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Full Length Research Paper

In vitro activities of *Eucalyptus camaldulensis* Dehnh. and *Eucalyptus torelliana* F. Muell. against non-tuberculous mycobacteria species

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The extracts of *Eucalyptus camaldulensis* Dehnh. and *Eucalyptus torelliana* F. Muell. (Myrtaceae) were screened against four non-tuberculous mycobacteria species: *Mycobacterium fortuitum* ATCC 684, *Mycobacterium smegmatis* ATCC 19420, *Mycobacterium phlei* ATCC 19240 and *Mycobacterium abscessus*. The agar diffusion method was used to investigate the activity of these plants at 1 and 2 mg/ml concentration. The methanol extracts exhibited the highest activity against the test organisms, the most susceptible being *M. fortuitum* ATCC 684. The minimum inhibitory concentration (MIC) value for the bioactive extracts ranged between 1 to 2 mg/ml while minimum bactericidal concentration (MBC) value was 2 to 4 mg/ml. Bactericidal activity of the methanol extracts of *E. camaldulensis* on *M. fortuitum* ATCC 684 was investigated using the viable counting technique. The leaf extracts at 4 mg/ml (4 × MIC) and 8 mg/ml (8 × MIC) gave 80.49 and 100% kill of the organism respectively at 24 h exposure time while the stem bark extracts at the same concentrations afforded 67.35 and 100% kill respectively after 24 h of exposure. The activities demonstrated by these plants support their use in the treatment of cough associated with most pulmonary diseases; and suggest that these plants may be of therapeutic importance for the treatment of infections caused by the non-tuberculous mycobacteria (NTM).

Key words: *Eucalyptus camaldulensis* Dehnh., *Eucalyptus torelliana* F. Muell., non-tuberculous mycobacteria, antimycobacterial activity, *in vitro*.

INTRODUCTION

Mycobacterium is a genus of Actinobacteria, given its own family, the Mycobacteriaceae. The genus is highly diverse with 85 known species and comprises both the strictly pathogenic species that are transmitted by human or animal reservoirs only and the non-tuberculous mycobacteria (NTM) (Le Dantec et al., 2002). Most of the non-tuberculous mycobacteria are environmental saprophytes, existing in various substrates including soil, water and plants; and are usually considered non pathogenic. However, an increasing number are being reported as opportunistic pathogens constituting a risk not only to the immunocompromised but also to the healthy individuals (Graham, 2002). These species are known to cause pulmonary and cutaneous diseases, lymphadenitis and other infections. They are non-susceptible to most available antimycobacterial drugs used to treat tuberculosis both *in vitro* and *in vivo* (Woods et al., 2000) hence most people in the developing countries depend on the use of herbs to treat infections caused by these organisms. Thus, there is a need to explore the plant kingdom to develop new and effective drugs for the treatment of diseases associated with these organisms. In Nigeria, medicinal plants have been used in the treatment of various infections and diseases including lymphadenitis, skin and soft tissue infections such as ulcer of the skin, and respiratory tract infections. Some of these medicinal plants include *Eucalyptus*

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camaldulensis and *Eucalyptus torelliana* (family Myrtaceae). The genus *Eucalyptus* is used to treat sore throat, wounds, ulcer and bacterial infections of the gastrointestinal, urinary and respiratory tract (Ashour, 2008; Olaniyi, 1982). The essential oil from the leaves has been used in the treatment of lung diseases. The leaves are also chewed for bad breath (Gill, 1992). The bark and the leaves of *Eucalyptus* species are used for cold and cough, influenza, toothaches, fevers, sore throat and other infections (Farah et al., 2002; Kim et al., 2001). Tannins, glycoside, flavonoid, ester and terpenes have been isolated from *Eucalyptus* species as reported by Oyedeji et al. (1999). Polyphenols, flavonols, flavonones, proanthocyanidins and ellagitannis (tannins) and essential oils were isolated from *E. camaldulensis* while terpene, triterpene and essential oils were isolated from *E. torelliana* (Brophy and Southwell, 2002). Extracts of *E. camaldulensis* and *E. torelliana* were found to inhibit the growth of *Helicobacter pylori in vitro* (Adeniyi et al., 2009). The aim of this study is to investigate the antimycobacterial effect of extracts of *E. camaldulensis* and *E. torelliana* on the non-tuberculous mycobacterial species.

