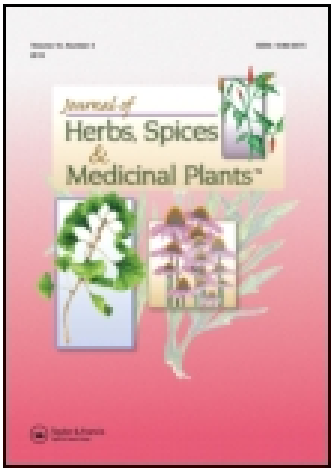

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The Effects of *Combretum zeyheri* Leaf Extract on Ergosterol Synthesis in *Candida albicans*

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The in vitro *effects of* Combretum zeyheri *leaf ethanol extract (CZLE) on ergosterol biosynthesis of* Candida albicans *were investigated by quantifying the ergosterol in* C. albicans *in the presence and absence of the extract using UV-visible spectrophotometric analyses. Miconazole was used as the positive control. CZLE showed ergosterol biosynthesis inhibition at sub-inhibitory concentration for growth of* C. albicans *and also at the MIC (0.08 mg.mL^{-1}). The decrease in ergosterol in* C. albicans *cells was dose-dependant with 67%, 79%, and 100% after growing in 20, 40, and 80 μg.mL^{-1} of CZLE, respectively. CZLE exerted its fungicidal effects by targeting the ergosterol biosynthesis in* C. albicans *and disrupting the membrane integrity.*

KEYWORDS Inhibitory effects, fungicidal, biosynthesis, 14 α*-demethylase*

INTRODUCTION

A common strategy in plant drug development is careful observation of the use of natural resources in folk medicine and making preparations for conventional laboratory testing (6). Many phytochemicals have been isolated

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from the genus *Combretum* including triterpenes, flavonoids, lignans, and non-protein amino acids, among others (1). In this study, the effect of *Combretum zeyheri* leaf ethanol extract on ergosterol, an important component of the membranes of *Candida albicans*, the leading cause of candidiasis, was examined. Ergosterol is important for membrane integrity, activity of many membrane-bound enzymes, and a major component of secretory vesicles, having a vital role in mitochondrial respiration (7). The rigidity, resistance to physical stress, and stability of the fungal membrane is dependent upon the presence of ergosterol. Loss and depletion, with concomitant accumulation of sterol intermediates of ergosterol, can result in destabilization of the membrane, alteration of the activity of membrane-bound enzymes, mitochondrial activities, susceptibility to drug of yeast cells, and membrane permeability (8).

The biosynthesis of ergosterol uses acetyl CoA as a precursor in a pathway involving a series of ~20 enzymatic reactions (4). This pathway is fungi-specific, and plasma membranes of other organisms are composed predominantly of other types of sterol such as the cholesterol in mammals. The ergosterol biosynthesis pathway has been the subject of intensive investigation as a target of antifungal drugs- for example, azole antifungals inhibit lanosterol 14-α-demethylase; allylamines such as terbinafine inhibit squalene epoxidase; and the polyene amphotericin B binds to ergosterol in the cell membrane (5). While a previous study reported antifungal effects of C. *zeyheri* (2), the current study examined how its leaf extracts may exert its fungicidal effects.

MATERIALS AND METHODS

Fungi and Reagents

All the chemicals used —absolute ethanol, glucose, Sabouraud dextrose agar, miconazole, sodium chloride, tryptone, peptone, and Sabouraud dextrose—were of high grade and obtained from Sigma Aldrich (Taufkirchen, Germany). *C. albicans (*ATCC 10231) was a gift from the Department of Biological Sciences, University of Botswana.

Plant Collection and Extract Preparation

The leaves from *C. zeyheri* were collected from the Norton (geographic coordinates of Norton, Zimbabwe: latitude, 17°52′59″ S; longitude, 30°42′00″ E; elevation above sea level, 1,360 m, Mashonaland West Province of Zimbabwe) and classified by a taxonomist, and a sample was kept at the Department of Biochemistry, University of Zimbabwe. The leaves were predried in a Labcon orbital incubator (Labotec Co., Cape Town, S.A.) at 40°C, ground in a two-speed blender (BL2, ABB, Moulinex, France), extracted in

ethanol, and filtered using a Whatman filter paper no.1 into a pre-weighed labeled container. The solvent in the extract was removed by air-drying under a fan, and a stock concentration of 20 $mg.mL^{-1}$ of the *C. zeyheri* was prepared.

