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# *In Vitro* Antifungal Activities of Selected Medicinal Plants from Zimbabwe against *Candida albicans* and *Candida krusei*

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## ABSTRACT

Thirty-eight Zimbabwean medicinal plant extracts were investigated for *in vitro* antifungal activity against *Candida albicans* and *Candida krusei.* These plants are used for the traditional treatment of various ailments, including fungal infections. The ethanol extracts were tested for antifungal activity using the agar disc diffusion method. The minimum inhibition concentrations (MICs) were determined for plant extracts that showed high efficacy against the tested microorganisms using the broth dilution method. The minimum fungicidal concentration (MFC) assay was carried out to determine if the fungal growth could irreversibly be inhibited by these plant extracts. The ciprofloxacin accumulation assay was carried out to investigate if these plant extracts could inhibit the activity of active drug efflux pumps in both *Candida* species. Nineteen plant extracts, among the 38 investigated, showed significant antifungal activity. MIC values ranged from 0.08–0.63 mg/ml for both *C. albicans* and *C. krusei.* MFCs ranged from 0.31–2.5 mg/ml. *Combretum zeyheri* extract had the highest antifungal activity in all cases. *Combretum zeyheri* and *Combretum molle* extracts were the most potent drug efflux pump inhibitors. The extracts from these *Combretum* species showed the greatest antifungal activity by both inhibition of growth and inhibition of drug efflux in *C. albicans* and *C. krusei*. *Combretum molle* and *Combretum zeyheri* provide a phytopharmacological basis for the traditional use of plant extracts against fungal infections.

**Keywords:** antifungal activity, *Combretum zeyheri*, *Combretum molle*, drug efflux, phytopharmacology
**Abbreviations:** **ATCC**, American type culture cell; **CFU**, colony forming units; **DMSO**, dimethyl sulphoxide; **ICD**, inhibitory concentrations in diffusion; **MFC**, minimum fungicidal concentration; **MIC**, minimum inhibition concentration; **MTT**, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **SDA**, Sabouraud dextrose agar

## INTRODUCTION

Since the discovery and exploitation of antifungal agents in the 20th century, the targeted selective toxicity of such agents has ensured their widespread and largely effective use to combat infection (Rahman *et al*. 2008). However, this use has paradoxically resulted in the emergence and dissemination of multi-drug resistant pathogens such as *Candida* yeasts (Woods-Panzaru 2009). *Candida albicans* is the most pathogenic and most causative agent, accounting for more than 90% of cases (Runyoro *et al*. 2006). It is a dimorphic fungus that exists as a commensal of warm-blooded animals, including humans, and it colonizes mucosal surfaces of the oral and vaginal cavities and the digestive tract (Perumal *et al*. 2007). Its ability to adhere to host tissues, produce secretory aspartyl proteases and phospholipase enzymes, and transform from yeast to hyphal phase are the major determinants of its pathogenicity (Lopes da Rosa 2010). It is also responsible for a variety of opportunistic human diseases manifestation ranging from superficial skin lesions to disseminate infection (Ramage *et al*. 2002; Cruz *et al*. 2007).

Candidiasis has become a major public health problem as an opportunistic infection of HIV/AIDS (Tanabe *et al*. 2007). Studies have shown that oral candidiasis, mostly commonly characterized by development of oral thrush, is the most frequent AIDS-associated opportunistic infection, with up to 90% of HIV-infected individuals suffering at least one episode during the course of their disease (Vazquez 1999). The high incidence of oral candidiasis in HIV/AIDS patients has made candidiasis a leading fungal infection in this immune-suppressed population (Jankowaska *et al*. 2001). Treatment of candidiasis is complicated by the emergence of strains of *Candida* that generally shows little permeability to a large variety of toxic compounds. This impermeability is believed to result to a large degree from the existence of an active permeability barrier (Kolaczkowski *et al.* 2009). The lack of entry of antifungal compounds into the cell prompts the need for development of new antifungal agents in order to widen the spectrum of activities against *Candida.*

For centuries, plants have been used by indigenous people to produce medicines that were used to treat different kinds of ailments such as (cancer, HIV/AIDS) because they produce wide array of bioactive molecules, most of which probably evolved as chemical defense against predation or infection (Samie *et al*. 2010). Almost 65% of the world's population relies on medicinal plants as part of their primary health care (Ndhlala *et al*. 2009). Medicinal plants are therapeutic resources used by the traditional population of the African continent specifically for health care and which may also serve as precursors for the synthesis of useful drugs (Okigbo *et al*. 2009). Many synthetic drugs owe their discovery and potency as a result of a mimic of structures from isolated natural products from plants rather than to the creativity and imagination of contemporary organic chemists (Zarrin *et al*. 2009). For example, the drug taxol, paclitaxel, one of the most powerful anticancer drugs known, first isolated from the bark of the yew tree *Taxus brevifolia* has yielded two approved drugs for breast and ovarian cancer (Jagessar *et al*. 2008). The compounds 5,6,7,8-tetramethoxyflavone, 5,6,7-trimethoxyflavone, and hydroxy-5,6,7,8-tetramethoxyflavone, were isolated from *Zeyheria tuberculosa* and are used as antifungals (Bastos *et al*. 2009).

