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Comparative Assessment of Antifungal Activity of Extracts from *Eucalyptus globulus* and *Eucalyptus citriodora*

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Abstract: A study was conducted to establish the antifungal properties of extracts from *Eucalyptus citriodora* and *Eucalyptus globulus*. Three fungal pathogens were identified and used in this study, *Trichophyton mentagrophytes*, *Microsporum gypsum* (filamentous forms) and *Candida albicans* (yeast) using the well diffusion method. Serial dilution of the extracts and essential oils was done in order to determine the lowest active concentration in comparison to that of 1% Clotrimazole, 50 mg mL^{-1} Griseofulvin and 1% life Nystatin. The activity of 100 and 50% of *E. citriodora* oil was greatly higher than that of *E. globulus* and standard drugs. Methanol extracts were less active, compared to essential oils ($p<0.05$). Gas chromatogram analysis of *E. citriodora* oil confirmed a total of 9 compounds. The *E. citriodora* oil presented the highest growth inhibition for all the microorganisms tested. The results indicate that *E. citriodora* and *E. globulus* have some antifungal properties which might be exploited as natural fungicide for the management of fungal diseases especially in this era of opportunistic diseases due to HIV/AIDS.

Key words: Antifungal activity, *Candida albicans*, *Eucalyptus citriodora*, *Eucalyptus globulus*, *Trichophyton mentagrophytes*, *Microsporum gypsum*

INTRODUCTION

The globe is witnessing an upsurge in the prevalence of opportunistic fungal infections due to long term use of broad spectrum antibiotics, immunosuppressive drugs and debilitating disease conditions such as HIV/Aids. Plants that are traditionally used in the treatment of fungal or related ailments could be good sources for new safe, biodegradable and renewable drugs (Njoroge and Bussmann, 2006; Kubo and Tuniguchi, 1993). *Eucalyptus citriodora* Hook, commouly known as the lemon scented gum and *E. globules* Labitt, from the family Myrtaceae are exotic trees to Kenya (Birnie and Tim, 1989). Eucalyptus trees are well known for their lemon scented and aromatic oils (Maxwell-Hudson, 1995), used in perfume industry (Taylor, 1984). Eucalyptus species' extracts are now entering into common herbal use for treatment of colds, chest pains, coughs. Eucalyptus leaf extracts have been used to treat influenza, chest problems, skin rashes and the vapor is inhaled for inflammation. Essential oils extracted from various plants have been shown to have significant antifungal properties (Sartorelli *et al*., 2006; Khalil and Dababneh, 2007). Most of the secondary activities of essential oils frequently reported are on bactericidal and bacteriostatic property (Mwangi, 1994). Antimicrobial activities have been attributed to the presence of 1, 8-cineol content (Dellacassa *et al*., 1989; Mwangi, 1991, 1994). Essential oils from several Eucalyptus species have been screened for their bioactivity against bacteria and have been found to be active against *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeroginosa*, *Staphylococcus aureus* and *Pseudomonas syringae* pr. Phaseolicola (Salari *et al*., 2006; Sartorelli *et al*., 2006; Dellacassa *et al*., 1989; Janseen *et al*., 1987; Banergee and

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Nigam, 1976). Treatment and control of *C. albicans* infections has been by use of 1% Clotrimazole, Amphotericin B and 1% life Nystatin. Some antifungal drugs such as Ketoconazole have serious side effects such as liver damage (Fromtling, 1984). The uses of plant material as fungicide are of great importance and needs more attention. Studies on antifungal activity of plant extracts are very few in Kenya. Due to these reasons, there is urgent need to search for inexpensive and safe, biodegradable alternative drugs possibly from plant origin. The present study was undertaken to isolate and to investigate the antifungal activity of extracts and essential oils from eucalyptus species, hence, establish the minimum inhibitory concentration of different plant extracts and fractions. We hypothesized that; extracts from different plants, plant parts and fractions exhibit different antifungal activities on fungal pathogens.

MATERIALS AND METHODS

Plant Materials

Plant materials used in the study were collected between February and June, 2006 from areas surrounding Maseno University in Kenya, latitude 0°'N-0° 12'S and longitude 34° 25'E-47'E. Voucher specimens were deposited in the Departmnent of Botany and Horticulture herbarium.