Ergosterol Extraction

A single *Candida* colony from an overnight Sabouraud dextrose agar plate was inoculated in a tube containing 20 mL Sabouraud dextrose agar (SDA) broth and incubated at 37°C overnight in a Lab Companion SI- 300 shaking incubator (Jeio Tech, Korea). Then 5 mL of cells from the overnight culture incubated was inoculated in 300 mL of SDA broth containing 0.5 $mg.mL^{-1}$ CZE, along with the positive (0.05 $mg.mL^{-1}$) and the negative controls. The cultures were incubated for 24 h with shaking at 170 rpm at 37°C. The stationery phase cells were harvested by centrifugation at 2,700 rpm for 5 min and washed once with sterile distilled water. The net cell of the pellets was determined. An aliquot (3 mL) of 25% alcoholic potassium hydroxide solution (25 g KOH and 35 mL sterile distilled water, in 100 mL of 99.9% ethanol) was added to each pellet and mixed for 1 min. Cell suspensions were transferred to 16- × 100-mm borosilicate glass screw-cap tubes, incubated at 85°C for 1 h, and cooled to room temperature. Sterols were then extracted by adding a mixture of 1 mL sterile water and 3 mL n-hexane followed by vigorous vortex mixing for 3 min and transferring the hexane layer to a clean tube and were stored at –20°C until use. For analysis, a 20-mL aliquot of sterol extract was diluted fivefold in 100% ethanol and scanned at 220 and 300 nm using a 2,800 UV/VIS spectrophotometer (UNICO, Dayton, OH, USA).

Ergosterol Quantification

The presence of ergosterol and the late sterol intermediate 24(28)-dehydroergosterol [24(28)-DHE] in the extracted sample results in the characteristic four-peaked spectra. The absence of detectable ergosterol in the extracted sample is indicated by a flat line. The sterol, 24(28)-DHE, shows intense spectra at 230 nm and the complex of ergosterol, and this intermediate shows maximum absorption at 281.5 nm. The ergosterol content was calculated as a percentage of the wet weight of the cell by the following equations:

$$\%\,\text{Ergosterol} + \%\,24(28)\,\text{DHE} = [(A_{281.5}/290) \cdot F]/\text{pellet weight}, \quad (1)$$

$$\%\,24(28)\,\text{DHE} = [(A_{230}/518) \cdot F]/\text{pellet weight}, \quad (2)$$

$$\%\,\text{Ergosterol} = [\%\,\text{ergosterol} + \%\,24(28)\,\text{DHE}] - \%\,24(28)\,\text{DHE} \quad (3)$$

Where F is the factor for dilution in ethanol and 290 and 518 are the extinction values (in percentages per cm) determined for crystalline ergosterol and 24(28)-DHE, respectively (3).

STATISTICAL ANALYSES

Data were analyzed by one-way analysis of variance followed by Dunnett's multiple range test ($p < 0.05$) using Graphpad Prism 5 software (Version 5.0, Graph pad Software Inc, San Diego, CA, USA).

RESULTS

Effect of *C. zeyheri* on Growth of *C. albicans*

Increase in CZLE did not affect the mass of cells; however, for miconazole, the mass of the cells decreased as the extract concentration increased (Table 1), reducing the mass of cells to 29% at 40 $\mu g.mL^{-1}$.

Effects of *C. zeyheri* on Ergosterol Content

A dose-dependent decrease in the content of ergosterol in the *Candida* isolates was observed in cells grown in CZLE and with miconazole (see Table 1).

TABLE 1 Comparative Effects of *Combretum zeyheri* Leaf Ethanol Extract and Miconazole on Ergosterol Synthesis in *Candida albicans* Cells