Traditions of collecting, processing and applying plants and plant-based medications have been handed down from

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**Table 1** Zimbabwean medicinal plants evaluated for antifungal activity.

| Family | Plant material (crude extract) | | Voucher | Part used | Antifungal activity | | Ethnomedicinal information |
|---|---|---|---|---|---|---|---|
| | Scientific | Local | | | *Candida albicans* | *Candida krusei* | |
| Leguminosae | *Xeroderris stuhlmannii* (Taub) Mendonca and E.P Sousa | *Murumanyama* | C4 E7 | leaves | + | - | Mastitis and backache (Ruffo 1991) |
| Chrysobalanaceae | *Parinari curatellifolia* Planch, ex Benth | *Muhacha* | C6 EI | roots | + | - | Skin rashes, tuberculosis, chronic diarrhea, herpes zoster, herpes simplex (Chigora *et al*. 2007) |
| | | | C6 E7 | leaves | + | - | |
| Combretaceae | *Combretum molle* Engl. & Diels | *Mudziyaishe* | C9 E7 | leaves | +++ | +++ | - |
| Anarcadiaceae | *Rhus lancea* Engl | *Muchokochiana* | C11 E10 | flowers | ++ | - | Headache (Maliwichi 2000) |
| | | | C11 E7 | leaves | ++ | - | |
| Myrtaceae | *Syzygium cordatum* Hochst.ex C.krauss | *Mukute* | C12 E10 | flowers | + | + | Herpes zoster, herpes simplex, skin rashes (Chigora *et al*. 2007) |
| | | | C12 E7 | leaves | +++ | +++ | |
| | | | C12 E4 | bark | +++ | +++ | |
| Proteaceae | *Faurea saligna* Harv | *Mutsatsati* | C13 E7 | leaves | + | - | Bilharzia and helminthiasis (Baerts and Lehmann) |
| Combretaceae | *Combretum zeyheri* Sond | *Muruka*, *mupembere-kono*, *muchenja* | N6 E7 | leaves | +++ | +++ | Coughs, diarrhoea, rectal prolapse, Snake bites and stomachache (Ruffo 1991) |
| Fabaceae | *Cajanus cajan* (L.) Millsp. | - | N8 E7 | leaves | +++ | - | Aphrodisiac, oral candidiasis (Ruffo 1991) |
| Myrtaceae | *Callistemon citrinus* | - | UZ2 E7 | leaves | ++ | ++ | - |
| Olacaceae | *Oxalis lutifolia* De Wild | *Gungwe*, *kahungwarara* | UZ8 E7 | leaves | + | - | Abdominal pain (Chinemana *et al*. 1985) |
| | | | UZ8 E1 | Roots | + | - | |
| Araliaceae | *Cussonia natalensis* Sond | *Mutobvi*, *mufenje*, *mushondya* | UZ9 E7 | leaves | +++ | - | - |
| Euphobiaceae | *Croton gratissimus* Burch. | *Gunukira*, *Mufandemenge*, *mugugu*, *mubvukuta* | UZ13 E7 | leaves | + | - | Respiratory disorders (Roodt1998) |
| Aloaceae | *Aloe vera barbadensis* Miller | *Gavakava* | UZ14 E7 | leaves | + | - | Wounds and inflammatory skin disorders (Gelfand *et al*. 1985) |
| Rubiaceae | *Catunaregum spinosa* Thunb | *Murovaduri* | C5 E7 | leaves | - | - | Intestinal worms, gonorrhoea and syphilis (Ruffo 1991) |
| Fabaceae | *Brachystegia boehmii* Taub | *Mupfuti* | N7 E7 | leaves | - | - | - |
| Euphorbiaceae | *Uapaca kirkiana* Muell. Arg. | *Muzhanje* | UZ15 E12 | fruit | - | - | - |
| Myrtaceae | *Syzigium cumini* (Linn.) Skeels | - | C15 E7 | leaves | - | - | - |
| Asteraceae | *Bidens pilosa* Linn. var. | *Tsine* | N1 E7 | leaves | - | - | Anti-inflamatory, anti-rheumatic (Wang *et al*. 2003). Wounds and relapsing fevers in children, oral candidiasis (Ruffo 1991) |
| Verbenaceae | *Lippia javanica* (Burm.f.) Spreng | *Zimbani* | C3 E7 | leaves | - | - | Coughs, colds and fever, influenza, measles, malaria and stomach ache (Chigora *et al*. 2007) |
| Malvaceae | *Abelmoschus esculentus* Moench | *Derere* | UZ17 E12 | fruit | - | - | - |
| Anarcardiaceae | *Mangifera indica* (L) | *Mumango* | UZ18 E7 | leaves | - | + | Astringent, gonorrhea, asthma, prolongs ejaculation, anthelmintic (Kadavul and Dixit 2009) |
| Rosaceae | *Prunus cerasoides* D. Don | - | UZ5 E7 | leaves | - | - | - |
| Verbenaceae | *Lantana camara* | *Mbarambati* | UZ1 E7 | leaves | - | - | Ring worm infections |
| Rhamnaceae | *Zyziphus mucronata* | *Muchecheni* | C7 E7 | leaves | - | - | Paste of leaves treats boils, carbuncles and swollen glands, leaf infusion is taken against chest complaints (Roodt 1998b) Snake bites and stomachache (Ruffo 1991) |
| Clusiaceae | *Garcinia huillensis* Welw | *Mutunduru* | C10 E7 | leaves | - | - | Treatment of cryptococcal meningitis (Chigora *et al*. 2007) |
| Celastraceae | *Gymnosporia senegalensis* Loes | *Chizhuzhu*, *musosawafa* | N5 E7 | leaves | - | - | Coughs, pneumonia and tuberculosis |
| Solanaceae | *Solanum mauritianum* Scop | - | UZ10 E7 | leaves | - | - | Remedy for stomach ache and diarrhea. It is also used to treat respiratory ailments and tuberculosis (Carolus and Porter 2004) |