MATERIALS AND METHODS

Plant collection and extraction

The leaves and stem bark of *E. camaldulensis* and *E. torelliana* were collected and authenticated at the Department of Botany, University of Ibadan and Forest Research Institute of Nigeria (FRIN) Herbarium. Voucher specimens were deposited at FRIN with the herbarium number (FHI 104908 and FHI 104909 for *E. camaldulensis* and *E. torelliana* respectively). The leaves and stem bark were air-dried and ground to coarse powder using an electric grinder.

Phytochemical screening of crude extract

The pulverized samples of the leaves and stem bark of *E. camaldulensis* and *E. torelliana* were examined for the presence of anthraquinones, tannins, saponins, alkaloids and cardenolides. The procedure used was as previously described by Harborne (1998).

Microorganisms

The microorganisms used were *Mycobacterium fortuitum* ATCC 684, *Mycobacterium smegmatis* ATCC 19420, *Mycobacterium phlei* ATCC 19240 and *Mycobacterium abscessus.*

Culture methods

Overnight culture of the test organisms was prepared by sub-culturing the organisms from stock cultures into sterile nutrient Tryptic Soy broth in tubes. The tubes were incubated at 37°C. A1:100 dilution of each culture was prepared and used for the study.

Screening method for antimycobacterial activities

This was carried out using the agar cup diffusion technique (Adeniyi et al., 2006; Adegoke et al., 2010). A 1 ml of a 1:100 dilution of an overnight culture of each bacterial isolates was used to seed sterile molten Mueller-Hinton agar medium maintained at 45°C. The seeded plates were allowed to dry in the incubator at 37°C for 20 min. A standard cork borer of 8 mm diameter was used to cut equidistant wells on the surface of the agar into which was added 0.1 ml solution of each fraction reconstituted with 50% ethylacetate to final concentrations of 1 and 2 mg/ml. The plates were incubated at 37°C for 24 h after which diameters of zones of inhibition were measured. 50% ethylacetate was included in each plate as a solvent control while Streptomycin (30 and 80 µg/ml) and Rifampicin (5 µg/ml) were used as positive control. The experiment was performed in triplicates.

Determination of minimum inhibitory concentration (MIC)

The MIC for the bioactive extracts was determined by the agar dilution method (Lajubutu et al., 1995). Different concentrations of the extracts were prepared to final concentration in the range of 4 to 0.125 mg/ml. Two milliliter of the extract from each dilution was mixed with 18 ml of molten Mueller-Hinton agar and poured into sterile Petri dishes allowing the agar to set. The surface of the agar was allowed to dry before streaking with overnight broth cultures of test organisms. The plates were incubated at 37°C for 24 h and examined for the presence or absence of growth. The lowest concentration preventing visible growth was taken as the MIC of the extract. All procedure was performed in triplicates.

Determination of minimum bactericidal concentration (MBC)

The MBC for the bioactive extracts was determined by a modification of the method of Aibinu et al. (2007). Concentrations higher than and equivalent to the MIC were prepared in Tryptic Soy broth, 0.5 ml of a 24 h culture of test organisms were added to 4.5 ml of the extracts solution in test tube. The mixture was incubated at 37°C for 24 h after which aliquots of samples were withdrawn. Ten-fold dilutions were made and 0.2 ml of 1:1000 dilutions was transferred onto extract-free sterile Mueller-Hinton agar in Petri dish. The agar plates were incubated at 37°C for 24 h and observed for absence or presence of growth. The minimum concentration preventing visible growth of the organisms was taken as MBC. All procedure was performed in triplicates

