1	**≤ 0.008**	**≤ 0.008**
Voriconazole	≤ 0.008	≤ 0.008	≤ 0.008	= 0.5	**≤ 0.008**	**≤ 0.008**

[a] GA- *G. africana* at 6.25 mg/mL.
[b] GA- *G. africana* at 12.5 mg/mL.
Values in bold outline the changes in MIC when drugs were combined with *G. africana*. MICs are presented as means of three replicates.

The results revealed that *G. africana* enhanced the antifungal activity of the antifungal drugs as seen by a decrease in the antifungal drug MIC against the *Candida* species. *G. africana* enhanced the antifungal activity of 5-flucytosine, amphotericin B, caspofungin, and fluconazole against *C. albicans*. In addition, *G. africana* enhanced the antifungal activities of amphotericin B, anidulafungin, caspofungin, fluconazole, itraconazole, micafungin, posaconazole, and voriconazole against *C. glabrata*. This was significant as the MIC of fluconazole alone against *C. glabrata* in the microdilution assay, checkerboard assay and Sensititre susceptibility method was ≥16 µg/mL, but reduced significantly in the combination assays.

3.6. Scanning electron microscopy (SEM)

Scanning electron microscopy was employed to examine the structural changes of *Candida* cells after treatment with the extract. Figure 3 shows the appearance of the *Candida* cells prior to treatment. The cells appeared rounded with smooth surfaces. Figures 4 and 5 represent scanning electron micrographs of *Candida* cells after treatment with *G. africana* at a concentration of 250 mg/mL. The structure of both *C. albicans* and *C. glabrata* cells appeared distorted and only remnants of other cells could be seen.

4. Discussion

The increase in *Candida* species that are resistant to numerous antifungal agents is due to the rise in the use of these antifungal agents (Pemmaraju et al., 2013). Oral candidiasis is an infection normally associated with HIV-positive patients as a result of their weakened immune system and results in high mortality rates (Dos Santos Abrantes et al., 2014; Pomarico et al., 2010). Treatment of candidiasis is usually achieved by the administration of amphotericin B and fluconazole, with fluconazole frequently being given as the first line of treatment when dealing with systemic infections. Unfortunately, *Candida* strains are developing resistance to fluconazole (Dos Santos Abrantes et al., 2014). The increase in multi-drug resistant organisms is influenced by altered gene expression (Kanafani and Perfect, 2008), impaired immune function, underlying disease, reduced drug bioavailability, and increased drug metabolism (Rex et al., 1997). Given that *Candida* species are resistant to various classes of antifungal drugs, such as azoles and echinocandins (Sanguinetti et al., 2015), there is need for the development of alternative treatment options. Medicinal plants possess numerous compounds with several health benefits that can be used in the development of new antifungal agents (Juca et al., 2020).

In this study, the antifungal activities as well as synergistic effects of *G. africana* and fluconazole were evaluated using the disk diffusion, broth microdilution, checkerboard assay, and Sensititre YeastOne colorimetric MIC procedure. The effect of *G. africana* on the structure of *Candida* cells was assessed using SEM. The results of the disk diffusion assay showed that the *G. africana* extract did not inhibit the growth of the *Candida* strains, even at a concentration of 250 mg/mL, as seen by the lack of large inhibition zones. This decrease in inhibitory activity could be attributed to the fact that natural plant products tend to diffuse out the disks much slower than conventional antimicrobial agents, (Klancnik et al., 2010) hence, the decreased antifungal activity observed. In addition, polarity also influences the rate of diffusion of a compound with more polar compounds diffusing out of the disk faster than less polar compounds (Moreno et al., 2006). The results of the broth dilution assay revealed that the dried *G. africana* was more effective than the ethanolic as seen by the lower MIC against the *Candida* strains. The MIC of the dried extract against *C. albicans* was 3.91 mg/mL whereas that of the ethanolic extract was 6.25 mg/mL. The MIC of the dried extract against *C. glabrata* was 1.95 mg/mL whereas that of the ethanolic extract was 6.25 mg/mL. However, when the MFC was determined for both the dried and ethanolic extracts, the dried extract was fungistatic, as seen by the presence of colonies when sub-cultured on SGA at even the highest concentration (15.62 mg/mL). On the other hand, the ethanolic extract was fungicidal at concentrations of 6.25 and 12.5 mg/mL for *C. albicans* and *C. glabrata* respectively. This result showed that the ethanolic extract had a greater ability to kill the yeast cells compared to the dried extract and it is for this reason that it was used in the checkerboard assay and Sensititre YeastOne colorimetric MIC procedure. The greater antifungal effect of the ethanolic extract compared to extracts prepared from other solvents can also be seen in other medicinal plants. A study carried out on ethanolic and methanolic leaf extracts of *Pogostemon parviflorus* Benth revealed that the ethanolic extract had greater antifungal activity compared to the methanolic extract. This could be seen from the mean MICs of the

