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Evaluation of Anti-fungal Activity of *Coleus* Species Extracts

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Abstract

Antifungal activity of 50%-methanol, water and absolute methanol extracts of *Coleus* species (whole plant) was evaluated using agar, and macro-broth dilution methods. The test microorganisms were strains of *Aspergillus, Rhizopus, Mucor, Rodotorula, Geotricum, Basidiobolus, Trichophyton, Microsporum, Epidermophyton* and *Candida*. The yields achieved with extracts were 24%, 22% and 29% for absolute methanol, water and 50% methanol extracts, respectively. The phytochemical analysis of the extracts revealed the presence of flavonoids, alkaloids, tannins, cardiac glycosides, carbohydrates, steroids, Proteins, glycosides and anthracene glycosides. All (100%) fungal strains tested were susceptible to all the extracts. The observation of the growth inhibition of the fungi tested lasted for 7-14 or more days of incubation on Sabouraud Dextrose agar plates. MIC range was 0.146-15.63mg/ml and *Candida albicans* was the most susceptible of the yeasts tested (MIC_{90} 0.78mg/ml), *Epidermophyton floccosum*, the most susceptible dermatophyte (MIC_{90} = 1.56mg/ml), while *T. tonsurans* was the least susceptible of the fungi tested (MIC_{90} 25mg/ml) and these results are consistent with the time kill observed with the extracts. Results authenticated the folklore medicinal usage of *Coleus species* for treatment of gastrointestinal, reproductive tract and skin infections.

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Introduction

Historically, man has always depended on plant sources for food, defense against enemies and for medicine. Every nationality and culture in the world has depended on herbal remedies, at one time or the other, for treatment of different diseases. The use of herbal remedies declined in favour of synthetic drugs particularly as the orthodox western medical practice gained ground worldwide. In recent times there has been resurgence of herbal treatment to the extent that herbal products (some distributed from the western countries), are seen everywhere neatly displayed on shelves, in markets and private clinics in Nigeria and other parts of the world. They have become a welcomed development given the rich plant diversities found in most countries where herbal remedies are patronized (Khare et al., 2011). Thus, these areas of the world are potential sources of indigenous natural products which could become important foreign exchange earners (Chirag et al., 2012) as well as help to reduce health care costs. Some of the issues in antimicrobial use of synthetic drugs include their inherent toxicity and frequent emergence of drug resistant strains of microorganisms. These factors have prompted the recent researches for therapeutic alternatives from plant sources (Ashok et al.,

2011; Chirag et al., 2012). The past two decades or so have witnessed renewed interest in medicinal plant research as source of new antimicrobial agents but many of the world's plant species have not been subjected to pharmacological and or biological tests for their biological activity and or antimicrobial activity (Senthilkumar et al., 2010).

In the Sub-Saharan African folk lore medicine, *Coleus species* and some members of this family Lamiaceae (Labiatae) have proven to have antiepileptic, antioxidant, anti-inflammatory, diuretic, nephroprotective activities, and anticlastogenic potency against anticancer drugs (Khare et al., 2011). They have been known to treat athlete's foot, skin, wound, gastrointestinal, respiratory, and urinary tract infections, bleeding, itching, fevers, asthma, allergies, sore throat, high blood pressure arthritis and infertility (Tarh et al., 2015). They have also been used as breast milk stimulant (Muhammad et al., 2013; Tarh et al., 2015). *Coleus vettiveroides* is used as a bitter cooling diuretic, trichogenous and antipyretic, also in hyperdipsia, vitiated conditions of pitta burning sensation, strangury, leprosy, leucoderma, vomiting, diarrhea and ulcers (Saraswathy and Lavanya, 2013). *Coleus kilimanschari* has been used to treat infections that destroy red blood cells (Mei et al., 2002). Murugesan et al. (2012) reported effect of *Coleus forskohlii* on intra-ocular pressure and hypotension, as well as it is anti-anaphylactic, amoebicidal, anti-platelet, gastroprotective, broncho-dilating, anti-cancer, anti- hair graying, antibacterial and antifungal activity. They also stated that it can be used as a research tool assessing the adenylate cyclase and cyclic AMP in cellular physiology, and as a condiment for heart ailments and stomach cramps .It was against this background that the work reported here set out to investigate the antifungal activity of *Coleus species*, with the aim of authenticating the folklore application of the plant in treatment of microbial infections.