Isolation of Essential Oils

Plant parts (fruits, flowers and barks) except the leaves were chopped into small pieces and left to dry in the shade at 20-27°C for one month before grinding to a fine powder. The powder was weighed and extracted twice with methanol for 72 h. The extract was decanted, filtered and dried with magnesium sulphate before vacuum evaporation. Essential oils were hydrodistilled for 3 h using Clevenger type apparatus according to the standard procedure described in the Sainte-Ruffine (1975) and dried using anhydrous magnesium sulphate and stored in dark screw-capped bottles under refrigeration.

Chemical Analysis of the Essential Oils

The chemical composition of the essential oils was determined with a G 800 B GCD system with an electron ionization detector (Hewlett-Packard Co, Polo All, CA) for high-resolution gas chromatography-mass spectrometry (GC-MS) analysis. Essential oils were injected into HP-5 fused silica capillary column (30 m = 0.25 mm) used with helium as the carrier gas (1 mL min^{-1}). The temperature programmed was 80°C for 2 min and 80-200°C at 4°C min^{-1}. MS were taken at 70 eV. The volatile components were identified by comparison of their Kovat indices data generated from a series of alkanes: C_9-C_{30} (Adams, 2001; Jennings and Shibamoto, 1980). Kovat indices of compounds with the area over 0.5% were calculated to obtain the name of the compound using the formula;

$$KI = \frac{[MX100 + Rt_x - Rt_a]}{Rt_b - Rt_a} \times 100$$

Where:

KI = Kovat index
Rt_x = Retention time of compound X
Rt_a = Retention time of immediate compound prior to X
Rt_b = Retention time compound after X
M = No. of carbons in the compound

General laboratory reagents were used to determine the presence of unknown functional groups present in the extracts. The presence of the phenolics group was tested using ferric chloride. The carbonyl group (-C = O) was tested with 2, 4, dinitrophenyl hydrazine while the presence of double or triple carbon bonds (unsaturation) were tested using bromine water.

Fractionation

About 2.5 g of *E. citriodora* oil was applied to two glass columns and left to run until they were just absorbed in to the silica gel without leaving the column to run dry. The column was first eluted with 250 mL of hexane followed by 250 mL of methanol. The eluted samples were dehydrated with magnesium sulphate, filtered and evaporated in a Buchi rotary evaporator at 40°C and weighed. The concentrate was analyzed by TLC on precoated silica plates using toluene/hexane (7:3).

Conducting *in vitro* Tests

The crude extracts and oils obtained were weighed and tested for antifungal activity using well diffusion method of Barry *et al.* (1979). The fungal strains of *Trichophyton mentagrophytes*, *Candida ablicans* and *Microsporum gypsum* were obtained from the school of biomedical sciences of Maseno University, Kenya. The susceptibility of the test organism to the extracts was done using the method described by Baker *et al.* (1983). One gram of each extracts and essential oil was serially diluted with sterile dimethyl sulfoxide (DMSO) to give 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 and 0.19% extract concentration. Sabourauds Dextrose Agar (SDA) was used as the basal medium for test fungi. Sixty two grams of SDA was dissolved in distilled water and autoclaved at 121°C for 15 min and left to cool to about 50- to 55°C. Between 20 and 25 mL was dispensed aseptically into a sterile 90 mm diameter petri dish and left to set uniformly in aseptic conditions before a 5 mm diameter well was punched at the center of the agar using a cork borer of 5 mm diameter. Fungicidal and fungistatic properties of the extracts were carried out by use of streak test for *Candida albicans* and the disk method for dermatophytes (Reiner, 1982). Minimum Inhibitory Concentration (MIC) which is the lowest concentration that gives an approximation to the least concentration of an antimicrobial needed to prevent microbial growth (Barry *et al*., 1981; Mostahar *et al*., 2007), was established.

Statistical Analysis

One factor ANOVA was used to determine whether there were significant differences in the activities of the extracts and those of the standards using SPSS statistical package.

RESULTS

Gas chromatogram analysis of the essential oil indicated a total of 9 compounds with 5 major ones (Table 1). The compound with the highest percentage content was n-hexyl acetate with percentage concentration of 35.89%. The other major compounds were iso-butyl formate (24.44%), allyl valerate (4.27%) and n-dodecane (7.85%). Unknown major compounds which were eluded at 21.58 min had a concentration of 22.23%. Major components of *Eucalyptus globulus* oil were reported earlier by Mustapha (2006) and contain 1, 8-cineole (29.5%), p-cymene (11.5%) and γ-terpineol (5.2%). The study of essential oils of *E. citriodora* indicated the presence of functional groups such as of phenolics compounds and double bonds (unsaturation) with a pH of 6.The carbonyl group was absent. The relative density of the essential oil of *E. citriodora* was 0.8958 and with refractive index of 1.4584.