ethanolic extract against *Candida* species of 5.7 mg/mL compared to that of 6.6 mg/mL of the methanolic extract (Najafi and Sadeghi-Nejad, 2010).

The checkerboard assay results showed that the interaction between *G. africana* and fluconazole against *C. albicans* was synergistic as evident by the FICI value of 0.36 which was < 0.5. This result is similar to a study carried out on glabridin (a compound found in *Glycyrrhiza glabra*) against *Canadida* species, such as drug-resistant *C. albicans*, which revealed that the combination of fluconazole and glabridin demonstrated synergistic effects (Liu et al., 2014). The interaction of *G. africana* and fluconazole against *C. glabrata* was indifferent as seen by the FICI value of 1.002. The Sensititre susceptibility method showed that the combination of *G. africana* and the antifungal drugs was more effective against the *Candida* strains than the antifungal drugs on their own. This could be seen in the reduction of the MIC of most of the antifungal agents for both *C. albicans* and *C. glabrata*. This was an important result because *C. glabrata* was showing less susceptibility to the antifungal drugs compared to *C. albicans*.

The microscopy analysis revealed that *G. africana* had an effect on the yeast cells. This could be seen by the distorted structure of the cells and the cell remnants that were present after treatment. These results were seen despite *G. africana* not showing much antifungal activity against the *Candida* strains in the disk diffusion assay. The results observed after microscopic analysis of yeast cells following treatment with *G. africana* are similar to the results obtained after treatment of *C. albicans* with *Euphorbia hirta* L. leaf extract. Transmission electron micrographs taken after treatment of *C. albicans* with the *E. hirta* leaf extract revealed significant alterations to the microstructure of the *C. albicans* cells. These alterations included changes in the morphology of the cells, lysis, and total disintegration of the cells after 36 h of exposure to the extract. The control cells, on the other hand, demonstrated the usual *Candida* morphology with a regular undamaged cell wall (Basma et al., 2011).

Furthermore, phytochemical analysis of the *G. africana* ethanolic extract revealed that flavonoids were the major secondary metabolites present (Ticha et al., 2015). Flavonoids are polyphenolic compounds widely present in plants, vegetables, beverages, and fruits. They can be classified as flavonols, flavones, flavanones, anthocyanins, isoflavones, and flavanols, and have been shown to possess several biological activities resulting in numerous health benefits. These include antioxidant, anti-inflammatory, immunomodulatory, anti-aging, antiviral, antibacterial, antiparasitic, and antifungal activities (Juca et al., 2020). The antifungal activity of the ethanolic extract of *G. africana* has been shown against *Alternaria* sp, *Botrytis cinerea*, *Cylindrocarpon* sp, *Eutypa lata*, *Fusarium equiseti*, *F. graminearum*, *F. oxysporum*, *F. solani*, *F. verticillioides*, *Penicillium expansum*, and *Phaeomoniella chlamydospora* (Ticha et al., 2015). The antifungal activity of flavonoids has also been demonstrated by their ability to inhibit fungal infections in humans. For instance, a flavone called baicalein has been shown to demonstrate antifungal activity against *C. albicans*, *C. tropicalis* and *C. parapsilosis*. In addition, baicalein exhibited a synergistic interaction with fluconazole against *Candida* infections (Serpa et al., 2012). Collectively, these results provide insight into the ability of *G. africana* to demonstrate antifungal activity against *C.*

albicans and *C. glabrata*, particularly owing to the biological activity of flavonoids present in the extracts.