-: no inhibitory activity, +: slight inhibitory, ++: medium inhibitory activity, and +++: high inhibitory activity.

generation to generation. In South Africa and also in many other African countries, traditionally used medicinal plants are sold in market places or prescribed by traditional healers in their homes (Fyhrquist *et al*. 2002). Because of this strong dependence on plants as medicines, it is important to study their safety and efficacy. Many medicinal plants produce a variety of compounds of known therapeutic properties. Substances that can either inhibit the growth of patho-

gens or kill them and have little or no toxicity to host cells are considered good candidates for developing new antimicrobial drugs (Woods-Panzaru 2009).

Zimbabwe has suffered from the AIDS pandemic (Mills *et al*. 2005). Many of the patients end up suffering from candidiasis and frequently turn to traditional medicines for cures. The most commonly used medicinal plant species to reduce the symptoms of AIDS are *Combretum*, *Callistemon* and *Faurea* and these plant species have been used for the treatment of coughs, diarrhea, rectal prolapsed and stomach ache (Ruffo 1991). Some of these plants could contain novel antifungal compounds.

The aim of this study was to investigate the *in vitro* antifungal activities of 38 Zimbabwean medicinal plant extracts against *Candida albicans* and *Candida krusei*. These plants were selected based on literature survey of their ethnomedicinal uses in the treatment of microbial infections and are listed in **Table 1**.

## MATERIALS AND METHODS

### Fungi and reagents

All chemicals used in this study including nutrient agar, Sabouraud dextrose agar (SDA), miconazole, ethanol and dimethyl sulphoxide were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA), and were of analytical reagent grade. The water used for all experiments was of triple distilled grade. Two different pathogenic and opportunistic fungi were used in this study. A clinical strain of *C. krusei* isolated from a patient with candidiasis at Parirenyatwa Hospital was provided by Prof. Robertson (Department of Microbiology, Medical School, Harare, Zimbabwe). *C. albicans* strain ATCC 10231 was a kind gift from Dr. K. Marobela (Department of Biological Sciences, University of Botswana).

### Plant collection

The plants used in this work were collected from three provincial localities of Zimbabwe, Norton (Mashonaland West), Centenary (Mashonaland Central) and University of Zimbabwe (Harare), Zimbabwe. The plants were classified by Mr. Chris Chapano, a taxonomist at the National Botanic Gardens, (Harare, Zimbabwe). Herbarium samples were kept at the Department of Biochemistry, University of Zimbabwe.

### Extraction

The preparation of plant extracts was described by Mukanganyama *et al*. (2011). Briefly, plant samples were ground in a two-speed blender (Cole Parmer Instrument Co., Vernon Hills, USA). Each sample was individually extracted by weighing an aliquot of 2 g of finely ground plant material and extracting with 10 ml of absolute ethanol in test tubes. Extracts were filtered through fine cloth and then through 0.45 µm pore size corning syringe filters (Sigma Aldrich, Germany). The filtrates were decanted into pre-weighed labeled containers. The solvent was removed under a stream of air in a fume cupboard at room temperature. The amount of solid extract was weighed and recorded.

### Culture of fungi

Each of the fungal species were cultured onto SDA plate and incubated overnight at 37°C. A single colony was then cultured in SDA at 37°C overnight. The density of the fungi culture required for the test was adjusted to $1\times10^6$ colony forming units/ml (CFU/ml).

### Antifungal susceptibility test by the disc diffusion assay

The plant extracts equivalent to 500 µg, dissolved in ethanol, were applied to sterile paper discs (6 mm diameter, cartridge susceptibility discs, Mast Diagnostics, Mast Group Ltd., Merseyside, UK). The solvent was allowed to evaporate from filters deposited on 96-well plates at room temperature. The discs were then deposited on the surface of the innoculated agar plates. Plates were incubated overnight at 4°C for 2 hrs and then at 37°C in a Labcon incubator (Gallenkamp, UK). The inhibition zone, which is the diameter of inhibition around each of the disc plus the diameter of the disc, was measured and recorded in mm. Miconazole, a known antifungal, was used as the positive control.

