

ANTIFUNGAL ACTIVITY OF ETHANOLIC LEAF EXTRACT OF *Eucalyptus camaldulensis* Dehn. AGAINST RINGWORM PATHOGENS

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ABSTRACT

Ethanollic leaf extract of *Eucalyptus camaldulensis*, dispersed in a concentrated sugar solution had marked fungicidal effect against clinical dermatophytic fungal isolates; *Microsporium gypseum* and *Trichophyton mentagrophytes*. *Microsporium gypseum* at an inoculum level of 4.8×10^3 cfu/ml and *T. mentagrophytes* at 6.3×10^3 cfu/ml were killed to 7 cfu/ml and 10 cfu/ml respectively within 4 hours in Sabouraud broth supplemented with 63% (w/w) of dextrose and containing 100% (w/w) of crude eucalypt leaf extract. Added organic matter (bovine serum) did not affect its antifungal activity. Although distilled H₂O extract conferred an equally significant ($P = 0.05$) antifungal activity, concentrated sugar solution provided a better vehicle for clinical application of the extracts. However the antifungal activity of the extract was dose-dependent. Minimum inhibitory concentrations (MIC) of 0.2mg/ml and 0.4mg/ml were recorded for *M. gypseum* and *T. mentagrophytes* respectively.

KEYWORDS: Antifungal, Eucalypt leaf extract, ringworm.

INTRODUCTION

Eucalyptus camaldulensis Dehn. belongs to the family *Myrtaceae*. It is among the prominent tree plants recommended for afforestation in Northern Nigeria; where it is grown mainly to check desert encroachment and for erosion control and aesthetics (Buckley, 1986).

The strongly scented narrow leaves of *E. camaldulensis* have been reported to be of high lipid content and could serve as a source of oils referred to as "Eucalyptol" (Verma 1974). Eucalyptol is of great commercial and medicinal importance. The oil is widely used in the treatment of common cold, sore throat and fever (Verma, 1974). Nicolae *et al.*, (1978) have reported that Eucalyptol oil is useful as analgesic and it is a good source of antiheadache ointments. It has also been reported that antivaricose antihemorrhoid ointments could be extracted from Eucalyptol for the treatment of varicose veins and hemorrhoids (Oita *et al.*, 1978). The essential oils of *E. camaldulensis* have been reported to be useful as preservatives of Tangerine (*Citrus reticulata*) fruits. (Anora and Pandey 1985).

In Nigerian ethnomedicine, fresh leaves of *E. camaldulensis* are eaten as a cure for tussis. Besides its antitussive properties, *Eucalyptus* leaf extract is rubbed on the skin as ointment against skin infections. However, the antitussive properties of the plant and its ability to prevent or cure skin irritations have not been reported. The

present study was designed to examine the antifungal activity of the ethanolic extract of *E. camaldulensis* leaves against ringworm pathogens.

MATERIALS AND METHODS

Preparation of Leaf Extract

The Eucalypt leaves used in this research were collected from fruiting stands of *E. camaldulensis*. Prior to chemical analysis. The leaves were dried under mild sunshine to avoid the escape of volatile components. Dried leaves were ground and sieved with 1mm² wire mesh. Milled samples were stored in air tight bottles at room temperature.

The active constituents of the leaves were extracted by soxhlet's extraction technique using 95% absolute alcohol (ethanol) as the extraction solvent (Garg and Saluja 1985). The extract was separated from the solvent by evaporation, contained in clean screw cap bottles and stored in the refrigerator until used.

Test Organism: Fresh pure cultures of *Microsporium gypseum* RAH-14 and *Trichophyton mentagrophytes* RAH-6 originating from clinical specimens were obtained from the medical laboratory unit of St. Lukes Hospital, Anua, Akwa Ibom State. The diphasic fungal isolates were maintained in Sabouraud dextrose broth at 37°C because at room temperature the organisms develop into filamentous forms.

