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Antifungal Activities and Preliminary Phytochemical Investigation of Combretum Species from Zimbabwe

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Abstract

Plants used by indigenous people to produce medicines to treat different kinds of ailments maybe a source of new pharmaceuticals. The *in vitro* antifungal activities of six *Combretum* species (*C. zeyheri*, *C. apiculatum*, *C. molle*, *C. kraussii*, *C. elaeagnoides* and *C. imberbe*) were investigated against *Candida albicans* and *Candida krusei* using agar disc diffusion. Broth dilution was then used to determine the minimum inhibition concentrations and minimum fungicidal concentrations of the plant extracts that had shown high efficacy against the tested microorganisms. For the plant species that had the most activity, qualitative and quantitative isolation of major phytoconstituents were determined. All extracts from the *Combretum* species showed antifungal activity and had MIC values ranging from 0.31 mg/ml-0.63 mg/ml for both *Candida* species. *Combretum zeyheri* extract had the highest antifungal activity in all cases with MIC values of 0.08 and 0.16 mg/ml. Partial bioguided fractionation of *C. zeyheri* extract by sephadex LH20 gel column chromatography enabled isolation of the active fractions, fraction 3 and flavonoids. The isolated fractions were found to have drug efflux pumping inhibitory activity. *Combretum* species, therefore, may serve as a potential source of lead compounds that can be developed as antifungal phytochemicals. Bioactive compounds from *C. zeyheri* may serve as drug efflux chemo-modulators for restoring drug sensitivity to *Candida* cells exhibiting resistance via antifungal drug efflux.

Keywords: *Candida albicans*; *Combretum spp*; Phytoconstituents; Bioguided fractionation; Susceptibility; Drug efflux

Introduction

The search for new drugs to combat diseases and infections has become the focus of research in herbal remedies [1]. For centuries, plants have been used by indigenous people to produce medicines that were used to treat different kinds of ailments. Plants produce wide array of bioactive molecules, most of which probably evolved as chemical defense against predation or infection [2]. It is estimated that only one percent of flowering plants on earth have been studied exhaustively for their chemical composition and potential important medicinal value [3]. Large scale evaluation of the local flora exploited in traditional medicine for various biological activities is a necessary first step in the isolation and characterization of the active principle and further leading to drug development [4]. The World Health Organisation defined a medicinal plant as any herbal preparation produced by subjecting plant materials to extraction, fractionation, purification, concentration or other physical or biological process which may be produced for immediate consumption or as a basis for herbal products [5].

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 [6,7]. For example, the drug taxol, (paclitaxel), one of the most powerful anticancer drug known, first isolated from the bark of the yew tree *Taxus brevifolia* has yielded two approved drugs for breast and ovarian cancer [8]. Several compounds including flavonoids, phenanthrenes, stilbenes, cyclobutanes and triterpenoids have been isolated from the *Combretaceae* species widely distributed in the tropical climes of Africa, South America and Asia. The *Combretaceae* species are important in traditional medical practice and some of them have been used in the treatment of syphilis, abdominal pains, conjunctivitis, diarrhoea and toothache, among other ailments [9,10].

Antifungal resistance is particularly problematic as initial diagnosis of systemic fungal infection. In humans, fungal infections range from

superficial to deeply invasive or disseminated, and have increased dramatically in recent years [11]. *Candida albicans* causes serious infections of the oral mucosa and severe oropharyngeal candidiasis afflicts many AIDS patients [12]. *C. krusei* is a naturally azole – resistant species which also occurs in AIDS patients or otherwise severely immunocompromised individuals [12]. Because of the resistance that pathogenic fungi build against antibiotics, there is a need for the search for new antifungal drugs from nature. Natural crude drug extracts isolated from plant species used in traditional medicine can be prolific resources for such new drugs [13]. Following previous work of screening for antifungal activity from a range of plants [14], six *Combretum* species used in Zimbabwe traditional medicine (*C. zeyheri* Sond, *C. apiculatum* Sond, *C. molle* Engl. and Diels, *C. kraussii* Hochst, *C. elaeagnoides* Klotzsch and *C. imberbe* Wawra) were studied for their antifungal properties.

Materials and Methods

Fungi and reagents

All chemicals used in this study including nutrient agar, Sabouraud dextrose agar, miconazole, ethanol and dimethyl sulphoxide were purchased from Sigma-Aldrich chemical company (St Louis, MO, USA), and were of highest purity available. The water used for all

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experiments was of triple distilled grade. Two different pathogenic and opportunistic fungi were used, clinical strain of *Candida krusei* isolated from a patient with candidiasis at Parirenyatwa Hospital was provided by Prof. V. Robertson (Department of Medical Microbiology, School of Health Sciences, Harare, Zimbabwe) and *Candida albicans* strain ATCC 10231, a kind gift from Dr. K. Marobela (Department of Biological Sciences, University of Botswana).