Determination of bactericidal activity of the methanol extracts of *Eucalyptus camaldulensis*

The viable counting technique was employed for this purpose (Lajubutu et al., 1995). An overnight broth culture in 4.5 ml of Tryptic Soy broth inoculated in a static growth condition of each organism was made. *M. fortuitum* ATCC 684 was used for this experiment. A 0.5 ml of each culture was subculture into a warm (37°C) 4.5 ml Tryptic Soy broth and incubated for 90 min using a Gallenkamp orbital incubator to give a logarithmic phase culture. A 0.1 ml of the logarithmic phase culture was then inoculated into a warm 4.9 ml of Tryptic Soy broth containing the tested compound to give 1 in 50 dilution of the culture (equivalent to approximately 1×10^7 colony forming units) and the required concentration of the extract. An appropriate quantity of the test sample (extract- culture mixture) was withdrawn immediately, diluted out in Tryptic Soy broth and 0.2 ml of 1:1000 dilution plated on an oven dried Mueller-Hinton agar to give control time 0 min count. Samples were taken at an interval of 30 min, 1, 2, 4, 6 and 24 h intervals.

The procedure was carried out in triplicate to ensure accuracy. Plates were incubated at 37°C for 24 h before counting the colonies. Control plates for negative and positive controls were also incubated. The number of colony forming unit were counted after

Table 1. Antimycobacterial screening of extracts of *E. camaldulensis*. Results showing diameter (mm) of zone of inhibition of triplicate experiments ± SEM.

Extract (mg/ml)	mEcL		cEcL		hEcL		mEcS		cEcS		hEcS		Strep (µg/ml)		RMP (µg/ml)	EtoAc
	1	2	1	2	1	2	1	2	1	2	1	2	30	80	5	50%
M. fort ATCC 684	17±2.0	21±5.0	14±2.0	16±2.0	15±1.0	14±0.0	16±0.0	18±2.0	20±0.0	-	-	-	-	-	-	-
M. smer ATCC 19420	16±2.0	16±2.0	-	-	16±2.0	18±2.0	20±2.0	21±3.0	-	-	15±1.0	17±6.0	-	-	12±1.0	-
M. absc	19±2.0	20±2.0	14±2.0	17±3.0	18±0.0	17±0.0	17±3.0	17±1.0	-	-	-	-	-	-	14±1.0	-
M. phlei ATCC 19240	20±0.0	24±0.0	-	-	-	-	-	20±0.0	-	-	-	-	-	-	12±0.0	-

Diameter of cork bore r – 8 mm, L - leaf, S - stem bark, m - methanol, c - chloroform, h - hexane, Ec - *Eucalyptus camaldulensis*, Strep - Streptomycin, RMP - Rifampicin, EtoAc - Ethylacetate, - = no zone of inhibition that is resistance, *M. fort - Mycobacterium fortuitum, M. smer - Mycobacterium smergmatis, M. absc - Mycobacterium absceccus, M. phlei -Mycobacterium phlei.*

Table 2. Antimycobacterial screening of extracts of *Eucalyptus torelliana* (Et). Results showing mean diameter (mm) of zone of inhibition of triplicate experiments ± SEM.

Extract (mg/ml)	mEtL		cEtL		hEtL		mEtS		cEtS		hEtS		Strep (µg/ml)		RMP (µg/ml)	EtoAc
	1	2	1	2	1	2	1	5	1	2	1	2	30	80	5	50%
M. fort ATCC 684	12±0.0	14±0.0	17±1.0	16±2.0	14±2.0	18±6.0	14±1.0	-	-	-	-	-	-	-	-	-
M. smer ATCC19420	-	-	-	-	21±0.0	24±0.0	18±0.0	13±1.0	-	-	-	-	-	-	12±1.0	-
M. absc	11±0.0	14±0.0	18±2.0	-	-	17±3.0	15±2.0	14±1.0	-	-	-	-	-	-	14±1.0	-
M. phlei ATCC19240	-	-	-	-	-	-	10±0.0	13±0.0	-	-	-	-	-	-	12±0.0	-