Preparation of Inocula

The dermatophytes were grown at 37°C for 72 hours in Sabouraud broth. The cultures were homogenised in a vortex apparatus and 0.1ml was added to 9.9ml of test solution (described below) in a screw cap tube previously heated to 37 ± 2°C in a temperature controlled water bath. The tubes were vigorously shaken by hand for 30 seconds to homogenize the contents. Homogenisation with this procedure had been reported to be effective (Jorgebriozzo *et al.*, 1989).

Test to Determine the spectrum of Antifungal Activity

The antifungal activity of the leaf extract against *Microsporium gypseum* RAH – 14 and *Trichophyton mentagrophytes* RAH-6 were determined using two basic methods as described by Garrod *et al* (1983) and Harrigan and McCance (1986).

- i. Viable cell enumeration method to estimate cell density after treatment with antifungal agent.
- ii. Diffusion method, to determine the level of inhibition induced by the antifungal agent.

Viable Cell Enumeration Method

The test solution consisted of Sabouraud broth (SB) supplemented with 63% (w/w) of sugar (dextrose), 10% normal bovine serum and the crude extract of eucalypt leaf. This was prepared as follows: To 8.9ml of sugar supplemented Sabouraud broth, 0.08ml of the leaf extract dissolved in polyethylene glycol 400 (PEG – 400) was added. Mixing gave a reasonably stable dispersion as no oil (extract) droplets (coalescence) were noticed in samples kept for 5 days at ambient temperature. The mixture was autoclaved at 10 psi/inch (115.2°C) for 10 minutes to avoid loss of active components of extract. After autoclaving 1ml of sterile bovine serum was added, homogenized, tempered to 37°C and 0.1ml of inocula incorporated. Samples were withdrawn at 4 hours interval and counted as described below. The time (4 hours) was measured from the addition of 0.1ml of the inocula to the test medium. Three replicate tests were performed for each microorganism.

Enumeration method

The samples taken from the test solutions for the two dermatophytes were diluted with 1% sabouraud broth containing 0.5% Tween 80 solution before viable counts were carried out. Three serial dilutions were made. These dilutions and the presence of Tween 80 neutralized the antifungal properties of the extract in the counting medium. Counts were made on sabouraud dextrose agar (SDA) plates by pour plate method

(Harrigan and McCance 1986, Fawole and Oso 1988). Inoculated plates were incubated at room temperature (28 ± 2°C).

Control Test: This was performed to

- i. determine to effectiveness and absence of antifungal activity of the inactivator (Tween 80 solution).
- ii. determine whether the sugar (dextrose) PEG 400 broth possess antimicrobial activity
- iii. determine the influence of added organic substance (bovine serum) and
- iv. to determine influence of vehicle (distilled H₂O) other than the sugar solution.

Effectiveness and absence of antifungal activity of the inactivator

Ethanollic leaf extract (0.08ml) dissolved (50%V) in PEG – 400 was added to 9.9ml of diluent containing the inactivator (0.5% Tween 80) left in contact for 10 minutes and 0.1ml of the fungal inocula incorporated and thoroughly mixed. A sample was withdrawn after 6 hours at 37°C and enumerated.

To determine the absence of antifungal activity of Tween 80, the fungal inocula were added to 9.9ml of diluent containing the inactivators, mixed, a sample was withdrawn after 6 hours and enumerated.

Antimicrobial Activity of Sugar (dextrose) and PEG – 400

Inocula (0.1ml) from the test organism were added to 9.9ml of dextrose solution (180g sugar/100g water) containing 0.04ml PEG-400, well mixed, and sample was withdrawn after 4 hours at 37°C and counted.

Influence of a vehicle (distilled H₂O) other than sugar solution

In this test 0.1ml of inocula, obtained from each of the two test organisms were added to 9.9ml of distilled H₂O, and to 9.9ml of sugar solution (180g of sugar/100g of water) containing 0.08ml of extract dissolved (50% V) in PEG – 400 well mixed and samples withdrawn after 4 hours at 37°C and counted. Oil droplets were not observed in the sample of distilled H₂O (no sugar added) during the experiment for 6 hours. Extract was seen to separate at the bottom of the distilled H₂O (density of extract higher than density of water). Distilled water was used as the test solution in the Agar-Diffusion technique.