Concentration of *C. zeyheri* ($\mu g.mL^{-1}$)	Mass of *C. albicans* cells (g)	Concentration of ergosterol ($\mu g.mL^{-1}$)	Ergosterol per mass of *C. albicans* cells
0	0.569 ± 0.000	0.056 ± 0.001	0.098 ± 0.002
10	0.415 ± 0.105	0.051 ± 0.016	0.122 ± 0.023
20	0.373 ± 0.128	$0.018 \pm 0.005^{*}$	$0.049 \pm 0.002^{*}$
40	0.302 ± 0.110	$0.012 \pm 0.001^{**}$	$0.041 \pm 0.010^{*}$
80	0.270 ± 0.091	$0.000 \pm 0.000^{**}$	$0.000 \pm 0.000^{**}$
Concentration of miconazole ($\mu g.mL^{-1}$)			
0	0.721 ± 0.000	0.076 ± 0.008	0.106 ± 0.015
5	$0.271 \pm 0.091^{**}$	0.064 ± 0.004	0.175 ± 0.025
10	$0.235 \pm 0.037^{**}$	$0.014 \pm 0.011^{***}$	0.040 ± 0.041
20	$0.236 \pm 0.028^{**}$	$0.000 \pm 0.000^{***}$	$0.000 \pm 0.000^{*}$
40	$0.226 \pm 0.075^{***}$	$0.000 \pm 0.000^{***}$	$0.000 \pm 0.000^{*}$

Note: Data represent the mean + *SD* for two independent experiments ($N = 2$). Mean separation by Dunnett's multiple range test ($p < 0.05$) compared to control.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Miconazole, the positive control, inhibited ergosterol synthesis with no ergosterol content observed at 20 $\mu g.mL^{-1}$. Typical absorption spectra profiles obtained are shown in Figure 1.

Effects of *C. zeyheri* on Ergosterol Content per Mass of Cells

Generally, as the concentration of CZLE or miconazole increased, the amount of ergosterol per mass of cells decreased (see Table 1). This showed that at this ratio, the extract diminished the ergosterol content since there was no decrease in the mass of the cells (see Table 1) as the extract concentration was increased. Increases in miconazole, on the other hand, decreased the mass of the cells, but the effects of this compound were more on ergosterol, as shown by the decrease of ergosterol/mass ratio (see Table 1).

DISCUSSION

Ergosterol is sterol found in eukaryotic fungi, and it is responsible for the membrane fluidity, rigidity, asymmetry, and activity of enzymes. Ergosterol has a concentration-dependent role: At lower concentration, it initiates growth while, at high concentration, it has a role in maintenance of the membrane (8). Several antifungal drugs target the ergosterol biosynthesis pathway. Azoles such as ketoconazole inhibit the enzyme responsible for the 14 α-demethylation of lanosterol, which results in concomitant accumulation in 14 α-sterol intermediates. Polyenes such as amphotericin B bind tightly to ergosterol and cause the formation of pores that cause leakage of ions and macromolecules. The growth sub-inhibitory concentrations of CZLE on *C. albicans* showed changes in ergosterol synthesis (see Table 1). The dose-dependent inhibitory effects of CZLE on ergosterol content were also seen after taking into account the mass of the fungal cells. The decreases in total ergosterol content were 67%, 79%, and 100% in the cells of *C. albicans* grown at 20, 40 and 80 $\mu g.mL^{-1}$ of CZLE, respectively. These results suggested that the fungicidal effects of CZLE were partly by interfering with the ergosterol synthesis pathway. This is in agreement with another report of plant-derived compounds against *C. albicans*- inhibited growth, viability. and ergosterol biosynthesis in *C. albicans* (5), which showed that the most potent compound, cinnamaldehyde, had an MIC of 0.06 $mg.mL^{-1}$ and an MFC of 0.25 $mg.mL^{-1}$ and inhibited ergosterol content by 59% at the MIC value. Miconazole, the positive control in the current study, is known to inhibit ergosterol biosynthesis by targeting the enzyme 14 α-demethylase. This compound showed a dose-dependent reduction in mass and sterol content (see Table 1). Complete blockage of ergosterol synthesis was observed at 20 and 40 $\mu g.mL^{-1}$ of miconazole. The decreases in total ergosterol were 16%, 81%, 100 and 100% in the *C. albicans* cells grown at 5, 10, 20, and 40 $\mu g.mL^{-1}$of

