Antifungal activity of G. africana extract against Candida strains

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Abstract: Candida infections have increased in recent years and are causing 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 anti-Candida 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 anti-Candida activity was evaluated using various techniques. The minimum inhibitory concentration (MIC) of the extract was 6.25 mg/ml for both Candida strains. After combining with FLC, G. africana exerted a strong synergistic effect 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 suggested that G. africana possessed antifungal activities 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: Drug resistance, Candida albicans, Candida glabrata, Galenia africana, Fluconazole, Broth microdilution, Checkerboard, Sensititre

1. Introduction

Fungal infection occurrence and frequency has been on the rise for a number of years now [1]. In spite of the availability of treatment options, they contribute considerably to the high morbidity and mortality rates, thus creating public health concern [2]. Candidiasis is the major cause of fungal infections especially in immunocompromised and hospitalized individuals. It is especially common in people suffering from cancer, diabetes, AIDS, as well as those with severe diseases and transplant patients [3]. Candidiasis is caused by Candida, a commensal fungus found in the gastrointestinal tract, oral mucosa, skin and vagina [4]. Candida species are capable of causing both superficial and systemic infections due to their ability to adapt to different environments. [5, 6].
Candida albicans is the leading cause of persistent and severe fungal infections [7]. However, the prevalence of infections caused by non-albicans species is on the rise [1] and these include Candida glabrata, Candida krusei, Candida tropicalis and Candida parapsilosis [8]. A North American study conducted in major medical centres showed that C. albicans was the most commonly isolated species followed by C. glabrata. The increase and spread of non-albicans infections could be attributed to severe immunosuppression, increased broad-spectrum antibiotic use and old age [7, 9]. Over the years, it has been observed that a large number of fungal species, including Candida species, are resistant to a variety of antifungal drugs worldwide [10]. This is especially seen in patients with other underlying medical conditions and those undergoing treatment with other therapeutic drugs [11, 12]. This is attributed to the ability of Candida species to form drug-resistant biofilms which significantly contributes to their disease causing characteristic in humans [13]. The advent of drug resistant species and the need for treatment options with fewer side effects paves the way for the development of new antifungal agents [14, 15].

Herbal medicines have been traditionally used in Africa, and around the world, to treat various diseases. This is because medicinal plants are widely available and are cheaper than conventional drugs [16]. Galenia africana (G. africana) is a medicinal plant commonly referred to as “kraalbos” [17]. It is documented that the indigenous Khoi-San chewed the plant to alleviate toothaches and it has also been used to treat wounds on both animals and humans [18, 19]. Other therapeutic options 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 [18, 19]. G. africana extracts have also demonstrated antifungal characteristics against Botrytis cinerea [20]. This was a preliminary study that mainly investigated the effect of G. africana alone and particularly in combination with fluconazole (FLC) against Candida albicans and Candida glabrata using different methods.

2. Results

2.1. Disk diffusion (Kirby-Bauer) 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 micro-colonies, 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 micro-colonies 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 micro-colonies. 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 micro-colonies. However, there was only a small zone of inhibition produced when used against C. glabrata.

2.2. Broth microdilution

The broth microdilution assay was used to evaluate the effect of both the dried and ethanolic G. africana extracts. The Minimum inhibitory concentration (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
The ethanolic extract produced an MIC of 6.25 mg/ml against both *C. albicans* and *C. glabrata*.

2.3. Minimum fungicidal concentration (MFC)

The minimum fungicidal concentration of the dried and ethanolic extracts was evaluated in order to establish whether *G. africana* was fungistatic or fungicidal. Figure 1 shows the results obtained after sub-culturing the aliquots from the negative wells (wells with no growth) in the microdilution assay. 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 Sabouraud glucose agar (SGA) plates.