Table 2a show that methanol bark extracts of *E. globulus* was active against all the three test organisms. The inhibitory activity was lower than that of the essential oil and same as that of the fruit extract. The study indicate that the activity of 100% bark and leaf extracts was as good as

Table 1: Percentage composition of oils present in *E. citriodora* and their respective Kovat Indices (KI)

Compounds	KI	Content (%)
Iso-butyl formate	953	24.43958
Di-iso-pentyl ether	1065	0.95760
Unknown	1148	22.22798
Limonene	1198	2.70895
n-dodecane	1202	7.85389
B-phellandrene	1219	0.70916
Allyl valerate	1257	4.27193
p-cymene	1271	0.93651
n-hexyl acetate	1310	35.89442

Table 2a: Growth inhibitions caused by *E. globulus* methanol extracts at the lowest active concentrations after 48 h for yeast and 72 h for filamentous forms

Bioactive material of *E. globulus* (% concentration)	Test organisms (% inhibitions)		
	C. ablicans	*T. mentagrophytes*	*M. gypsum*
Fruit extract			
(a) 100%	13	25	16
(b) Lowest conc.	5 (50%*)	3 (313%*)	5 (25%*)
Bark extract			
(a) 100%	14	25	20
(b) Lowest conc.	11 (25%*)	4 (12.5%*)	11 (50%*)
Essential oils			
(a) 100%	18	23	46
(b) Lowest conc.	6 (12.5%*)	4 (6.25%*)	7 (3.13%*)
1% Clotrimazole	42	45	51
Griseofulvin	13	43	40
1% Nystatin	35	15	32

*: Lowest active concentration

Table 2b: Growth inhibitions caused by *E. citriodora* methanol extracts at the lowest active concentrations after 48 h for yeast and 72 h for filamentous forms

Bioactive material of *E. citriodora* (% concentration)	Test organisms (% inhibitions)		
	C. ablicans	*T. mentagrophytes*	*M. gypsum*
Leaf methanolic extract			
(a)100%	18	16	35
(b) 50%	18	9	18
(c) Lowest active conc.	8 (1.56%*)	4 (25%*)	4 (6.25%*)
Essential oils			
(a) 100%	100	100	100
(b) 50%	34	100	100
(c) Lowest active conc.	9 (3.13%*)	8 (1.56%*)	8 (1.56%*)
Bark methanolic extract			
(a) 100%	13	17	15
(b) 50%	7	9	8
(c) Lowest active conc.	4 (25%*)	5 (12.5%*)	5 (25%*)
1% Clotrimazole	40	50	46
50 mg mL^{-1} Griseofulvin	9	47	31
1% Nystatin	38	16	39

*: Lowest active concentrations

at 50 mg mL^{-1} Griseofulvin against *C. albicans* and better than 1% Nystatin against *T. mentagrophytes*. The activity was however much lower than that of 1% Clotrimazole against all the test micro-organisms. The activity of 100% essential oils was higher than 1% Nystatin against dermatophytes and slightly higher than 50 mg mL^{-1} Griseofulvin against *C. albicans* and 1% Nystatin against *M. gypsum*, respectively. The essential oils were fungicidal against all the test organisms. Table 2b shows that *E. citriodora* essential oil was active against dermatophytes and yeast. *Candida ablicans* was completely inhibited at 100% of oil concentration.

Significant differences occurred between *E. citriodora* extracts and the standard antifungal drugs. *Eucalyptus citriodora* essential oil was extremely effective at 100% concentration on all test fungi compared to all the standards ($p<0.05$). The filamentous fungi were highly inhibited at 50-100% concentration of the oil. The oil was a more potent fungitoxic drug than 50 mg mL^{-1} of Griseofulvin. Dermatophytes responded similarly to essential oil at the same lowest inhibitory concentration of 1.56% with 7% inhibition. *Candida ablicans* was less susceptible to essential oil, with a higher inhibitory concentration at 3.13%.