Plant collection

The plant leaves were collected from the Botanical and Herbal garden in Harare, Zimbabwe and were classified by Mr. Christopher Chapano, a taxonomist. Herbarium samples were kept at the Department of Biochemistry, University of Zimbabwe.

Screening for antifungal activities of the plant species extraction

Plant leaves 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 leaves 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, Steinheim, 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.

Antifungal susceptibility test by the disc diffusion assay

To determine antifungal activities of the plant extracts, cells of *Candida albicans* and *Candida krusei* were grown in nutrient broth (Oxoid Ltd, Basingstoke, Hampshire, England) for 24 hours. The cultured cells were diluted to approximately 10⁶ cfu/mL then inoculated into sterilized nutrient agar. The mixture was poured into petri dishes and gave a solid plate. The plant extracts were dissolved in absolute ethanol and 500 µg of each plant extract was applied on sterile paper discs (6 mm diameter, cartridge susceptibility discs, Mast diagnostics, Mast Group Ltd, Merseyside, UK). The sterile paper discs were deposited on 96-well plates and ethanol was allowed to evaporate from the discs under a stream of air. The discs were then deposited on the surface of the inoculated agar plates. Miconazole (Sigma-Aldrich Chemical Co., St Louis, MO, USA) was used as positive control and ethanol and DMSO as negative controls. Plates were then incubated at 4°C for 2 hours and then at 37°C, overnight. The inhibition zones which were taken as the diameter of inhibition around each of the disc.

Minimum inhibitory concentration and minimum fungicidal concentration determination

Minimum inhibition concentrations (MIC's) were determined using (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 [15]. The plant extracts were dissolved in ethanol to a final concentration of 10 mg/ml and were serially diluted 2-fold with water in 96-well microtitre plates. Cultures of *Candida* species were transferred into fresh nutrient broth and 100 µl of fresh culture was added to each well. Miconazole was used as the positive control and appropriate solvent blanks were included as negative control. Micro plates were incubated for 24 hours 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 three hours. Metabolically viable cells convert the MTT into colored insoluble

crystal formazan. These crystals were dissolved in dimethyl sulphoxide (DMSO) and the intensity of the purple colour formed was measured spectrophotometrically at 560 nm. MIC was then recorded as the lowest concentration that showed no growth. The minimum fungicidal concentration (MFC) was determined by selecting concentrations of MIC values. The organisms from these wells were sub-cultured and incubated on agar for 24 h at 37°C after which they were observed for growths. The lowest concentration of the extract that showed no growth of *Candida* was considered as the MFC.

Bioguided fractionation of *C. zeyheri*

In order to isolate fractions with antifungal activity from the plant species which showed high efficacy against the tested microorganisms, the plant extract that was found to have the potent antifungal activity was fractionated by solvent-solvent partitioning then by SEPHADEX gel LH -20 column chromatography. The fractions obtained were then investigated for their antifungal activity.

Extraction

The leaves of *Combretum zeyheri* were ground in a two-speed blender (Cole Parmer Instrument Co, USA) and the sample was extracted with ethanol on an orbital shaker (Bibby Scientific Limited, Staffordshire, UK) with a 4: 1 solvent to dry weight ratio for 24 hours. The extract was filtered through cotton and the filtrate was decanted into pre-weighed labeled container. The water in the extract was removed by evaporation in a vacuum rotator evaporator (Buchi Labortechnik Essen, Germany) and then weighed. The ethanol extract was further fractionated using the solvent-solvent fractionation procedure recommended by the USA National Cancer Institute as described by Suffness and Douros (1979) and adapted by Eloff (1998a). The dried ethanol extract was dissolved in 30% methanol and the partitioning was done by adding 100 ml of the separating solvent 4 times, in order of their increasing polarity. The solvents used for partitioning were distilled petroleum ether, chloroform, ethyl acetate and butanol subsequently. All extracts were then taken to dryness in a vacuum rotator evaporator and were weighed. To determine antifungal activities of the separated fractions, the antifungal susceptibility test was carried out as indicated above.

Isolation of active compounds from ethyl acetate fraction

SEPHADEX LH-20 column chromatography is a technique which separates constituents of the extract by size, and normally used to remove unwanted substances such as chlorophyll [16]. The ethyl acetate fraction from *C. zeyheri* plant extract was loaded onto SEPHADEX LH-20 column chromatography (glass column, 3 x 25 cm) and separation was performed using 400 ml of methanol:chloroform (1:1) as the solvent. The procedure was repeated three times. The fractions obtained were dried using a vacuum rotator evaporator (Buchi Labortechnik Essen, Germany) and weighed. To determine antifungal activities of the separated fractions obtained by SEPHADEX LH-20 column chromatography, the antifungal susceptibility test was carried out as explained before. Fraction 3 was found to have the most potent antifungal activity using broth dilution method, therefore, further work was done with this fraction in order to determine growth inhibition parameters.