Diameter of cork borer – 8 mm, L - leaf, S - stem bark, m - methanol, c - chloroform, h - hexane, Et - *Eucalyptus torelliana*, Strep - Streptomycin, RMP - Rifampicin, EtoAc - Ethylacetate, - = no zone of inhibition i.e. resistance, *M. fort - Mycobacterium fortuitum, M. smer - Mycobacterium smergmatis, M. absc - Mycobacterium absceccus, M. phlei - Mycobacterium phlei.*

the period of incubation. The numbers of surviving bacterial cells per ml were calculated by taking into consideration the dilution factor and the volume of the inoculum. All the procedure was repeated for 2 × MIC, 4 × MIC and 8 × MIC. A graph of percentage viable count against time in hour was plotted to show the rate of kill of the test organisms after triplicate experiments.

RESULTS AND DISCUSSION

Phytochemical screening of the plant samples revealed the presence of tannins, saponins, cardenolides and anthraquinones (only in *E. torelliana* leaf). Antimycobacterial screening showed that the plants extracts were active against the test organisms except *M. phlei* ATCC 19240 which was resistant to most of the extracts. The most susceptible of the organisms was *M. fortuitum* ATCC 684 (Tables 1 and 2). The highest antimycobacterial activity was observed in the extracts of *E. camaldulensis* leaves as shown by the diameter of zone of inhibition (Table 1). The activity demonstrated by the fractions was dose-dependent as shown by the diameter of zone of inhibition for most of the active extracts. The results of the MIC and MBC of the bioactive extracts are shown in Table 3. The MIC value for the bioactive extracts ranged between 1 to 2 mg/ml while MBC value was 2 to 4 mg/ml. The test organisms in this study were susceptible to most of the extracts even though they were resistant to Streptomycin and partially sensitive to Rifampicin (the drug controls). This observation is in accordance to other reports on their resistance to most available antimycobacterial drugs used to treat tuberculosis both *in vitro* and *in vivo* (Woods et al., 2000). The non-tuberculous mycobacteria (NTM) are abundantly available in nature and are associated with various pulmonary and cutaneous diseases in immunocompromised as well as healthy individuals (Graham, 2002).

These rapidly-growing mycobacteria such as *M. fortuitum* are the usual causes in persons with aspiration secondary to esophageal disease; and occasionally cause localized dermatologic and rheumatologic infections that require a high

Table 3. Minimum inhibitory concentration and minimum bacterial concentration of bioactive extracts in mg/ml.

Extract (mg/ml)	mEcL		mEcS		hEtL		mEtS		Strep		RMP (μg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
M. fort ATCC 684	1	2	1	2	1	2	2	4	R	R	R	R
M. smer ATCC 19420	1	2	1	1	1	2	2	4	R	R	5	10
M. absc	1	2	1	2	2	2	2	4	R	R	5	5
M. phlei ATCC 19240	1	2	2	4	2	2	R	R	R	R	5	10

mEcL- methanol *Eucalyptus camaldulensis* leaf, mEcS- methanol *Eucalyptus camaldulensis* stem bark, hEtL- hexane *Eucalyptus torelliana* leaf, mEtS- methanol *Eucalyptus torelliana* stem bark, *M. fort* - *Mycobacterium fortuitum, M. smer -Mycobacterium smergmatis, M. absc - Mycobacterium absceccus, M. phlei -Mycobacterium phlei,* Strep - Streptomycin, RMP - Rifampicin, R – resistance.