Materials and methods

Coleus species, commonly known by the Anyang-Kendem people of Cameroon as *Osem-antuoh*, are edible aromatic perennial and mostly succulent herbs with square stems, toothed and opposite leaves, belonging to the mint family, Labiatae, and contains about 150 species (Tarh et al., 2015). *Coleus blumei* (Hybrid Coleus) has been reclassified under *Solanostemon* or *Plectranthus* genera and family Lamiaceae, a family of perennial plants native to tropical Africa, Asia, Australia, East Indies, Malay Archipelago and the Philippines (Ratnakaram et al., 2011). Variegated species of *Coleus* are very colourful, with scapploped or fringed, green, pink, maroon, bronze, yellow, red, crimson leaves, (sometimes confused with the unrelated *Caladium*). Example, *Coleus fredericii* has deep purple flowers in late winter or early spring, with thick brittle, fuzzy green leaves that have a strong aroma. *Coleus* species can grow up to 3-4 feet tall in moist drained soils. They do less well in subtropical areas than shaded areas, where their foliage colour tends to intensify, and are heat tolerant. They are typically grown as house hold plants and become leggy and unattractive with age (Ratnakaram et al., 2011). These famous plants belong to the peppermint and *Basil* family and have been known as kitchen, edible and aromatic perennial herbs

Collection of plant materials

Coleus species plants were collected from Kendem in the south west region of Cameroon as whole plants. The identification and authentication was done in the Department of Botany, University of Nigeria, Nsukka where voucher specimen was deposited. They were washed with distilled water, cut into tiny pieces and air dried in the dark. They were then ground to powder in a mortar, weighed and stored in plastic bags in the dark, dry place until they were used in the experiments that followed.

Extraction of plant materials

A 100 g weight of powdered plant material was soaked in 400 ml of solvent (absolute methanol, water or 50% methanol in water) in a 1 L conical flask covered with cotton wool plugs. The flask was shaken vigorously at first and then intermittently for 24 hrs leaving it in a water bath maintained at 40°C between the intervals of shaking. The mixture was filtered, first through three layers of clean muslin cloth, and then through Whatman no. 1 filter-paper. The filtrates were evaporated to dryness in a water bath at 56°C and the percentage yields of the crude extract determined (Tarh et al., 2015).

Phytochemical analysis of plant extracts

This analysis, which includes detecting the secondary metabolites present in the plant extracts, was carried out according to the methods described by Trease and Evans (1996).

Test organisms

The fungi species used for the study were *Trichophyton mentagropytes, Trichophyton tonsurans, Trichophyton violaceum, Microsporum gypseum, Microsporum canis, Epidermophyton floccosum, Candida albicans*, *Aspegillus species, Basidiobolus, Rodotorulla, Mucor, Rhizopus* and *Geotricum*. They were cultured on Sabouraud Dextrose Agar plates at 25-35°C for 48 hrs or more and the resultant pure mature colonies were sub-cultured on Sabouraud Dextrose Agar slants and stored as stock cultures.

Standardization of fungal inocula

The fungal inocula were prepared from the 7 to 14 days stock cultures grown on Sabouraud Dextrose Agar at 25-35°C. Matured colonies were covered with 2ml of distilled water and suspensions were made by gently probing the colonies with a sterile loop or the tip of a Pasteur pipette. The resulting suspensions were transferred to sterile test tubes. Heavy particles were allowed to settle for about 3-5 minutes and the resultant supernatant homogenous mixtures or suspensions drained into sterile bottles. The colony forming units (CFU) in the suspensions were counted using a haemocytometer and then the suspension diluted with Sabouraud dextrose broth to corresponds to the final standard inocula suspension (spores or yeast cells) of approximately 1×10^5 colony forming unit per ml.

Susceptibility testing of fungi by pour-plate method

A 2.0ml amount of each reconstituted plant extract at the concentration of 1000mg/ml was pipetted into two sterile glass test tube containing 18 ml of molten Sabouraud Dextrose Agar (at about 45°C).The mixtures were swirled carefully for the contents and agar to be thoroughly mixed. Then 100µl of the standard fungal inocula were seeded onto each of the tubes. Again they were thoroughly mixed and poured into each of the plates and allowed to set. They were then incubated at about 25-35°C.

A sterile plate without the extract served as the positive control for growth while another plate containing 2.0ml of 16µg/ml voriconazole as the negative control. As soon as growth was observed at the positive control plates the test plates were checked for growth daily and the period of inhibition of growth was recorded in days.