### Minimum inhibitory concentration and minimum fungicidal concentration determination

Minimum inhibition concentrations (MICs) were determined using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay, a colorimetric method which is used to measure the activity of enzymes that reduce MTT to formazan, giving a purple colour. The plant extracts were dissolved in ethanol to a final concentration of 10 mg/ml and were serially diluted with water in 96-well microtitre plates. Cultures of *Candida* species (100 µl) were added to each well. Miconazole was used as the positive control and appropriate solvent blanks were included as negative control. Microtitre plates were incubated for 24 hrs at 37°C and 100% relative humidity. As an indicator of growth 25 µl of 2 mg/ml MTT was added to each of the microtitre plate wells and incubated for 3 h. A purple colour was observed where there was growth of the *Candida* species and a yellow colour was observed where there was no growth. The MIC was then recorded as the lowest concentration that showed no growth. The MFC was then carried out to investigate if the fungi could be killed completely or their growths could only be inhibited. The basis of selection was on the MIC wells and the preceding wells. The organisms from these wells were sub-cultured and incubated for 24 h at 37°C after which growth was observed. The lowest concentration of the extract without growth was considered as the MFC.

### Effects of plant extracts on drug efflux

Accumulation of ciprofloxacin in the *Candida* species was measured by the method of Xia *et al*. (2002) with a few modifications. Fungi were grown in nutrient broth at 37°C to an $A_{660}$ of 0.6-0.8 and harvested by centrifugation at 8000 rpm for 5 min. Fungi were then washed once with 50 mmol/L sodium phosphate buffer (pH 7.0) at 4°C and resuspended in the same buffer at about 40 mg (dry weight) cells/ml. The cells were placed in a water bath set at 30°C for 15 min. Samples were split into sample A and B and maintained at the same temperature. Ciprofloxacin was added to both samples to a final concentration of 20 mg/L. Aliquots of 750 µL were removed at 0, 1, 2, 3, 4, 5, 10, 15, 30, 45 and 60 min after the addition of ciprofloxacin to the fungi from both A and B. The active efflux of ciprofloxacin was determined in the presence of reserpine, an ATP binding cassette efflux pump inhibitor, 50 mg/ml stock solution in DMSO (Morita *et al*. 1998) which was added only to sample B to a final concentration of 100 µmol/L (Abdallah *et al*. 2007) but not to sample A. Volume samples of 750 µL were removed at 15, 30, 45 and 60 min after the addition of reserpine. Samples were diluted immediately into 750 µL chilled sodium phosphate buffer (pH 7.0) on ice, and then centrifuged in a micro centrifuge (Hermle Labortechnik, Germany) at 8000 r/min, at 4°C for 5 min. The fungi were washed again in the chilled buffer and re-centrifuged for 5 min. The cell pellet was suspended in 3.0 ml glycine hydrochloride (0.1 mol/L, pH 3.0) for 2 h at 30°C, and centrifuged at 8000 rpm for 10 min. The supernatant was centrifuged for another 5 min. The active efflux of ciprofloxacin was determined at the excitation and emission wavelengths of 270 nm and 452 nm, respectively using an RF-1501 Shimadzu spectrofluorometer (Shimadzu Cooperation, Tokyo, Japan). The active efflux of ciprofloxacin from the *Candida* species was also examined in the presence of ethanolic extracts from the plants *Syzigium cordatum*, *Cussonia natalensis*, *Combretum zeyheri* and *Combretum molle.* Initial screening results had shown that extracts from these plants had the most antifungal effect and, hence, there was need to determine if the antifungal effects observed was due to inhibition of efflux activity.

**Table 2** Minimum inhibitory concentrations and minimum fungicidal concentrations of extracts towards the *Candida* species.

| Plant species (crude extract) | | *Candida albicans* | *Candida krusei* |
|---|---|---|---|
| *Combretum zeyheri* (leaves) | Zone of inhibition[a] | 18.5 ± 0.7 mm | 18 ± 0.2 mm |
| | MIC | 0.08 mg/ml | 0.16 mg/ml |
| | MFC | 0.31 mg/ml | 0.31 mg/ml |
| *Combretum molle* (leaves) | Zone of inhibition | 17 ± 0.1 mm | 15 ± 0.3 mm |
| | MIC | 0.31 mg/ml | 0.31 mg/ml |
| | MFC | 0.63 mg/ml | 1.25 mg/ml |
| *Cussonia natalensis* (leaves) | Zone of inhibition | 16 ± 1.4 mm | - |
| | MIC | 0.31 mg/ml | |
| | MFC | 1.25 mg/ml | |
| *Syzigium cordatum* (leaves) | Zone of inhibition | 15 ± 0.1 mm | 12 ± 0.1 mm |
| | MIC | 0.63 mg/ml | 0.63 mg/ml |
| | MFC | 1.25 mg/ml | 2.5 mg/ml |
| *Syzigium cordatum* (bark) | Zone of inhibition | 15 ± 0.1 mm | 12 ± 0.6 mm |
| | MIC | 0.63 mg/ml | 0.63 mg/ml |
| | MFC | 1.25 mg/ml | 2.5 mg/ml |
| Positive control (miconazole) | Zone of inhibition | 20 ± 0.8 mm | 22.5 ± 0.7 mm |
| | MIC | 0.31 mg/ml | 0.63 mg/ml |
| | MFC | 0.31 mg/ml | 0.63 mg/ml |
| Negative control (DMSO) | Zone of inhibition | 6 mm | 6 mm |