Determination of antifungal activity of the leaf extract

The eucalypt leaf extract was also screened for antifungal activity using the cup-

plate agar diffusion technique (BSI 1981 and Garrod *et al* 1983). 2g of the crude extract was dissolved in 5ml of distilled H₂O to give 400mg/ml standard solution from which 0.25, 0.5, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2mg/ml concentrations were prepared and used for the antifungal analysis. This test was carried on a plate of Mueller Hinton sensitivity agar (oxoid) and with wells of 2mm diameter incubated for 96 hours at 37° before examination. The degree of sensitivity was expressed as a measure of the diameter of inhibition of growth in millimetres. A diameter of inhibition of 10mm or higher was considered as an indication of the sensitivity of the dermatophytes to the extract.

The minimum inhibitory concentration (M.I.C) of the extract was determined according to the method reported by Bryant (1981), BSI (1981), and Garrod *et al* (1983). A tube containing only the Mueller Hinton sensitivity agar was also prepared to serve as control.

RESULTS AND DISCUSSION

Results of the antifungal activity test of eucalypt leaf extract on dermatophytic fungi are summarized in Table 1. The test solution containing the extract showed a strong antifungal effect on *Microsporium gypseum* RAH-14 and *Trichophyton mentagrophytes* RAH-6. Apparently *M. gypseum* RAH-14 exhibited a higher level of sensitivity to extract treatment than *T. mentagrophytes* RAH-6. In *M. gypseum* RAH-14 the kill rate was sufficient to reduce viable counts to less than 10cfu/ml in 2 hours, while 10.3cfu/ml were recorded for *T. mentagrophytes* RAH-6 under the same period of treatment. This variation in response to extract treatments may be attributed to the biologically (genetically) different status of the organisms. Similar observations have earlier been reported by Morris *et al* (1979) between two biologically different bacteria, *Staphylococcus aureus* and *Escherichia coli* treated with clove oil.

It was observed that the inactivator (Tween 80) adequately neutralize the antifungal properties of the eucalypt extract while lacking in antifungal potency against the test organisms. Therefore the results presented in Table 1 showed high level of eucalypt extract fungicidal activity. The rapid killing of *M. gypseum* RAH- 14 may be attributed to the addition of the extract since it is well known that the concentrated sugar solutions alone do not have the observed rapid lethal effect on fungi (Chirife *et al* 1983, Selwyn and Durodie 1985). It was also observed that the addition of 10% of liquid human serum to the sugar solution does not impair the antifungal activity of the extract against the test organisms. This result is of value in relation to potential

application of sugar eucalypt extract for the treatment of ringworms.

The results in Table 2 indicate the influence of a vehicle other than the sugar solution on the antifungal activity of extract against the dermatophytic fungi. The antifungal properties of the extract seem to be similar in distilled H₂O and sugar solution. Nevertheless, the concentrated sugar solution provided a good vehicle in the sense that a relatively more stable dispersion of the extract was easily obtained. This was not the case with distilled H₂O since oil (extract) droplets were observed 4 hours after the extract was dispersed. This aspect is important in relation to future application of the ethanolic eucalypt extract as an antifungal agent.

Analysis by Agar-Diffusion technique further confirmed the antifungal potential of eucalypt leaf. The leaf extract showed significant ($P = 0.05$) antifungal activity against the dermatophytes (Table 3). A mean growth inhibition zone diameter of 28.3mm was observed against *M. gypseum* RAH - 14 and 24.5mm against *T. mentagrophytes* RAH-6 after treatment with crude ethanolic extract at 37°C. The antifungal activity of the extract however decreases with decrease in concentrations (Table 4). The minimum inhibitory concentrations (MIC) of the extract obtained were 0.2mg/ml and 0.4

Table 1. Antifungal activity of eucalypt leaf extract on dermatophytes after 4hours exposure at 37°C.