Determination of minimum inhibitory concentration (MIC) of the plant extracts on the fungi by Macro-broth dilution method

A two-fold serial dilution of the plant extract was carried out in tubes of Saboraud dextrose broth to obtain dilutions ranging from 200mg/ml down to 0.39mg/ml. The 11th tube, which was used as positive growth control culture, did not contain any extract. The control antimycotic agent, voriconazole, was similarly serially diluted but to attain concentrations ranging from 0.125-128µg/ml. Each dilution was seeded with 100µl of the standardized suspensions of the test fungal spores and incubated at 25-35°C for ≥48 hrs. The lowest dilutions without visible growth in the tube cultures, compared to the positive and negative controls were considered as the MICs. The tests were carried out in quadruplets and the means of the MICs calculated.

Determination of minimum concentration at which 90% fungal growth inhibition was observed (MIC_{90}) from broth dilution tubes

A 100µl volume of the fungal test suspensions were taken from all the tubes showing no visible fungal growth and sub-cultured on Sabouraud Dextrose agar plates. These were incubated at 25-35°C. Positive growth control plates were also included and checked simultaneously in comparison with the test plates for fungal growth. Any dilution showing 90% growth inhibition was recorded as MIC_{90} (Sanguinetti et al., 2007). The Minimum Fungicidal Concentration (MFC) was considered as the minimum concentration of the test substances that yielded no visible growth on sub-culture of 100 µl of serial dilutions and incubation at 35°C for more than 48 hrs.

Determination of the effects of plant extracts on viable colony forming units (CFU) of the fungi using Time-Kill (inhibition) assay

The effects of 50% methanloic extracts of *Coleus* species were evaluated by a time-kill assay using the macrobroth dilution technique. The extracts were reconstituted in 20% Dimethyl Sulfoxide (DMSO) and appropriately diluted to the required concentrations. The inoculums size was determined according to the type of fungus, (e.g. 1×10^6 for *Candida albicans* and 1×10^5 for dermatophytes). About 1.00ml of the extract was added to 9ml of Saboraud dextrose broth, seeded with the appropriate concentrations of the test fungus

to achieve concentrations equivalent to 0.5×MIC, 1×MIC, 2×MIC, or 4×MICs values. Two sets of control tubes were included for each experiment. One set was seeded with the organism in broth without extract, and the other set contained broth without organism and extract. The control drug voriconazole was similarly diluted. All the fungal cultures were incubated at 25-35°C for ≥48 hrs. Immediately after inoculation of the tubes, aliquots of 100 µl of the negative control tubes contents were taken, serially diluted in saline and seeded on nutrient agar plates to determine the zero hour counts. The same was done for the tubes which contained the test fungi after 0 hr, 3 hrs, 6 hrs, 24 hrs and 48 hrs, respectively. After incubation, the emergent colonies were counted and the mean count (CFU) of each test organism was determined and expressed as $\log_{10}$. The Minimum Lethal Concentrations (MLCs) of the extract were the lowest concentrations that gave 99.9% to 100% killing.

Statistical analysis

The data obtained was subjected to analysis of variance (ANOVA) using the Randomised Complete Block Design (Two-way analysis of variance) using SPSS version 13.0 (SPSS, 2003). Duncan's New Multiple Range Test was used to separate the means that were significantly different.

Results

Absolute methanol gave a yield of 24%, 22% for the water, while 50%-methanol gave 39%. Phytochemical analysis of *Coleus species* (whole plant) extracts showed the presence of alkaloids, flavonoids, tannins, cardiac glycosides, carbohydrates and steroids, glycosides, proteins and anthracene glycosides. No reducing sugars and saponins, were found in any of the plant extracts (Table 1).

Table 1. Phytochemical composition of whole plant extracts of *Coleus species.*

Phytochemicals	*Coleus species* (whole plant)extracts		
	Absolute methanol	Water	50% methanol
Alkaloids	+	+	++
Flavonoids	+++	++	+++
Tannins	++	++	+++
Saponins	–	–	–
Cardiac glycosides	+	+	+
Glycosides	+	+	+
Proteins	+	+	+
Carbohydrates	+	+	+
Reducing sugars	–	–	–
Steroids	+	+	++
Antracene glycosides	+	+	+

+++ = Strongly Positive; ++= moderately positive; + = Weakly positive; - = Negative

The 50% methanolic extract of *Coleus species* inhibited the growth of the test fungi, namely, dermatophytes, *Basidiobolus haptosporus, Rhodotorula species, Candida albicans*, species of *Aspergillus, Mucor*, *Rhizopus* and *Geotricum* for ≥14 days. The absolute methanolic extract as well as the aqueous extract inhibited the fungal growth for ≥12 days (Table 2). The control antimycotic agent (voriconazole) inhibited the growth of all the fungal isolates tested for over 14days (Table 2).