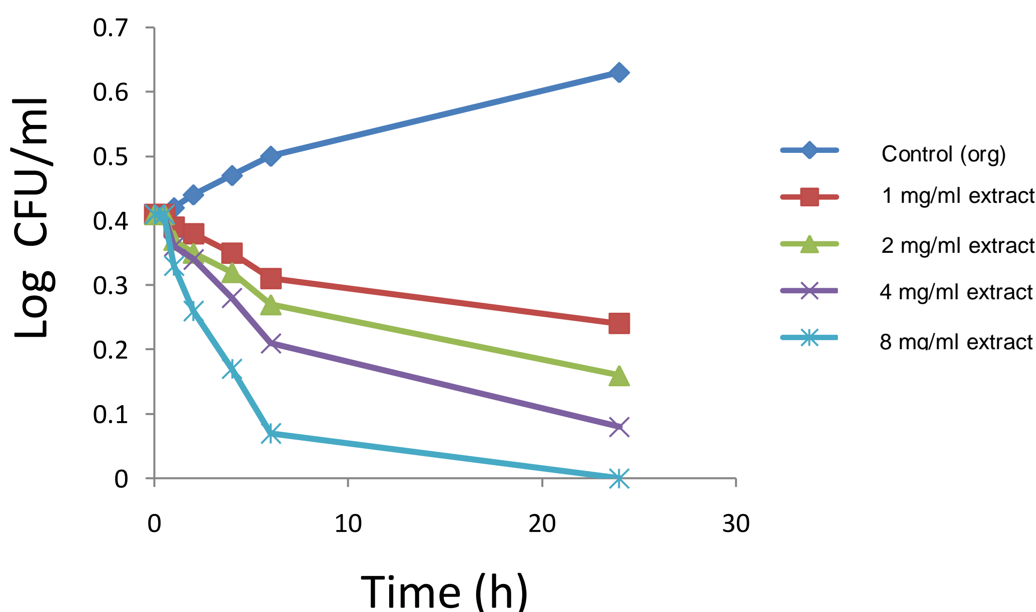

Figure 1. Time-kill study of methanol extract of *E. camaldulensis* leaf *M. fortuitum* ATCC 684 where control (org) - organism control, 1 mg/ml - MIC, 2 mg/ml - 2 × MIC, 4 mg/ml - 4 × MIC, 8 mg/ml - 8 × MIC of extract.

index of suspicion for correct diagnosis and appropriate therapy. The clinical presentation of non-tuberculous mycobacterial infection of the lungs is usually nonspecific and varies to some extent depending on the microorganism and underlying conditions (Bryan, 2008). The most predictably effective therapy for infections due to rapidly growing mycobacteria is surgical removal of all involved tissues. Thus, the use of herbs in the treatment of diseases caused by these organisms is widespread especially in non-industrialized nations.

The antimycobacterial activities exhibited by the extracts of *E. camaldulensis* and *E. torelliana* on the non-tuberculous mycobacterial species used in this study are significant because the outer membrane of the mycobacteria is reported to preclude the entrance of substances into the cell thus rendering most antibiotics and antimicrobial agents ineffective (Brennan and Nikaido, 1995). The time-kill study of the methanol extracts of *E. camaldulensis* leaf and stem bark on *M. fortuitum* ATCC 684 as presented in Figures 1 and 2 revealed a dose-dependent reduction in inoculum size of the test organism. The leaf extracts at 1 mg/ml (MIC), 2 mg/ml (2 ×MIC) and 4 mg/ml (4 × MIC) gave 41.46, 60.98 and 80.49% kill of the test organism respectively at 24 h exposure time while the stem bark extracts at 1 mg/ml (MIC), 2 mg/ml (2 × MIC) and 4 mg/ml (4 × MIC) afforded 38.78, 44.89 and 67.35% kill respectively after 24 h of exposure to the extracts. A 100% kill was only achieved at a very high concentration of 8 mg/ml (8 × MIC), this is because the non-tuberculous mycobacteria species are known to be more resistant to antimicrobial agents than the tubercle bacilli; and this poses a threat in the treatment of infections caused by these species of mycobacteria (Woods et al., 2000). The bactericidal