Isolation of phytoconstituents

Phytochemical screening: The leaves of *Combretum zeyheri* were evaluated qualitatively and quantitatively for the determination of major phytoconstituents namely; alkaloids, flavonoids, tannins, saponins and cardiac glycosides as described by Kaur and Arora, [17].

Qualitative screening

Alkaloid detection was carried out by extracting powdered sample with methanol and 2N HCl; and then treating the filtrate with Meyer's and Wagner's reagents [18,19]. The samples were scored positive on the basis of turbidity or precipitation. Flavonoids were tested by heating powdered sample with ethyl acetate over a steam bath (40-50°C) for 5 min; filtrate was treated with dilute ammonia. A yellow colour demonstrated a positive test for flavonoids. The presence of tannins was confirmed by boiling powdered sample in distilled water, followed by addition of drops of 5% ferric chloride (FeCl_3) to the filtrate. Development of brownish-green or blue-black colour is a positive test for the presence of tannins. Saponins content was determined by boiling powdered sample in distilled water for 15 min and after cooling, the extract was shaken vigorously to observe froth formation. Cardiac glycosides were identified by extracting sample in methanol. This methanolic extract was treated with glacial acetic acid containing a drop of 5% FeCl_3 solution. This solution was transferred to the surface of concentrated sulphuric acid. The formation of reddish brown ring at the junction of two liquids was indicative of cardenolides/cardiac glycosides.

Bioautography of extracts

Qualitatively isolated group of compounds were also assessed for their antifungal potential using agar disc diffusion assay. Alkaloids were isolated by mixing powdered sample with 10% (v/v) ammonia solution and extracted with methanol for 10 min on water bath at 40°C [17]. The mixture was then filtered through Whatman filter paper No. 1 and the filtrate was concentrated under a stream of air. Isolation of flavonoids was achieved by heating powdered sample with methanol in a water bath at 40°C for 10 min. The filtrate was then concentrated to 25% of its original volume. For saponins, powdered sample was extracted with methanol by heating on a water bath at 40°C for 10 min. The extract was filtered and evaporated to concentrate, mixed with water and then extracted thrice with n-butanol. The n-butanol phase was evaporated and concentrated. Tannins were obtained by treating powdered sample with 2 M hydrochloric acid (HCl) and hydrolyzing in boiling water bath for 30 min. The solution was filtered, mixed thoroughly with ethyl acetate, and the ethyl acetate layer was then discarded. Drops of amyl alcohol were added and shaken thoroughly. The alcoholic layer was retained and used for antifungal testing. Cardiac glycosides were isolated by extracting powdered sample with 50% (v/v) methanol and 10% (w/v) lead (II) acetate solution by heating on water bath at 40°C for 10 min. The filtrate was cooled to room temperature and then extracted twice with dichloromethane/isopropanol (3:2) solution. The combined lower phases were filtered over anhydrous sodium sulphate and evaporated to dryness. The residue was dissolved in dichloromethane/isopropanol (3:2) and this solution was further used for antifungal investigations. The five isolated groups of compounds were applied on sterile paper discs (6 mm diameter, cartridge susceptibility discs, Mast diagnostics, Mast Group Ltd, Merseyside, UK) and the discs were then air-dried and used for antifungal activity testing.

Quantitative analysis

Alkaloids were quantitatively determined according to the method of Harborne [20], Acetic acid (10%) in ethanol was added to powdered sample, covered and allowed to stand for 4 h. The filtrate was then concentrated on a water bath to 25% of its original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed

to settle; collected precipitates were washed with dilute ammonium hydroxide and then filtered. The residue was dried, weighed and expressed as the alkaloids.

To estimate flavonoids quantitatively, powdered sample was extracted twice with 80% aqueous methanol at room temperature. The solution was filtered through Whatman filter paper No.1, the filtrate was later transferred into crucibles, evaporated to dryness on a water bath to a constant weight.

Quantitative determination of saponins was done according to Obadoni and Ochuko, [21]. Twenty grams of each powdered sample was added to 20% aqueous ethanol and kept in a shaker for 30 min. The samples were heated over a water bath for 4 h at 55°C. The mixture was then filtered and the residue re-extracted with another 20% aqueous ethanol. The combined extracts were reduced over water bath at 90°C. The concentrate was then extracted twice with diethyl ether. Ether layer was discarded while aqueous layer was retained and n-butanol was added to it. Then n-butanol extracts were washed twice with 5% aqueous sodium chloride. The remaining solution was heated in a water bath and after evaporation the samples were dried in oven at 40°C to a constant weight. The saponin content was calculated as percentage of the initial weight of sample taken.