Dermatophyte	Mean viable cell count (cfu/ml)	
	Pre-exposure	Post exposure
<i>M. gypseum</i>	4.8×10^3	7.0
<i>T. mentagrophytes</i>	8.3×10^3	10.00

Table 2. Effect of vehicle medium on the antifungal activity of leaf extract after 4 hour exposure at 37°C

Dermatophyte	Incubation	Mean viable cell count (cfu/ml)	
		Dist. H ₂ O	sugar solution
<i>T. gypseum</i>	Pre-	2.4×10^2	2.5×10^2
	Post-	14	8
<i>T. mentagrophytes</i>	Pre-	3.0×10^2	3.0×10^2
	Post-	21	11

Table 3. Antifungal activity of eucalypt leaf extract on dermatophytes at 37°C incubation.

Dermatophyte	Mean diameter of inhibition (mm)	
	crude leaf extract	Sugar solution (control)
<i>M. gypseum</i>	28.3	4.8
<i>M. mentagrophytes</i>	24.5	2.3

Table 4: Influence of extract concentration (mg/ml) on the antifungal activity of Eucalypt leaf.

Dermatophytes	Mean zone of intubition (mm)							
	Concentration							
	0.25mg/ml	0.5mg/ml	0.1mg/ml	0.2mg/ml	0.4 mg/ml	0.8 mg/ml	1.6 mg/ml	3.2 mg/ml
<i>M. gypseum</i>	0	5.0	7.8	*11.0	14.5	15.0	16.8	20.1
<i>M. mentagrophytes</i>	0.1	2.0	4.2	8.0	*14.0	14.7	16	16.1

* Point at which the minimum inhibitory concentration was derived

mg/ml for *M. gypseum* RAH-14 and *T. mentagrophytes* RAH-6 respectively. The apparently low MICs obtained is an indication of the high antifungal potency of the extract at diluted concentrations. Concentrations which are 4 times the MIC resulted in a very remarkable inhibition of the growth rates of test organisms.

Most studies on the antimicrobial activity of plants and their essential oils sought alternative methods of food preservation (Hargreaves *et al.* 1975). For this reason minimal inhibitory concentrations (MIC) were usually determined rather than biocidal effect. Little or no studies (to the best of the authors knowledge) have been carried out on the antimicrobial and preservative properties of *E. camaldulensis* leaves in Nigeria. Thus, it is difficult to compare the results of the present study with those of other workers. Nevertheless some references can be made to studies by Moreira *et al.* (1980) and Nicolae *et al.* (1978) who made comprehensive review of the medicinal and preservative properties of Eucalyptol (essential oil). They showed that Eucalyptol has preservative, antiheadache, analgesic and antimicrobial potentials, confirmed basically by the presence of naturally occurring antioxidative compounds such as beta-pinene, limonene, 2-pentene cineole and alpha-tarpinene (Moreira *et al.*, Anora and Pandey 1985 and Carman *et al.*, 1985).

The present work was designed basically to ascertain the antimicrobial potential of *E. camaldulensis* leaf extract, in attempt to increase the leaf extract therapy for certain clinical infections. The results obtained in vitro is in support of the claims of the ethno-medicine practitioners on the therapeutic efficacy of eucalypt plants. The results have also indicated an enhanced extract antifungal activity when added to concentrated sugar solution (63% w/w). In sugar-test solution, the extract demonstrated marked germicidal effect against fungi pathogenic to humans. The effect was so much that only 4 hours were sufficient to kill *M. sypseum* RAH-14 from about 5.0×10^3 cfu/ml to 7cfu/ml. It was also demonstrated that sugar was not necessary for this test of antifungal activity, which was due mainly to the ethanolic extract. It was also

obvious that distilled H₂O as test solution equally gave remarkable fungicidal activity, but with a shorter stability because of poor extract solubility in water. However before eucalypt leaf extract can be applied to clinical situations further investigation into the phytochemical properties of eucalypt leaves is necessary to identify the active constituents of the extract and ascertain the appropriate concentrations for higher efficacy and safety.

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