[a]Results are the average (± SD) of two separate antifungal susceptibility test (each antifungal susceptibility test was followed by a disk diffusion assay done in quadruplicate).
MIC, minimum inhibition concentration
MFC, minimum fungicidal concentration

## Statistical analysis

A comparison of the efflux activity of the samples with the standard efflux inhibitor, reserpine was evaluated by applying one way ANOVA Dunnet's Multiple Comparison Test. All values are expressed as the mean ± standard deviation and $P < 0.05$ values or less were considered to indicate statistically significant differences. Numerical data were analysed using Graphpad™ version 4 for Windows (Graphpad™ Software Inc., San Diego, California, USA).

# RESULTS

## Screening by disc diffusion assay

**Table 1** summarizes the inhibitory activity of 38 ethanol crude plant extracts collected from three different localities in Zimbabwe against *C. albicans* and *C. krusei*, 19 of which had significant antifungal activity against *C. albicans* by the disc diffusion assay, and only seven against *C. krusei*. The ethanol extracts of leaves of *Combretum molle*, *Combretum zeyheri* and *Syzigium cordatum* showed remarkable antifungal effects against *C. albicans* (17 ± 0.1, 18.5 ± 0.7 and 15 ± 0.1 mm; n = 4) and *C. krusei* (15 ± 0.3, 18 ± 0.2 and 12 ± 0.1 mm; n = 4), respectively. The positive control, miconazole showed a zone of inhibition of 21 mm. The other plants showed mild to moderate antifungal activities against *C. albicans* with zones of inhibition ranging from 7-10 mm *C. krusei* was less sensitive to most crude plant extracts than *C. albicans*. **Table 1** also shows information about the popular plant names, parts used as well as their ethnomedicinal use and in parentheses are the sources or references of the information.

## Determination of MIC and MFC

**Table 2** shows the antifungal activities (MIC values in mg/ml) of five plant extracts which were found to have the most potent antifungal activity by the disc diffusion assay. Most plant extracts were devoid of antifungal activity up to 0.63 mg/ml. As shown in **Table 2** the MFC values ranged from 0.31 mg/ml as the most potent to 2.5 mg/ml as the least potent against the two tested pathogens. All plant extracts were most potent against *C. albicans*, with an MFC < 2.5 mg/ml. Only *Combretum zeyheri* and *Combretum molle* showed potent fungicidal activity against *C. krusei*.

## Ciprofloxacin accumulation assay

Overexpression of ATP binding cassette (ABC) transporters has been proposed as a major mechanism contributing to drug resistance in *C. albicans* (Holmes *et al*. 2008). The aim of this part of the study was, therefore, to determine the effects of the five most potent antifungal extracts screened by the disc diffusion assay, on drug efflux pumps in *C. albicans* and *C. krusei.*

*Combretum zeyheri* had the highest drug efflux pumps inhibitory effect against both *C. albicans* and *C. krusei*, as shown from the ciprofloxacin accumulation results (**Figs. 1, 2**). This effect was concentration-dependent as there was a corresponding decrease in inhibition of drug efflux pumping activity when the concentration was reduced by 50% (**Table 3**). *Combretum molle* and *Syzigium cordatum* leaf extracts also showed to be inhibitors of the efflux pumps in *Candida* species compared with the standard inhibitor, reserpine. *Cussonia natalensis* extract had no effect on *C. albicans* efflux pumps. The extent of inhibition of the extracts was calculated as the percentage control of ciprofloxacin concentration at 60 min and results are recorded in **Tables 3** and **4**.

To rule out interference due to the plant extracts, their fluorescence was determined separately at the same wavelengths and the results are shown in **Fig. 3**. Results show that all the plant extracts at the same concentration as reserpine (61 µg ml) did not fluoresce at the excitation and emission wavelengths. Reserpine fluoresced but its fluorescence was not significant compared to the fluorescence of ciprofloxacin. Reserpine was added at an inhibitor concentration of 61 µg/ml whilst the concentration of ciprofloxacin added to the cells was 20 mg/L.

# DISCUSSION

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality in immunocompromised patients in developing countries (Chevreuil *et al*. 2006; Yang *et al*. 2006). Although a large number of antimicrobial agents have been discovered, pathogenic microorganisms are constantly developing resistance to these agents (Rahman *et al*. 2008; Bastos *et al*. 2009). In recent years, attempts have been made to investigate indigenous drugs against infectious diseases in order to help develop safer antimicrobial drugs (Rahman *et al*. 2008).