![Image](image1.png)

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

The results obtained after sub-culturing the aliquot from the negative 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 ethanolic extract against *C. glabrata* was 12.5 mg/ml. This showed that the MIC=MFC=6.25 mg/ml for *C. albicans* whereas the MIC=6.25 mg/ml and MFC=12.5 mg/ml for *C. glabrata*.
Figure 2. MFC results of *Candida* species treated with the *G. africana* ethanolic extract: (a) *C. albicans*; (b) *C. glabrata* treated with the ethanolic extract made up in RPMI media. A1-A4 represent the different *G. africana* concentrations as follows: A1- 25 mg/ml; A2- 12.5 mg/ml; A3- 6.25 mg/ml and A4- 3.12 mg/ml.

The MFC results demonstrated that the ethanolic 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. The dried extract was fungistatic and not fungicidal even at a concentration of 15.63 mg/ml (the highest concentration used). It is for this reason that the ethanolic extract was used in the checkerboard and Sensititre susceptibility testing methods to assess the combination effects.

2.4. 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 fractional inhibitory concentration (FIC) index value 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 FIC index value 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.

2.5. 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 are shown in Table 1 as outlined by the CLSI.
Table 1. MIC interpretive criteria (µg/ml) for Candida species as per CLSI M27.

<table>
<thead>
<tr>
<th>Antifungal drug</th>
<th>C. albicans</th>
<th>C. glabrata</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td>Anidulafungin</td>
<td>≤0.25</td>
<td>0.5</td>
<td>≥1</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>≤0.25</td>
<td>0.5</td>
<td>≥1</td>
</tr>
<tr>
<td>Micafungin</td>
<td>≤0.25</td>
<td>0.5</td>
<td>≥1</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>≤0.12</td>
<td>0.25-0.5</td>
<td>≥1</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>≤2</td>
<td>4</td>
<td>≥8</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>≤0.06</td>
<td>–</td>
<td>&gt;0.06</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>≤0.12</td>
<td>0.25-0.5</td>
<td>≥1</td>
</tr>
<tr>
<td>5-Flucytosine</td>
<td>≤4</td>
<td>8-16</td>
<td>≥32</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>&lt;1</td>
<td>–</td>
<td>≥1</td>
</tr>
</tbody>
</table>

S - sensitive; I - intermediate; R - resistant.

Table 2 represents the results obtained after the treatment of C. albicans and C. glabrata with different antifungal agents alone as well as in combination with G. africana. The results revealed that G. africana enhanced the antifungal activity of the antifungal agents as seen by a decrease in the antifungal 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, itraconazolém 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 test was ≥16 µg/ml, but reduced significantly in the combination assays.

Table 2. This is a table. Tables should be placed in the main text near to the first time they are cited.

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>C. albicans</th>
<th>C. glabrata</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Extract</td>
<td>Drugs + GA</td>
</tr>
<tr>
<td>5-Flucytosine</td>
<td>= 0.5</td>
<td>≤0.06</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>= 0.5</td>
<td>≤0.12</td>
</tr>
<tr>
<td>Anidulafungin</td>
<td>≤0.015</td>
<td>≤0.015</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>= 0.03</td>
<td>≤0.008</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>= 0.25</td>
<td>≤0.12</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>≤0.015</td>
<td>≤0.015</td>
</tr>
<tr>
<td>Micafungin</td>
<td>≤0.008</td>
<td>≤0.008</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>≤0.008</td>
<td>≤0.008</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>≤0.008</td>
<td>≤0.008</td>
</tr>
</tbody>
</table>

*GA - G. africana at 6.25 mg/ml.

Values in bold represent changes in MIC observed after addition of the extract.

2.6. 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.

**Figure 3.** Scanning electron micrographs of untreated *Candida* species: (a) *C. albicans*; (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; (b) 10 000 X 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; (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.