5. Conclusion

The results obtained in this study indicate that *G. africana* is a potential source of new antifungal agents. The fungicidal and synergistic effects exhibited by the *G. africana* extract, alone and in the combination with antifungal drugs, support the growing need for the use of medicinal plant extracts as therapeutic agents in the fight against multi-drug resistant pathogens. Toxicity assessment of medicinal plants already in use, including the ones not yet commercialized, is vital in evaluating their safety and sensitizing potential at dosages for which these formulations are being used. Acute toxicity studies of the ethanolic extract of *G. africana* revealed that the extract did not result in any *in vivo* danger (Ng'uni et al., 2018). Despite acute toxicity studies being conducted, pharmacological and biochemical investigations will be essential in elucidating the mechanism of action and will be beneficial in utilizing this plant as a therapeutic agent. Furthermore, a detailed experimental analysis of chronic toxicities associated with *G. africana* will be beneficial to support these findings. Clinical trials have not yet been performed on this medicinal plant and will be essential. This study supplies additional information on the activity of *G. africana* and contributes to the knowledge of antimicrobial properties of plants commonly found in South Africa. However, additional research such as exploring the mechanism of action and performing *in vivo* studies to corroborate the antimicrobial potential of the extract alone and in combination with antifungal agents will have to be conducted. Given that this is a preliminary study, future studies will be developed and performed based on these results.

Acknowledgments

The authors wish to thank the Technology Innovation Agency (TIA) and National Research Foundation (NRF) for the funding. The authors also wish to thank Prof Charlene WJ Africa (Microbial Endogenous Infections Studies (MEnIS) Research Laboratories, Department of Medical BioSciences, Faculty of Natural Sciences, University of the Western Cape, Cape Town, South Africa) for providing the *Candida* type strains used in this study.

Funding

This research was funded by the Technology Innovation Agency (TIA) and the National Research Foundation (NRF) of South Africa. Any opinions, findings and conclusions or recommendations expressed in this manuscript are those of the authors and therefore the TIA and NRF do not accept any liability in regard thereto.

Competing interests

The authors declare no competing interests.

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599

600 **Figure 1.** MFC results of *Candida* species treated with the dried *G. africana* extract. **a** *C.*
601 *albicans* (left) and **b** *C. glabrata* (right) treated with the dried extract made up in RPMI
602 media. A1-A4 represent the different *G. africana* concentrations as follows: A1-15.63
603 mg/mL; A2-7.81 mg/mL; A3-3.91 mg/mL and A-4-1.95 mg/mL.
604

605 **Figure 2.** MFC results of *Candida* species treated with the *G. africana* ethanolic extract. **a**
606 *C. albicans* (left) and **b** *C. glabrata* (right) treated with the ethanolic extract made up in RPMI
607 media. A1-A4 represent the different *G. africana* concentrations as follows: A1-25 mg/mL;
608 A2-12.5 mg/mL; A3-6.25 mg/mL and A-4-3.12 mg/mL.
609

Figure 3. Scanning electron micrographs of untreated *Candida* species. **a** *C. albicans* (left) and **b** *C. glabrata* (right) at a magnification of 10 000 x. Cells are rounded and smooth with signs of budding.

Figure 4. Scanning electron micrographs of *C. albicans* treated with 250 mg/ml *G. africana* extract and cells viewed at different magnifications. **a** 5 000 x magnification (left) and **b** 10 000 X (right) magnification. Cells appeared desiccated and craggy after treatment. The structural appearance was rough compared to the untreated cells.

Figure 5. Scanning electron micrographs of *C. glabrata* treated with 250 mg/ml *G. africana* extract and cells viewed at different magnifications. **a** 5 000 x magnification (left) and **b** 10 000 X (right) magnification. Some cells appeared distorted while only remnants of other cells could be seen. The structural appearance of the cells was rough compared to the untreated cells.