The lowest inhibitory concentration of methanol leaf extract against dermatophytes was much higher than of the essential oil and lower for yeast. The activity of the bark extract was lower than that of the leaf ($p<0.05$). The bark extract was only active at 12.5-100% concentration compared to 1.56-100% from the leaf extract. The activity of 100% concentration of leaf and bark methanol extracts was lower compared to 1% Clotrimazole. The activity of the leaf extract was comparable with that of 1% Nystatin against dermatophytes and better than 50 mg mL^{-1} Griseofulvin against *M. gypsum* and *C. ablicans* (Table 2b).

The bark extract was active at 12.5-100% concentration while the oil was active at 3.13-100% (Table 3). The polar fraction was found to be inactive at all concentrations against all the test organisms. Table 4 shows the activity of *E. citriodora* whole oil against *C. ablicans* was significantly higher than that of fractionated oil ($p = 0.0073$). The test organisms were completely inhibited at 100%

Table 3: Percentage growth inhibitions of *E. globulus* essential oil compared with that of *E. citriodora* bark extracts

		Growth inhibition of fungi (%)		
Plants	Extract concentration (%)	*C. ablicans*	*T. mentagrophytes*	*M. gypsum*
E. globulus	0.190	0.0	0.0	0.0
	0.390	0.0	0.0	0.0
	0.780	0.0	0.0	0.0
	1.560	0.0	0.0	0.0
	3.125	0.0	0.0	5.0
	6.250	0.0	2.0	14.0
	12.500	3.0	13.0	23.0
	25.000	7.0	17.0	29.0
	50.000	16.0	19.0	38.0
	100.000	18.0	21.0	43.0
E. citriodora	0.190	0.0	0.0	0.0
	0.390	0.0	0.0	0.0
	0.780	0.0	0.0	0.0
	1.560	7.0	5.0	5.0
	3.125	7.5	5.5	5.5
	6.250	18.0	14.0	18.0
	12.500	23.0	16.0	23.0
	25.000	25.0	22.0	27.0
	50.000	33.0	100.0	100.0
	100.000	100.0	100.0	100.0

Table 4: Growth percentage inhibition exhibited by *E. citriodora* fractionated and non fractionated oil after 48 h for yeast and 72 h for filamentous form

	Test organisms (% inhibitions)		
Bioactive materials (% conc.)	*C. ablicans*	*T. mentagrophytes*	*M. gypsum*
Fractionated oil			
100%	100	100	100
Lowest active conc.	5 (12.5%*)	8 (1.56%*)	8 (6.25%*)
Whole oil			
100%	100	100	100
Lowest active conc.	12 (3.13%*)	7 (1.56%*)	10 (1.56%*)

*: Lowest active concentrations

of the oils, the lowest active concentration of fractionated oil was much higher against *C. ablicans* and *M. gypsum* compared to that of whole oil. There were no significant differences in the activity of the two oils against *T. mentagrophytes*. The activity of *E. citriodora* essential oil was fungicidal against both filamentous and yeast fungi at the lowest active concentration.

DISCUSSION

The study indicated that the plants extracts were effective on the fungi tested. The leaf, bark and fruit extracts exhibited different antifungal properties. This is probably because of different concentrations of the active compounds in the different plant parts. Lack of activity in the fruit extracts could mean that the active compounds are absent in the fruit.

The essential oils were more effective than methanol extracts (Table 2a, b). The essential oil from *E. citriodora* was the more effective fungitoxin in comparison to *E. globulus*. The essential oil completely inhibited filamentous (*M. gypsum* and *T. mentagrophytes*) fungi at 100% concentration (Table 2a, b). The effectiveness of the essential oils could suggest the presence of strong active principles among their constituents. These results are in agreement with the previous works (Salari *et al*., 2006; Low *et al*., 1974). The antimicrobial activity of plant extracts can be attributed to not only a single bioactive principle but also due to the combined action of other compounds (Sunayana *et al*., 2003). Previously, the activity of eucalyptus essential oil has been attributed to 1, 8-cineol content (Maxwell-Hudson, 1995). However, the work by Dellacassa *et al*. (1989) and Mustapha (2006) indicated no relationship between 1, 8-cineol content and inhibition zones of the test organisms. Although *E. citriodora* and *E. globulus* belong to the same species, the activity of *E. citriodora* was significantly higher than that of *E. globulus* (Table 3). The differences in inhibition activity of these plants could be explained by their different essential oil components. Antifungal properties of many plants have been established to be due to, at least partially, to their essential oils, lethal effect, growth inhibition or growth retardation (Mustapha, 2006).