The MICs and MIC_{90}s were evaluated using the 50% methanolic extract (the most potent or efficacious of the extracts) and *Candida albicans* emerged as the most susceptible fungus (MIC 0.146mg/ml and MIC_{90} of 0.78mg/ml). This was closely followed by *B. haptosporus* with MIC of 0.49mg/ml and MIC_{90} of 1.56mg/ml. Among the dermatophytes tested, *E. floccosum* was the most susceptible with MIC 0.98mg/ml and MIC_{90}, 1.56mg/ml while *T. tonsurans* was the least susceptible with MIC of 15.63 and MIC_{90} of 25mg/ml (Table 3).

For the control drug (voriconazole), *C. albicans* (MIC 0.313 µg/ml, MIC_{90} of 0.5µg/ml), and *T. rubrum* (MIC 0.375 µg/ml, MIC_{90} of 0.5µg/ml), were the most susceptible fungi while the least susceptible of the fungi was *B. haptosporus* at MIC of 1.25µg/ml, and MIC_{90} of 4µg/ml (Table 3).

Table 2. Fungal growth inhibition (in days) by *Coleus species* extracts.

Fungal species Tested	***Coleus species* extracts (100mg/ml) and the number of days the inhibition of growth lasted**			
	Absolute methanol	Aqueous	50% Methanol	Voriconazole (8μg/ml)
Basidiobolus haptosporus	>12	12	>14	>14
Rhodotorulla species	>12	12	>14	>14
Candida albicans	>12	12	>14	>14
Trichophyton mentagrophytes	>12	12	>14	>14
Trichophyton rubrum	>14	12	>14	>14
Trichophyton tonsurans	>12	12	>14	>14
Trichophyton violaceum	12	12	>14	>14
Microsporum gypseum	12	12	>14	>14
Microsporum canis	12	12	>14	>14
Epidermophyton floccosum	>14	12	>14	>14
Aspergillus flavus	14	14	>14	>14
Aspergillus niger	14	14	>14	>14
Aspergillus fumigatus	14	14	>14	>14
Mucor species	14	14	>14	>14
Geotricum species	14	14	>14	>14
Rhizopus nigricans	14	14	>14	>14

Table 3. Effects of 50% methanol extract of *Coleus species* on fungi.

Fungi species	***Coleus species* whole plant 50% methanol extract (mg/ml)**		**Voriconazole (μg/ml)**	
	MIC±SD (mg/ml)	**MIC_{90} (mg/ml)**	**MIC±SD (μg/ml)**	**MIC_{90} (μg/ml)**
Candida albicans	0.146±0.05	0.78	0.313±0.13	0.50
Rhodotorulla species	0.39±0.00	1.56	0.75±0.29	2.00
Basidiobolus haptosporus	0.49±0.195	1.56	1.25±0.50	4.00
Epidermophyton floccosum	0.98±0.39	1.56	0.313±0.13	1.00
Trichophyton mentagrophytes	1.37±0.59	3.13	0.189±0.07	1.00
Trichophyton rubrum	1.95±0.78	3.13	0.375±0.14	0.50
Microsporum canis	2.73±0.78	3.13	0.25±0.00	0.50
Trichophytonton surans	15.63±6.30	25	0.25±0.00	1.00
Trichophyton violaceum	1.37±0.39	3.13	0.5±0.00	1.00
Microsporum gypseum	7.80±3.13	12.50	0.75±0.29	2.00
Aspergillus favus	1.95±0.78	1.56	0.25±0.00	0.50
Aspergillus niger	1.95±0.78	1.56	0.25±0.00	1.00
Aspergillus fumigatus	1.37±0.59	1.56	0.25±0.00	1.00
Rhyzopus nigrcans	1.95±0.78	1.56	0.189±0.07	1.00
Mucor species	0.98±0.39	3.13	0.375±0.14	1.00
Geotricum species	0.49±0.195	3.13	0.75±0.29	2.00

Time-Kill (inhibition) assay

The exposure of *C. albicans* to varied concentrations of 50%-methanol extract of *coleus species,* showed that 4MIC concentration (1.17mg/ml) and Voriconazole (0.5µg/ml) cleared *Candida albicans* viable cells in 24 hrs and 48 hrs, respectively (Fig. 1). Fungicidal activity was also observed even with the sub-inhibitory concentrations, i.e.0.5 MIC (0.14mg/ml).