Figure

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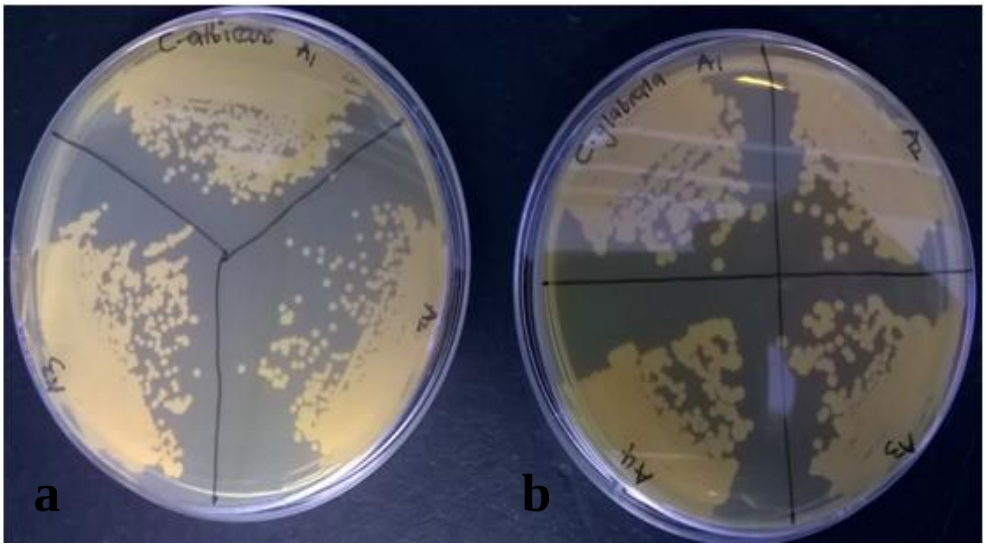

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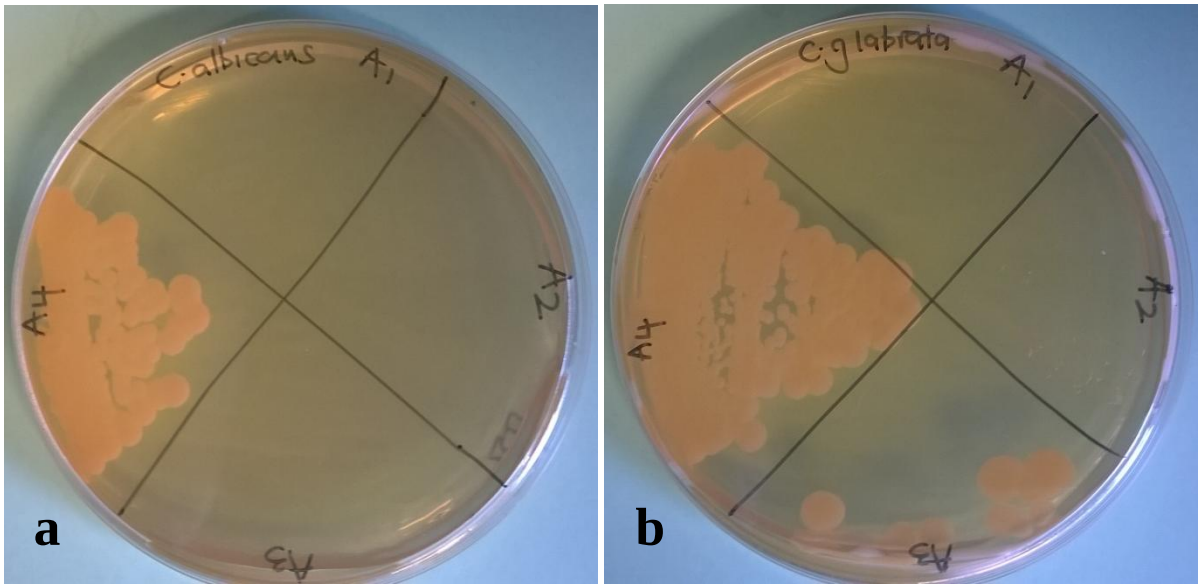