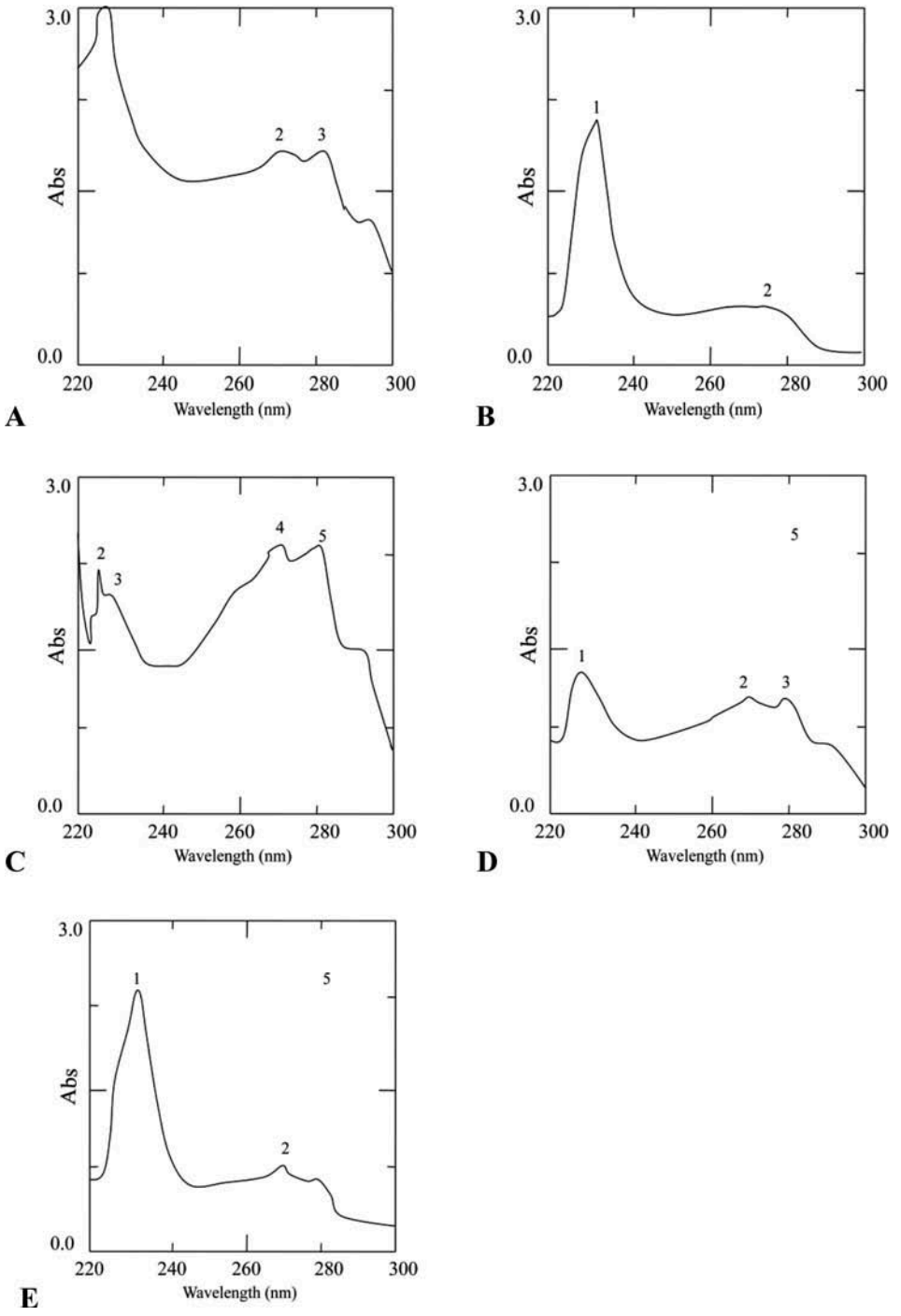

FIGURE 1 UV spectrophotometric sterol profiles of *Candida albicans* isolates. Isolates were grown for 24 hours in Sabouraud dextrose agar broth containing (A) 0, (B) 5, (C) 10, (D) 20, (E) 40 $\mu g.mL^{-1}$ of *Combretum zeyheri* extract.

miconazole, respectively. However, more in-depth study is needed to establish whether any intermediate sterols of the ergosterol biosynthesis are being produced upon growing of the fungus in the presence of the extract. This will assist in determining the level of effect by the extract. Miconazole was more potent in reducing the ergosterol content than the ethanolic leaf extracts of *C. zeyheri*.

C. zeyheri leaf extracts were antifungal and these extracts and/or their phytoconstituents inhibited drug efflux from *C. albicans* (2). Enzymes of the ergosterol biosynthetic pathway are important targets of several classes of antifungals used to treat candidiasis. The use of plant *C. zeyheri* extracts to treat candidiasis may be advantageous as it may target multiple mechanisms essential for the development and growth of *C. albicans*.

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