The present study showed that 19 ethanolic plant extracts had profound antifungal effects when compared with the positive control, miconazole and, therefore, may have potential use as antifungal agents (**Table 1**). The clinical

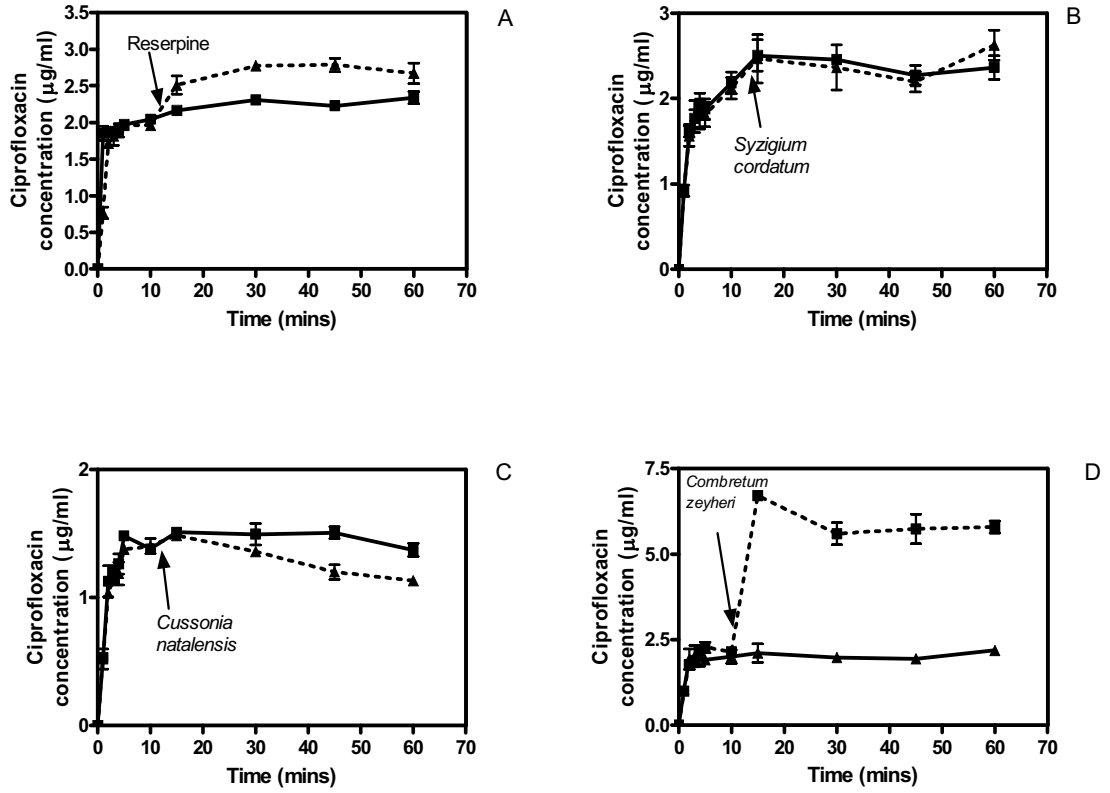


**Fig. 1 Inhibition of drug efflux pumps in *Candida albicans* by a standard influx pump inhibitor, reserpine (A), and crude ethanolic extracts from *Syzigium cordatum* (B), *Cussonia natalensis* (C) and *Combretum zeyheri* (D).** The tubes containing either *C. albicans* cells and ciprofloxacin (20 mg/L), (control) or *C. albicans* cells, ciprofloxacin and the plant extract (100 µmol/L) (test sample), were incubated at 37°C. The active efflux of ciprofloxacin was then examined at time intervals in all tubes. The arrow points at the time when the plant extract was added to the cells incubated in the presence of ciprofloxacin.