Determination of tannin content was performed according to the method of Van-Burden and Robinson [22], with some modifications. Distilled water was added to the sample, taken in a 500 ml flask and kept in shaken for 1 h. It was filtered into a 50 ml volumetric flask and made up to the mark. The filtrate was then dispensed into a test tube and mixed with 0.1 M FeCl_3 in 0.1 N HCl and 0.008 M potassium ferrocyanide. Absorbance was measured at 605 nm within 10 min.

Drug accumulation and efflux assays

The concept of parameter IC_{50} is fundamental to pharmacology. It is the concentration of an inhibitor where the response to an effector is reduced by half. In this study, IC_{50} was determined in order to measure the effectiveness of *C. zeyheri* fractions as drug efflux pump inhibitors. This was done by measuring the accumulation of ciprofloxacin in the *Candida* species. Fungi were grown in nutrient broth (NB) at 37°C to an A_{660} of 0.6-0.8 and harvested by centrifugation at 8 000 r/min for 5 minutes. Fungi were then washed once with 50 nmol/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 37°C for 15 minutes. Cells were then split into aliquots of 1.5 ml and maintained at the same temperature. Ciprofloxacin was added to cells to a final concentration of 0.02 mg/ml. The plant extracts were then added to all the tubes to a final concentration of 0-0.1 mg/ml. The concentrations of the plant extracts were prepared by 2.5 fold serial dilution. Aliquots of 750 μL cells were removed at 60 minutes after the addition of ciprofloxacin to the fungi from all microtubes. Cells were diluted immediately into 750 μL chilled sodium phosphate buffer (pH 7.0) on ice, and then centrifuged in a micro centrifuge (Hermle Labortechnik, Steinheim, Germany) at 8000 r/min, at 4°C for 5 minutes. The fungi were washed again in the chilled buffer and re-centrifuged for 5 minutes. The cell pellet was suspended in 3.0 ml glycine hydrochloride (0.1 mol/L, pH 3.0) for 2 hours at 30°C, and centrifuged at 8000 r/min for 10 minutes. The supernatant was centrifuged for another 5 minutes. 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).

Results

Disk diffusion assay

The inhibitory activity of six *Combretum* plant extracts against *Candida albicans* and *Candida krusei* are shown in Table 1. All of the six plant extracts tested were active against *Candida albicans* by disc diffusion assay, with zones of inhibition varying from 7 to 18.5 mm, and only four were active against *C. krusei*. The ethanol extracts of leaves of *Combretum zeyheri*, *Combretum imberbe*, *Combretum*

apiculatum and *Combretum elaeagnoides*, showed significant antifungal effects against *Candida albicans* and *Candida krusei*. *Combretum molle* and *Combretum kraussii* extracts showed mild antifungal activity against *C. albicans* and no activity against *C. krusei*. Miconazole at a concentration of 10 mg/ml was used as a positive control while ethanol was used as a negative control. Dimethylsulfoxide was also tested to rule out the effect of the solvent.

All the five fractions obtained (Figure 1) by solvent-solvent

Plant material (crude extract)				Zone of inhibition (mm)		Ethnomedicinal information
Family	Scientific	Local	Part used	<i>Candida albicans</i>	<i>Candida krusei</i>	
Combretaceae	<i>Combretum molle</i> Engl. & Diels	Mudziyaishe	leaves	7.7 ± 0.7	6	Used medicinally to treat fever and stomach complaints
Combretaceae	<i>Combretum imberbe</i> Wawra	Muchenarota, Monzo	leaves	15.5 ± 0.5	16.2 ± 0.2	Coughs. Colds, diarrhoea (Rogers and Verotta, 1996)
Combretaceae	<i>Combretum apiculatum</i> Sond	Chikukute, Mugodo, Bonda	leaves	10.0 ± 0.1	10.3 ± 0.6	Snake and scorpion bite, bloody diarrhoea, leprosy, abdominal disorders, conjunctivities and weak body. (Rogers and Verotta, 1996)
Combretaceae	<i>Combretum zeyheri</i> Sond	Muruka, mupembere-kono, muchenja	leaves	18.5 ± 0.5	18.0 ± 0.3	Coughs, diarrhoea, rectal prolapse, Snake bites and stomachache (Ruffo, 1991)
Combretaceae	<i>Combretum elaeagnoides</i> Klotzsch	Muswati, Mukapo	leaves	12.20 ± 0.7	11.4 ± 0.3	-
Combretaceae	<i>Combretum kraussii</i> Hochst	-	leaves	7 ± 0.8	6	Antidiuretics, lotions for eye infections, as well as antiseptics
	Miconazole			15.5 ± 0.5	14.4 ± 0.7	
	Dimethylsulfoxide (DMSO)			6	6	
	Ethanol			6	6	

^aResults are the average (± SD) of two separate antifungal susceptibility test (Each antifungal susceptibility test was followed by a disk diffusion assay done in quadruplicate at 500 µg of sample per disk). Miconazole was used as a positive control and DMSO and ethanol were used as negative controls

Table 1: Antifungal activity of plant extracts by the agar disc diffusion method.