Trichophyton mentagrophytes was killed in 3 hrs by the 4MIC (5.48mg/ml) concentration of 50%-methanol extract of *Coleus species,* and in 48hrs by the control drug (voriconazole 1µg/ml) (Fig.2).

M. gypseum viable cells were totally undetectable after exposure to 4 MIC and 2 MIC (31.25mg/ml and 15.6mg/ml) concentrations of 50% methanol extract of *Coleus species* in 3hours and by the control drug at 2µg/ml in 48 hrs (Fig. 3). The fungicidal activity of the 50% methanol extract of *Coleus species* on *E. floccosum* was more prominent with the 4MIC and 2MIC

(3.9mg/ml and 1.96mg/ml) concentrations (Fig. 4). These concentrations inhibited *E. floccosum* totally in 3hours and 1µg/ml of the control drug inhibited the fungal cells in 48hrs).

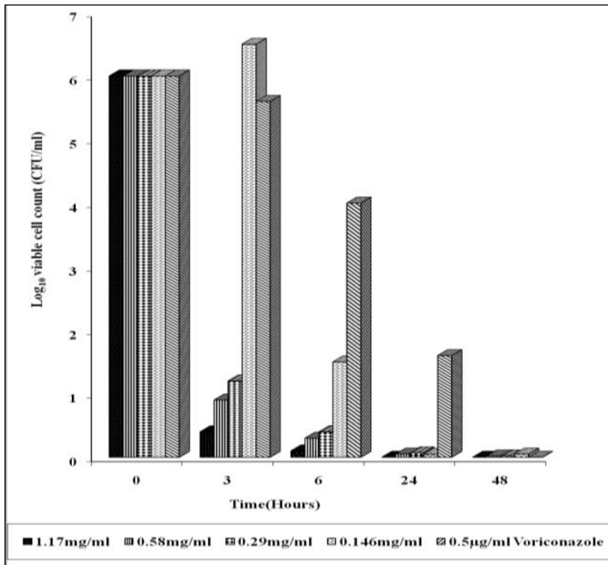

Fig. 1: Effect of 50% methanol extract of *Coleus species* (whole plant) on viable cell count of *Candida albicans*.

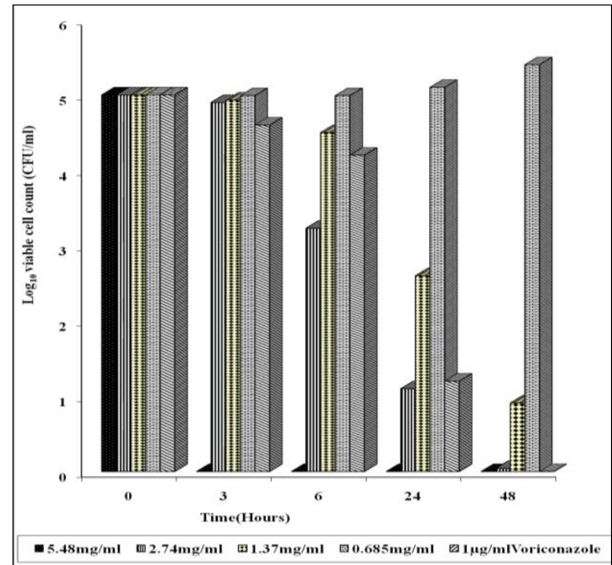

Fig. 2: Effect of 50% methanol extract of *Coleus species* (whole plant) on viable cell count of *Trichophyton mentagrophytes*.

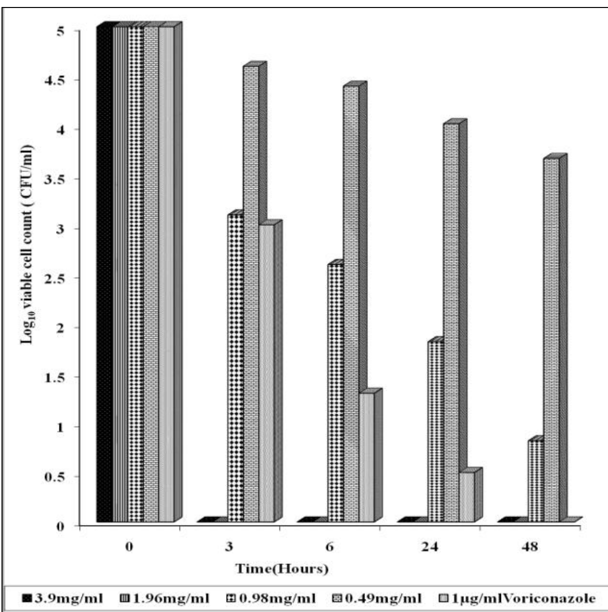

Fig. 3: Effect of 50% methanol extract of *Coleus species* (whole plant) on viable cell count of *Microsporum gypseum*.