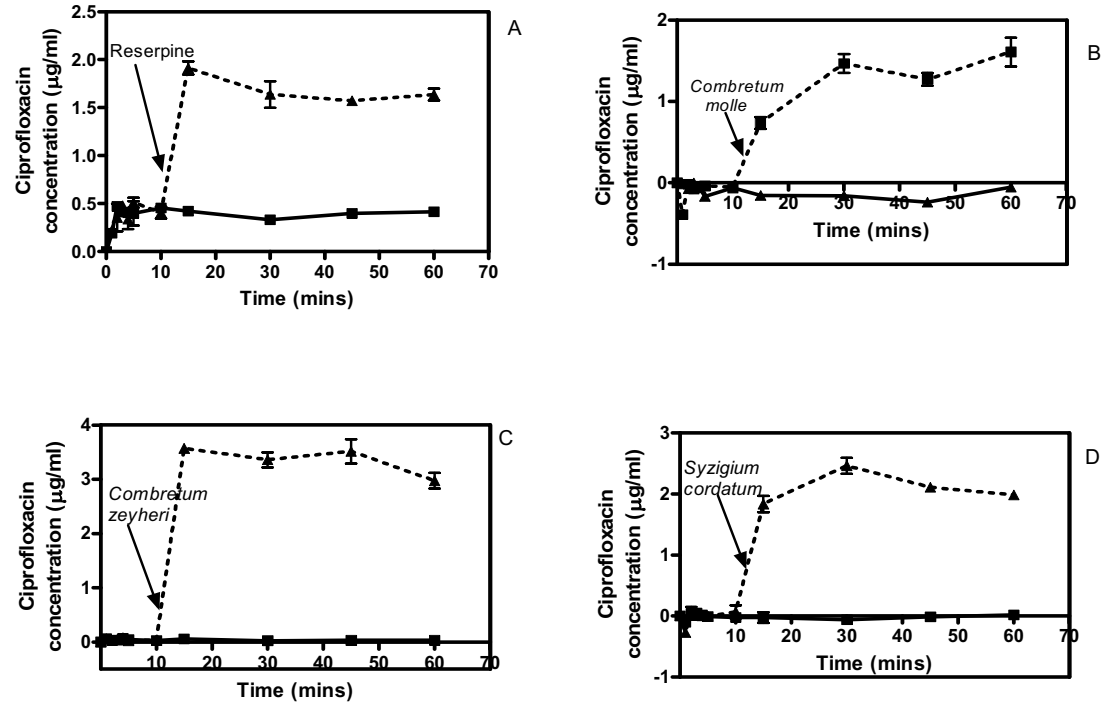


**Fig. 2 Inhibition of drug efflux pumps in *Candida krusei* by a standard influx pump inhibitor, reserpine (A), and crude ethanolic extracts from *Combretum molle*, (B) *Combretum zeyheri* (C) and *Syzigium cordatum* (D).** The tubes containing either *Candida krusei* cells and ciprofloxacin (20 mg/L), (control) or *Candida krusei* cells, ciprofloxacin and the plant extract (100 µmol/L), were incubated at 37°C. The active efflux of ciprofloxacin was then examined at time intervals in all tubes. The arrow points at the time when the plant extract was added to the cells incubated in the presence of ciprofloxacin.

isolate *C. krusei* was less sensitive to plant extracts than the *C. albicans* (ATCC) sample, supporting the reported literature data about the higher resistance of recently isolated clinical strains (Kersun *et al*. 2008). Recently, *Candida* species associated with candidemia have shifted from *C. albicans* to non-*Candida albicans* species in approximately half of the reported cases (Kersun *et al*. 2008).

Although *Bidens pilosa* has been reported as a treatment

**Table 3** Effects of plant extracts on *Candida albicans* drug efflux pumps.

| Plant extract | Effect on efflux of Ciprofloxacin | % control of [Ciprofloxacin] at 60 min[a] |
|---|---|---|
| *Combretum zeyheri* (0.06 mg/ml %) | Inhibition | 529 ± 12.4** |
| *Combretum zeyheri* (0.03 mg/ml) | Inhibition | 273 ± 0.3** |
| *Combretum molle* | Inhibition | 145 ± 0.7* |
| *Syzigium cordatum* (leaves) | Inhibition | 142 ± 13.1* |
| *Syzigium cordatum* (bark) | No effect | - |
| *Cussonia natalensis* | Activation | 80 ± 3.8* |
| Reserpine (positive control) | Inhibition | 112 ± 8.5 |

[a]The inhibition of the drug efflux pumps in *Candida albicans* by extracts as the percentage control of ciprofloxacin concentration at 60 min.
Results are the average (± SD) of two separate drug efflux pump inhibitory test (Each test was followed by a ciprofloxacin accumulation assay done in duplicate).** P<0.01;* P<0.05 against the control

**Table 4** Effects of plant extracts on *Candida krusei* drug efflux pumps.

| Plant extract (compound) | Effect on efflux of Ciprofloxacin | % control of [Ciprofloxacin] at 60 min[a] |
|---|---|---|
| *Combretum zeyheri* | Inhibition | 5120 ± 107** |
| *Syzigium cordatum* (bark) | Inhibition | 3669 ± 95.2** |
| *Combretum molle* | Inhibition | 3097 ± 97.3** |
| *Syzigium cordatum* (leaves) | Inhibition | 1392 ± 25.2** |
| Reserpine (positive control) | Inhibition | 393 ± 18.7 |

[a]The inhibition of the drug efflux pumps in *Candida krusei* by extracts as the percentage control of ciprofloxacin concentration at 60 min.
Results are the average (± SD) of two separate drug efflux pump inhibitory test. Each test was followed by a ciprofloxacin accumulation assay done in duplicate.
** P<0.01 against the control reserpine.

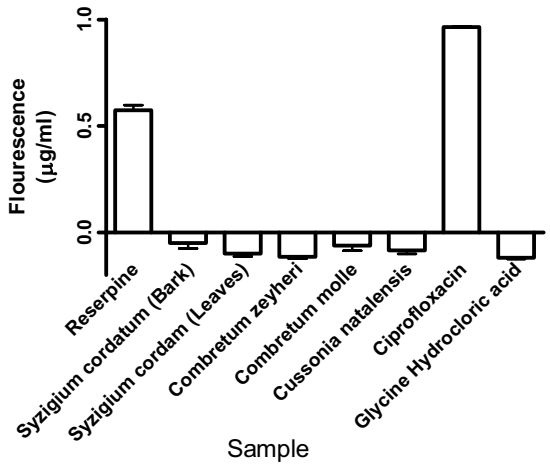


**Fig. 3 Baseline fluorescence of the extracts.** Fluorescence of each extract, at a concentration of 61 ng/ml, the same as the standard inhibitor reserpine, was determined at 254 nm emission wavelength 270 nm excitation wavelength.

of oral candidiasis (Ruffo 1991), no antifungal activity was observed during this study when *C. albicans* and *C. krusei* were used. However, this could be due to differences in biological activities. The absence of antifungal activity from *Bidens pilosa* may also suggest that the extracts may act in an indirect way; the active ingredient may exist as a precursor, which requires activation in the body by some as yet unknown mechanism. It is possible that the extract may achieve an effect via an immunopharmacological mechanism (Olila *et al.* 2001). No activity was demonstrated against *C. krusei* with *Cajanus cajan* although it has been reported to exhibit some antifungal activity (Ruffo 1991). This may be due to the fact that plants growing in different geographical locations may contain different phytochemicals (Clarkson *et al*. 2004).