Previous research works have indicated that essential oil antifungal activity is not associated only with major components (Reuveni *et al*., 1984; Low *et al*., 1974). Antimicrobial activities of some essential oils such as those of eucalyptus have been reported by Singh *et al*. (1987) and Janseen *et al*. (1987). The oil composition of plants has also been shown to be dependent of the phenologic stage (Sartorelli *et al*., 2006). For instance, *E. saligna* in the vegetative phase, the major constituents were p-cymene (54.2%) and γ-terpinene (43.8%), while during the blossoming α-pinene became the major constituent followed by p-cymene (22.5%). Previous studies have reported high variation in quantity and composition of essential oils of wild plant populations and clones depending on the location of the plants stands (Karaman and Cömlekçioğolu, 2007).

Eucalyptus citriodora was chosen for fractionation than *E. globulus* because it was found to have a complete inhibition of all the test organisms at 100% concentration. The polar fraction of the oil was found to be inactive against all the test organisms unlike the non polar fraction which was active. The activity of *E. citriodora* against *C. ablicans* was significantly higher compared to that of fractionated oil ($p = 0.0073$). The non polar fractions of the fractionated oils were active while the fractionated ones were not. The results may suggest that the active compounds are the non polar components. Some authors have confirmed that the antifungal activity of essential components decreases with their degree of water solubility (Knobloch *et al*., 1988; Zygadlo and Grosso, 1995). It has been reported that methanol is a better solvent for consistent extraction of antimicrobial substances from plants as compared to other solvents such as aqueous ethanol and hexane (Lin *et al*., 1999). The yeast (*C. ablicans*) was completely inhibited by 100% concentration of both the oil and the lowest active concentration of the whole oil was at 3.13% compared to 12.5% for fractionated oil (Table 4). The reduction in activity of fractionated oil may be due to reactivity of silica gel with hydrocarbons and

oxygenated fractions (Banthorpe and Charlwood, 1990). Keto-rich oils are more effective than oxide-rich oil (Mustapha, 2006). Some of the compounds could have been retained in the gel or could have changed in their properties. There is also a possibility of some of the active compounds being lost during vacuo-evaporation process.

The oil contained phenolic compounds and double bonds (unsaturation) with a pH of 6. Phenolics compounds are flavonoids (Mostahar *et al*., 2007) and they protect plants against disease causing microorganisms. The presence of high phenolic coefficient in some plant essential oil has been associated with their antifungal property (Stitcher *et al*., 1989). Biological activities of many other flavonoids, polyphenols or phenolics compounds were also reported (Edenharder and Grunhage, 2003; Wang *et al*., 2003). Their reactions with proteins, enzymes and other biological processes in the cells make them toxic or to serve as growth inhibitors (Murrey *et al*., 1988; Malterud *et al*., 1985). They are stable compounds which can be extracted by use of water, methanol or acetone (Harbone, 1984). Phenolics compounds are found on external surfaces of leaves and bark and rarely on fruits. This may explain the absence of activity of the fruit extracts on the test organisms. They are less polar compounds requiring less polar solvents for extraction (Harborne, 1984) and could contribute to the antifungal activity of the non polar fractions of the fractionated oils.

Physico-chemical and structural comparisons of the compounds revealed that some compounds shared some similarities with the commercial antifungal drugs. For example Nystatin, contains a system of a conjugated double bonds and a separate hydroxylated sections (Gale, 1977). The presence of hydroxyl groups in the extracts could enhance the likelihood of intra-molecular reactions such as the formation of cyclic acetals and anhydroethers (Mann, 1994). Many pharmacological effects of plant extracts could be associated with inhibition of nucleic acid, protein and membrane phospholipids biosynthesis (Shelton, 1991).

CONCLUSION

The study confirmed antifungal properties of essential oils from *Eucalyptus globulus* and *Eucalyptus citriodora*.The stem bark contained more active compounds than the leaves and fruits. Methanol extracts were less active compared to essential oils. Gas chromatogram analysis of *E. citriodora* oil confirmed a total of 9 compounds. The *E. citriodora* oil presented the higher growth inhibition for all the microorganisms tested. The results indicate that *E. citriodora* and *E. globulus* might be exploited as natural fungicide for the management of fungal diseases especially in this era of opportunistic diseases due to HIV/AIDS.

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