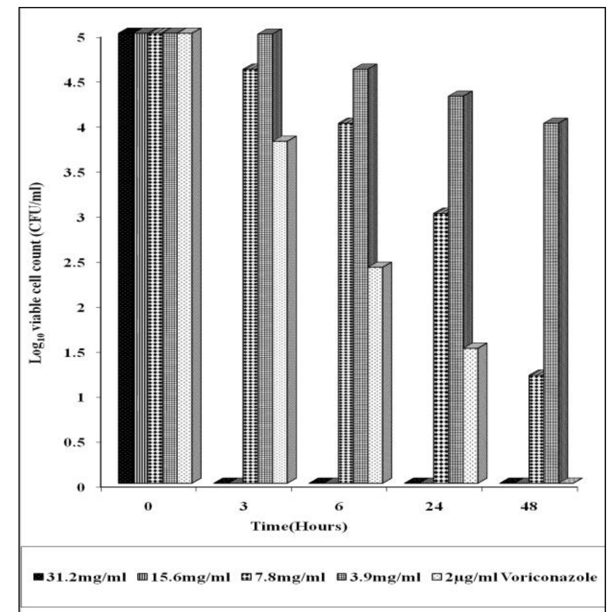

Fig. 4: Effect of 50% methanol extract of *Coleus species* (whole plant) on viable cell count of *Epidermophyton floccosum*.

Discussion

The comparatively high yield of extracts (22%, 24% and 39% for water, Methanol and 50% methanol, respectively) was consistent with the observation reported in Tarh et al. (2015) and compares favorably with the yields of 17.5% and 22.3% obtained by Majee et al. (2013) from respective water and methanol extracts of *Coleus aromaticus* leaves. The methanol extracts (50% and absolute) inhibited growth of test fungal strains for longer period than the aqueous extract suggesting that they contained more efficacious and/or more stable bioactive compounds. Majee et al. (2013) observed a similar superior antimicrobial activity of hydro-alcohol extracts of *Coleus aromaticus* Bioactivity has also been reported for alcohol extracts of other

Coleus species (Khare et al*.,* 2011; Murugesan et al*.*, 2012; Majee et al*.,* 2013) against bacteria and or fungal strains. This is interesting given that in Kendem, south-west Cameroon where the plant materials were collected concoctions made by macerating parts of this plant in locally distilled gin is consumed for treatment of various ailments including microbial infections. Thus, the results of this work authenticate the use of this plant, particularly as alcohol preparation in ethno-medicinal practice in the region. The aqueous extract showed the least activity on all the test organisms which may imply that the water soluble constituents are not comparatively bioactive or the bioactivity may have been destroyed by hydrolysis.

In this study, the MIC_{90} values obtained, showed that the yeasts were significantly more susceptible (*Candida albicans*, F_{cal} = 0.896, *Rhodotorula,* F_{cal} = 0.993, *p*=0.322) than the dermatophytes (*E. floccosum,* F_{cal} = 1.053; *T. mentagrophytes,* F_{cal} = 2.927 and *M. gypseum,* F_{cal} = 14.641, respectively, *p*=0.001). Sub-inhibitory concentrations of some of the plant extracts killed *Candida albicans*. There was no significant difference between the values obtained by the macro broth dilution method and the agar dilution method ($p<0.05$). This is unlike Guilhem et al. (2007) who reported that test methods influenced the results obtainable from susceptibility assays.

The rate at which the extracts killed the fungal cells varied with concentration of the extract, duration of exposure and test organism. The 100% killing of the viable cells recorded at different concentrations of the extracts and exposure intervals is very uncommon for crude plant extracts (Ibrahim et al., 1998). The fungicidal or fungistatic activity recorded at these concentrations for the extracts might have been dependent on the duration of exposure, and could be a combined effect of various constituents in the plant.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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