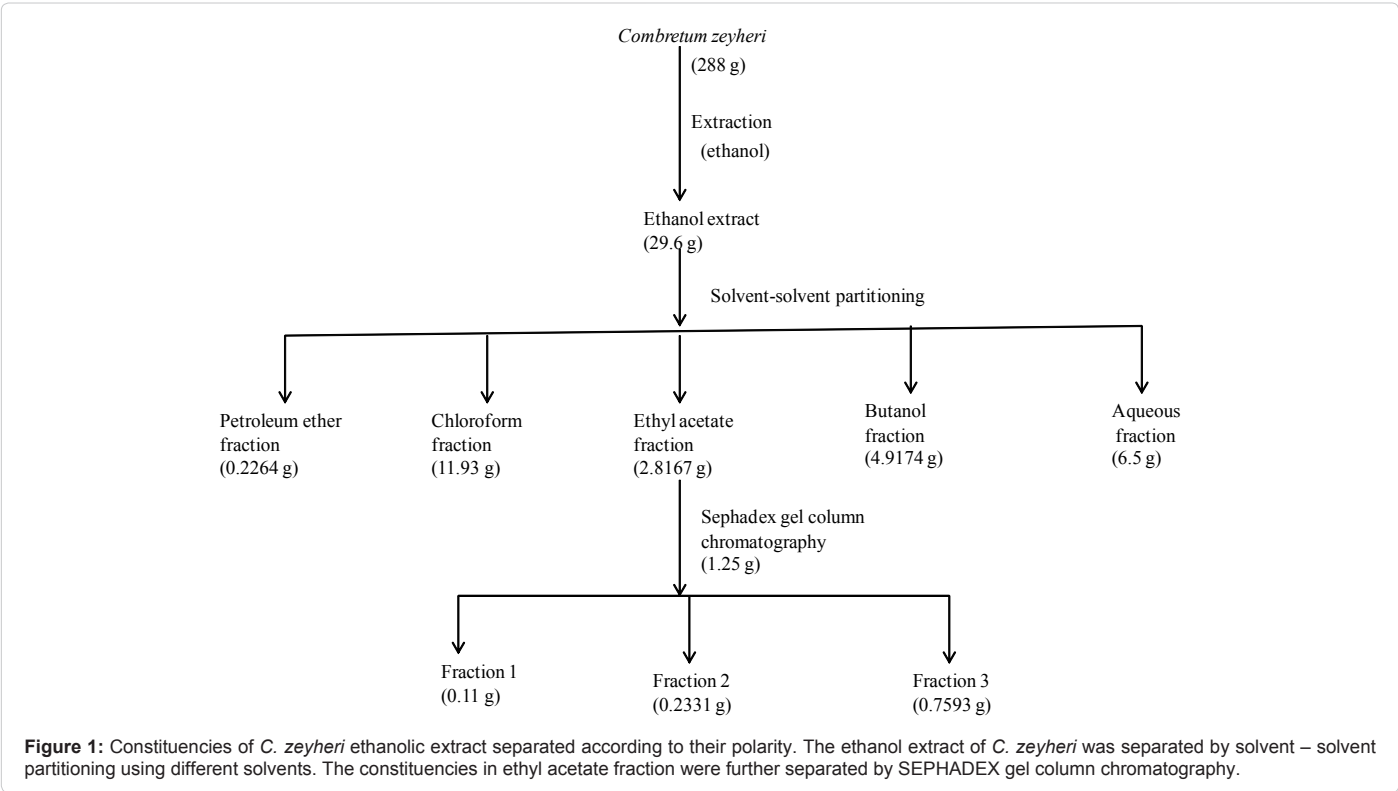


Figure 1: Constituencies of *C. zeyheri* ethanolic extract separated according to their polarity. The ethanol extract of *C. zeyheri* was separated by solvent – solvent partitioning using different solvents. The constituencies in ethyl acetate fraction were further separated by SEPHADEX gel column chromatography.

partitioning were tested for their antifungal activity against *C. albicans* and *C. krusei* and results are shown in Table 3. The ethyl acetate fraction had the highest antifungal activity against both *Candida* species tested. The results obtained with ethyl acetate fraction were comparable with those obtained with the standard antifungal drug, miconazole. No activity was found in the aqueous extract. Constituencies of the ethyl acetate fraction separated by SEPHADEX LH-20 gel column chromatography were also tested for their antifungal activity and results are shown in Table 4. The results show that fractions 3 had the highest antifungal activity as compared to all the fractions tested. *Combretum zeyheri* phytoconstituents, alkaloids and saponins also demonstrated their antifungal effect against *Candida* species (Table 6) while tannins and flavonoids did not show inhibitory activity. This study shows that the