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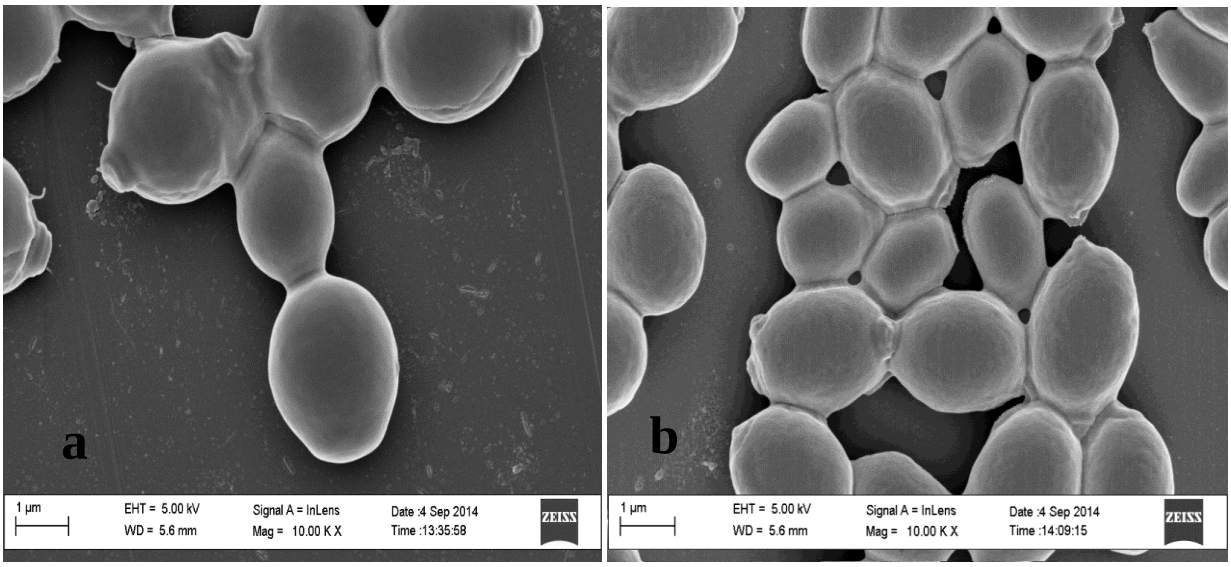

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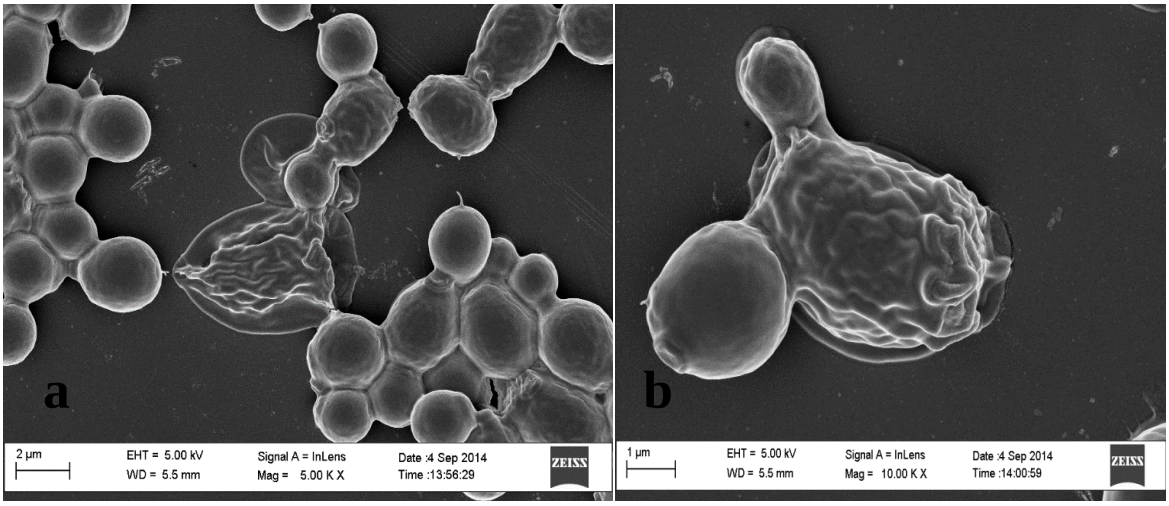

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