3. Discussion

The appearance of Candida species that are resistant to a number of antifungal agents is as a result of the rise in the use these antifungal agents [25]. An example of an infection caused by Candida species, particularly C. albicans, is oral candidiasis. This infection is normally associated with HIV-positive patients as a result of their weakened immune system. In addition, oral candidiasis can result in high mortality rates [26, 27]. 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 [26]. The rise is multi-drug resistant organisms prompted health institutions to propose the use of combination therapy in an effort to prevent these organisms from developing resistance and increase the effectiveness of the drugs against infectious agents [28]. However, resistance to various drugs has been observed and hence the need for alternative treatment options. Medicinal plants possess numerous health beneficial compounds that can be used in the development of antimicrobial agents [28].

In this study, the antifungal activities and 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 [29]. 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 [30]. 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 determine
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 [31].

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 *Candida* species, such as drug-resistant *C. albicans*, which revealed that the combination of fluconazole and glabridin demonstrated synergistic effects [32]. The interaction of *G. africana* and fluconazole against *C. glabrata* was indifferent as seen by the FICI value of 1.002. The Sensititre susceptibility test showed that the combination of *G. africana* and the antifungal agents was more effective against the *Candida* strains than the antifungal agents 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 agents 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 [33].

4. Materials and Methods

4.1. Preparation of the *Galenia africana* ethanolic plant extract

The plant material was collected from Komaggas farmers in Namaqua District, Northern Cape Province. 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 is 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.

4.2. Preparation of *C. glabrata* (ATCC 26512) and *C. albicans* (ATCC 90028)

The *Candida* type strains (*C. glabrata* and *C. albicans*) 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. Type strains were used in this study as they served as a good reference point for studying the effect of the extract on *Candida* species due to their prevalence. 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 x 10^8 microorganisms per ml using a nephelometer. This technique uses known densities of microorganism suspensions that are used for standardization [34]. In the broth microdilution assay, cell suspensions were standardized using Sabouraud dextrose broth (SDB) to the desired concentration. Confirmation of the two strains was done 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.

4.3. Disk diffusion (Kirby-Bauer) assay

The disk diffusion assay was conducted according to the Clinical and Laboratory Standards Institute (CLSI) [35] 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 extract dilutions. 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 and 48 h. All experiments were performed in triplicate. Observations of antifungal sensitivity and resistance were done after 24 and 48 h of incubation by measuring zones of inhibition.

4.4. Broth microdilution assay

The minimum inhibitory concentration (MIC) of *G. africana* was determined by the broth microdilution assay and performed according to the CLSI [36]. Briefly, the dried and ethanolic *G. africana* extracts were prepared by diluting them in RPMI 1640 broth (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 10^6 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 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 3-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.

4.5. MFC determination

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). This was incubated for a further 18-24 h at 37°C. The concentration that yielded no single bacterial colony on the solid medium was taken as the MFC [37].

4.6. Checkerboard assay

The checkerboard assay was performed to assess the interaction between the *G. africana* ethanolic extract and fluconazole as previously described [38, 39]. 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 FICI was used to estimate the interaction between *G. africana* and fluconazole in the checkerboard 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 [40].

4.7. Sensititre YeastOne colorimetric MIC procedure

This Sensititre susceptibility test was utilized to evaluate the interaction between *G. africana* and various antifungal agents. This colometric microdilution assay relied on a colometric indicator to indicate yeast cell growth and was performed as previously described [41]. 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 both *Candida* species) of approximately 1.5-8 x 10^3 CFU/ml was 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 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 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 [41].

4.8. 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. Specimens were fixed onto a glass cover slip using 2.5% gluteraldehyde 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.
5. Conclusions

The results obtained in this study indicate that G. africana is a potential source of new antifungal agents. The fungicidal effects as well as the synergistic effects exhibited by the G. africana extract, alone and in the combination studies, support the growing need for medicinal plant extracts to be used as therapeutic agents in the fight against multi-drug resistant pathogens. 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 the chronic toxicities is important to support these findings. Toxicity assessment of medicinal plants already in use, including the ones not yet commercialized, is important in evaluating their safety and sensitizing potential at dosages for which these formulations are being used. 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.

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