Plant species	<i>C. albicans</i>		<i>C. krusei</i>	
	MIC	MFC	MIC	MFC
<i>Combretum zeyheri</i>	0.08	0.31	0.16	0.63
<i>Combretum imberbe</i>	0.31	0.63	0.63	1.25
<i>Combretum apiculatum</i>	0.31	0.31	0.63	0.63
<i>Combretum elaeagnoides</i>	0.31	>10	0.63	>10
Miconazole	1.25	1.25	1.25	1.25

^aAll results are the average (± SD) of two separate MTT assays and are in mg/ml

Table 2: Minimum inhibitory concentrations and minimum fungicidal concentrations of different Combretum extracts.

	<i>C. albicans</i>	<i>C. krusei</i>
Fraction	^b Zone of Inhibition (mm)	
Petroleum ether	8 ± 0.1	10 ± 1.2
Chloroform	10 ± 0.2	12 ± 1.8
Ethyl acetate	11 ± 0.8	21 ± 1.8
Butanol	10 ± 0.3	20 ± 0.8
Aqueous	6	6
Ethanol	6	6
Miconazole	15 ± 1.1	22 ± 1.4

^bResults are the average (± SD) of two separate antifungal susceptibility test (Each antifungal susceptibility test was followed by a disk diffusion assay done in quadruplicate at 500 µg of sample per disk). Ethanol was used as the negative control and miconazole as the positive control.

Table 3: Effect of fractions of *C. zeyheri* extract on *C. albicans* and *C. krusei*.

Fraction	^c Zone of inhibition (mm)	
	<i>C. albicans</i>	<i>C. krusei</i>
1	12 ± 0.7	11 ± 0.3
2	12 ± 0.3	12 ± 0.6
3	15 ± 1.0	15 ± 1.4
Ethanol	6 ± 0	6 ± 0
Miconazole	15 ± 1.2	17 ± 1.8

^cResults are the average (± SD) of two separate antifungal susceptibility test (Each antifungal susceptibility test was followed by a disk diffusion assay done in quadruplicate AT 500 µg of sample per disk). Ethanol was used as the negative control since all the tested samples were dissolved in ethanol, and miconazole as the positive control

Table 4: Effect of fractions separated by Sephadex column chromatography on *C. albicans* and *C. krusei*.

Plant name	<i>C. albicans</i>		<i>C. krusei</i>	
	MIC	MFC	MIC	MFC
<i>Combretum zeyheri</i>				
Ethanol extract	0.08	0.31	0.16	0.63
Fraction 3	0.31	1.25	2.5	2.5
Miconazole	1.25	1.25	1.25	1.25

^aAll results are the average (± SD) of two separate MTT assays and are in mg/ml

Table 5: MICs and MFCs of ethanol crude extract and fraction 3 of *C. zeyheri* against *C. albicans* and *C. krusei*.

Phytoconstituencies	Percentage yield in plant %	^d Zone of Inhibition (mm)	
		<i>C. albicans</i>	<i>C. krusei</i>
Flavonoids	2.3	10 ± 0.1	9 ± 0.9
Alkaloids	1.08	9 ± 0.3	9 ± 0.2
Saponins	0.8	8 ± 0.1	7 ± 0.3
Tannins	21.5	6	6
Cardiac glycosides	ND	6	6
DMSO		6	6
Ethanol		6	6
Miconazole		14 ± 0.2	14 ± 1.6

^dResults are the average (± SD) of two separate antifungal susceptibility test (Each antifungal susceptibility test was followed by a disk diffusion assay done in quadruplicate). Ethanol was used as the negative control and miconazole as the positive control

The percentage yield was calculated using the formula below

$$\text{Percentage yield (\%)} = \frac{\text{Mass of powdered plant extract (g)}}{\text{Mass of plant material (g)}} \times 100$$

Table 6: Phytochemical evaluation of leaves of *C. zeyheri* and effect of these phytoconstituents on *C. albicans* and *C. krusei*.

crude extracts contain a number of phytoconstituents whose isolation and purification may yield significant novel antimicrobial agents. However, *C. zeyheri* crude extract exhibited better activity in terms of zone of inhibition relative to its fractions and phytoconstituents.

MICs and MFCs of plant extracts

The four plant extracts of *Combretum zeyheri*, *Combretum imberbe*, *Combretum apiculatum* and *Combretum elaeagnoides*, which showed a significant antifungal activity, comparable to the antifungal activity of miconazole against both *Candida* species, were selected further for determination of their minimum inhibition concentrations (MIC) and their minimum fungicidal concentrations (MFC) using the broth dilution method. Table 2 shows the MICs and MFCs of the plant extracts against *Candida albicans* and *Candida krusei*. MIC values ranged from 0.31 mg/ml – 0.63 mg/ml for both *Candida albicans* and *Candida krusei*. *Combretum zeyheri* plant extract had the most antifungal activity as shown in the table, with an MIC of 0.08 mg/ml against *C. albicans* and 0.31 mg/ml against *C. krusei*.