This study shows that *Combretum molle* and *Combretum zeyheri* extracts possess remarkable antifungal activity against *C. albicans* and *C. krusei*. At a concentration of 0.31 mg/ml, *Combretum zeyheri* was able to completely kill both pathogens (**Table 2**). *Combretum molle* had fungicidal activity against *C. albicans* and *C. krusei* at 0.63 and 1.25 mg/ml, respectively. Therefore, this study demonstrates that the ethanolic extract of *Combretum zeyheri* was the most potent plant material examined against both fungal pathogens. These results concur with published literature, where *Combretum zeyheri* is known as a potent antimicrobial against *C. albicans*, *Cryptococcus neoformans*, *Microsporium canis*, *Sporotrichum schenckii* and, *Aspergillus fumigatus* (Masoko and Eloff 2006) and where *Combretum molle* leaf extracts was found to have an inhibitory effect against *Candida krusei* (Fyhrquist 2007). Therefore, these results show that *Combretum* species might be beneficial in treating oral *Candida* infections often occurring in immunocompromised patients such as those suffering from AIDS. Also, punicalagin isolated from *Combretum molle* were found to inhibit HIV-1 replication in MT-4 cells (Asres *et al*. 2005). It has also been reported that when compounds isolated from the stem bark of *Combretum molle* were tested for anti-HIV activity against immunodeficiency virus type 1 (HIV-I) and type II (HIV-II), punicalgin and CM-A displayed selective inhibition of HIV-1 replication and afforded cell protection of viral induced cytopathic effect (Ares and Bucar 2005). The MICs for all the plant extracts which were found to be most potent by the broth dilution assay (*Combretum molle*, *Combretum zeyheri*, *Cussonia natalensis*, *Syzigium cordatum*) against both tested *Candida* species were 0.63 mg/ml or less (**Table 2**). Therefore, these results add value to the ethnomedicinal use of these plants in Zimbabwe.

To determine if extracts had any effect on the drug efflux pumps in *Candida* species, accumulation of ciprofloxacin was determined in the presence 61 ng/ml of each extract and in the presence of reserpine. As expected, extracts which were found to be most potent antifungals inhibited the *Candida* efflux pumps (**Figs. 1, 2**). However, *C. krusei* accumulated more ciprofloxacin in the presence of all extracts than *C. albicans* (**Figs. 1, 2**). These results suggest that overexpression of multi-drug efflux transporters, including ABC transporters, could be major mechanism for the drug resistance for this clinical isolate. Among ABC transporters, Cdr1p and Cdr2p are major drug efflux pump proteins, which play a key role in azole resistance of *Candida* species. Other studies so far have confirmed that the overall drug resistance activity of *Candida* isolates depends on the level of expression of genes such as CDR1 and CDR2 (Kolaczkowski *et al.* 2009). Cdr1p and Cdr2p have typical features of ABC transporters, such as two hydrophobic transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) exposed to the cytoplasmic surface. The fungi accumulated high concentrations of ciprofloxacin in the presence of extracts from *Combretum molle* and *Combretum zeyheri* and, thus, confirms the inhibition of the drug efflux pumps in *Candida*. However, the results obtained from *Cussonia natalensis* were not expected because this extract proved to be a growth inhibitor of *C. albicans* by the broth dilution assay (MIC = 0.31 mg/ml). A plant showing antifungal activity would be expected to also show inhibition of drug efflux but this was not the case with *C. natalensis* and, therefore, this extract may contain other compounds that inhibit growth and at the same time, enhance the efflux of ciprofloxacin (**Fig. 1C**).

The results of the present study provide an ethnopharmacological basis for the use of plant extracts for infectious diseases, especially against fungal infections. Not only are these plant extracts able to inhibit fungal growth but they are also able to enhance the accumulation of drugs inside *Candida*. This activity may contribute to the suppression of drug resistance to existing antifungals. Therefore, the antifungal activity of these plants may be due to direct effects on growth as well as indirect effects to do with inhibition of the efflux of compounds from these cells. These results also confirm the potential of plants used by traditional healers in Zimbabwe as a source of bioactive compounds. Further investigations on isolation and characterization of antifungal bioactive agents from these plants are in progress.

## CONCLUSION

Six plant extracts (*Syzigium cordatum* (leaves), *Syzigium cordatum* (bark), *Syzigium cordatum* (flowers), *Combretum molle*, *Combretum zeyheri* and *Callistemon species*) had antifungal activity against both *Candida* isolates examined. Traditional cures and remedies that rely on the antifungal properties of aqueous extracts of these plants may serve as a source of phytochemicals that have antifungal activity.

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