Further work was done to determine the MIC of *C. zeyheri* fraction 3 and then compare with the MIC of the crude extract. The results show that *C. zeyheri*, fraction 3 had a higher MIC against *C. albicans* than the crude extract. The MFC of the fraction 3 was also determined using broth dilution method and results recorded in Table 5.

Qualitative and quantitative analysis of leaves for their phytoconstituents

Qualitative phytochemical analysis of *C. zeyheri* showed the presence of alkaloids, flavonoids, tannins, saponins and cardiac glycosides and the data for their quantitative determination is shown in Table 6.

Plant extracts as efflux pump inhibitors

Combretum zeyheri ethanol extract, fraction 3 and flavonoids were able to act as inhibitors of drug efflux pumps in *Candida* species, results shown in Figure 2. Fraction 3 of *Combretum zeyheri* was the most potent as it reduced the activity of the drug efflux pumps in *Candida albicans* by 50% at 0.73 µg/ml while crude ethanolic extract reduced it at 1.4 µg/ml. Reserpine, a standard drug efflux inhibitor reduced activity of the efflux pumps in *C. albicans* by 50% at 0.1 mg/ml.

Discussion

In the past two decades, the prevalence of life-threatening infections has increased significantly [23]. Among them, candidiasis is the most frequently fungal infection causing morbidity in seriously debilitated and immunocompromised hosts [24]. Although a large number of antimicrobial agents have been discovered, pathogenic microorganisms are constantly developing resistance to these agents [25,26]. In recent years, attempts have been made to investigate the indigenous drugs against infectious diseases in order to help developing safer antimicrobial drugs [26,27].

The present study showed that four *Combretum* plant extracts had profound antifungal effects against *Candida albicans* and *Candida krusei* when compared with the positive control, miconazole and, therefore, may have potential use as antifungal agents. MIC and MFC results showed that *Combretum zeyheri* and *Combretum apiculatum* plant extracts had the ability to kill fungal cells at a very low concentration as compared to all the tested plant extracts and the standard inhibitor miconazole (Table 2). This fungicidal activity gives credit to these plants as traditional medicine for treatment of different ailments [28]. A number of antibacterial compounds including flavonoids (apigenin; genkwanin; 5-hydroxy-7,4'-dimethoxyflavone, rhamnocitrin; kaempferol; quercetin-5,3'-dimethylether; rhamnazin) were isolated from *Combretum* species [10]. Martini and Eloff (1998) demonstrated the presence of at least 14 different unidentified bacterial inhibitors, of widely differing polarity, in leaves of *Combretum erythrophyllum*. MacGaw et al. [29], have investigated some of the biological activity which include anti-inflammatory, anthelmintic, antischistosomal and DNA-damaging effects of *Combretum* species. Significant activity in more than one bioassay was exhibited by *C. apiculatum*, *C. hereroense*, *C. molle* and *C. mossambicense*. Breytenbach and Malan [30], isolated three antibacterial compounds from *Combretum zeyheri* and proposed structures for two of them.

Fractionation of *C. zeyheri* extract by solvent-solvent partition produced fractions that had antifungal activity and of these the ethyl acetate fraction was found to have the most antifungal effects when compared with the positive control, miconazole (Table 3). These results show that ethyl acetate was able to extract the compounds with most antifungal activity and these could be flavonoids since they have been previously reported to have antifungal effects. Most antifungal constituents were extracted in intermediate polarity solvent (ethyl acetate), fewer in high (butanol) and least in low polarity (petroleum ether and chloroform). However, compared to the activity of the crude extracts, the antifungal

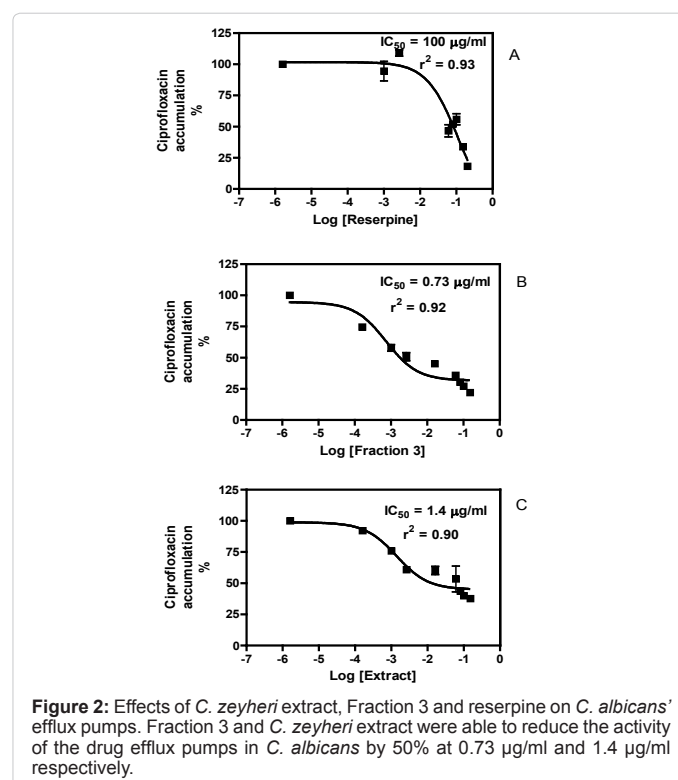


Figure 2: Effects of *C. zeyheri* extract, Fraction 3 and reserpine on *C. albicans*' efflux pumps. Fraction 3 and *C. zeyheri* extract were able to reduce the activity of the drug efflux pumps in *C. albicans* by 50% at 0.73 µg/ml and 1.4 µg/ml respectively.

activity of fractions obtained by solvent-solvent partition was less potent and this could be due to loss of active compounds during fractionation. Further fractionation of the ethyl acetate extract was performed by a SEPHADEX LH-20 gel filtration to give three major constituents namely fraction 1, fraction 2 and fraction 3. The antifungal activities of these three constituents were determined by a dilution assay and of these, fraction 3 inhibited growths of *C. albicans* and *C. krusei* with MIC values of 0.31 and 2.5 mg/ml respectively (Table 5). These results are a general agreement to the published literature, where *Combretum zeyheri* fractions isolated by bioassay-guided fractionation are known to have potent antimicrobial activity [31].

Combretum zeyheri plant extract was subjected to various phytochemical tests to identify the chemical constituents present and the results showed the presence of flavonoids, saponins, alkaloids, cardiac glycosides and tannins. Of these phytoconstituents, flavonoids were found to have the most antifungal activity. Previously, flavonoids have been reported to possess antimicrobial activity [32]. It has been reported that the antimicrobial activity of flavonoids is due to their ability to complex with extracellular and soluble protein and to complex with fungal cell [33]. The results of antifungal activity against *Candida albicans* and *Candida krusei* have indicated that ethanol extract of *C. zeyheri* was found to be significantly active exhibiting the highest potency with MICs of 0.08 and 0.16 mg/ml respectively. This activity may be attributed to the rich flavonoids content of the plant.

Overexpression of multi-drug efflux transporters, including ABC transporters, has been shown to be a major mechanism for the drug resistance for *Candida* species [34]. Among ABC transporters, Cdr1p and Cdr2p have been reported as major drug efflux pump proteins, which play a key role in azole resistance of *Candida* species [35]. Mukhopadhyay et al. [36], examined the functionality of Cdr1p drug extrusion pump by measuring the efflux of rhodamine (R6G), a known

substrate of Cdr1p and similarly, R6G was used as the substrate of efflux pumps in this study. The results of the study show that the whole extract and fraction 3 of *C. zeyheri* had inhibitory effects on drug efflux to an extent that was significantly higher than reserpine. R6G has been reported to be a substrate of drug efflux pumps in *Candida* [12], and this was also shown in this study. Other studies have shown that ciprofloxacin is a substrate for bacterial ATP-dependent efflux pumps [37,38]. This study also shows that ciprofloxacin is a substrate of ATP-dependent drug efflux pumps in *Candida*. The inhibition of drug efflux transporters in *C. albicans* would be expected to restore azole sensitivity to drug resistant forms of *Candida albicans* whose mechanism of azole resistance is via drug efflux. The results of the present study provide a pharmacological basis for the traditional use of *Combretum* species for infectious diseases especially in therapeutic applications against fungal infections. These results may also confirm the great potential of plants of the Zimbabwe ethnomedicine for production of bioactive compounds. Further investigations on isolation and characterization of antifungal bioactive agents from these plants are in progress. It is also necessary to determine the composition of fraction 3 from *C. zeyheri*.

Conclusion

Combretum species are a source of bioactive antifungal compounds. *Combretum zeyheri* was found to have the most potent antifungal activity and some of its fractions isolated were also found to have antifungal activity and to reduce the activity of *Candida albicans* efflux pumps. However, *C. zeyheri* crude extract exhibited better activity in terms of zone of inhibition relative to its fractions and phytoconstituents. This study has identified *C. zeyheri* plant as a plant with potential antifungal activity and partial isolation of active agents has shown that it can be used as a source for the isolation of active compounds that may serve as lead compounds in antifungal drug development.

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