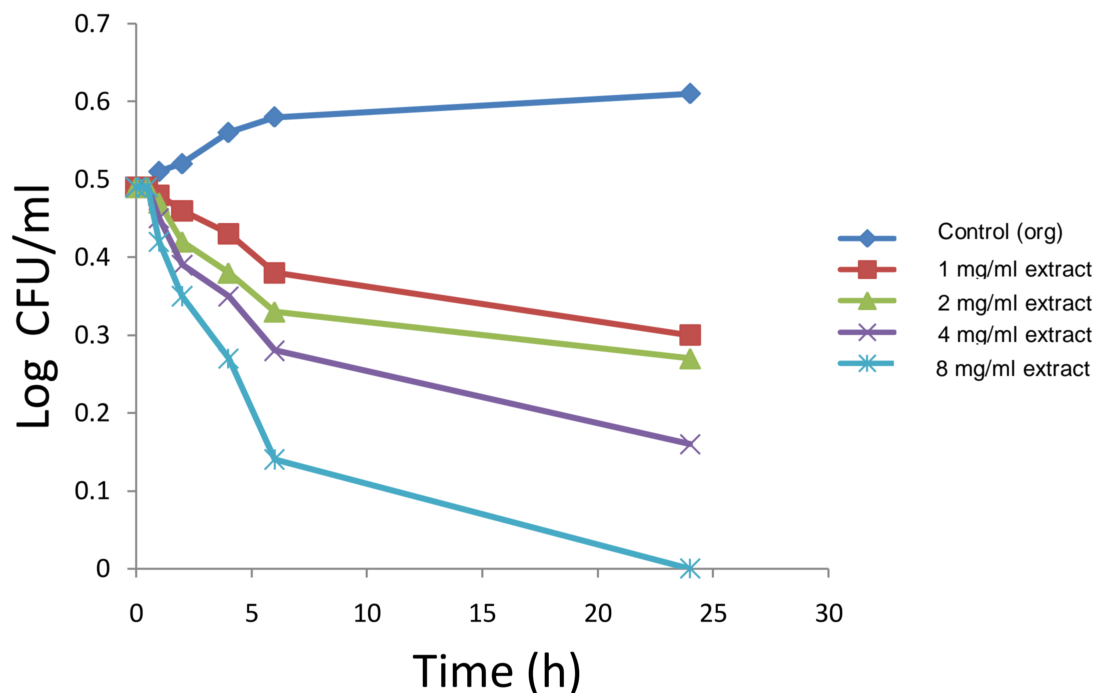

Figure 2. Time-kill study methanol extract of *E. camaldulensis* stem bark on *M. fortuitum* ATCC 684 where control (org) - organism control, 1 mg/ml - MIC, 2 mg/ml - 2 × MIC, 4 mg/ml - 4 × MIC, 8 mg/ ml - 8 × MIC of extract.

activity observed was time and dose dependent as the percentage reduction in inoculum increased with increase in exposure time and concentration of the extracts.

In this study the methanol extract of *E. camaldulensis* leaf demonstrated the highest activity against the test organisms as seen with the diameter of zone of inhibition, the MIC, MBC and time-kill experiments. The present study revealed the ability of the extracts to penetrate the waxy mycobacterial cell wall to interact with the cellular components thus inhibiting their growth and multiplication. The antimycobacterial activity exerted against the NTMs by these plants is due to the presence of chemical compounds such as tannins, saponins, cardiac and anthraquinone glycosides in the plants as revealed by the phytochemical screening. These secondary compounds are known to possess strong antimicrobial activity against a broad spectrum of microorganisms. Antimycobacterial terpenoids isolated from *Juniperus communis* inhibited the growth of *M. fortuitum* and *M. phlei* (Gordien et al., 2009). The antimycobacterial activities of naphthoquinones from *Diospyros canaliculata, Diospyros crassiflora* and *Euclea* species against *Mycobacterium smegmatis* and *M. fortuitum* have also been reported (Kuete et al., 2009; McGaw et al., 2008). The antimicrobial activity of Diospyrin, Isodiospyrin and Bisisodiospyrin from the root of *Diospyros piscatorial* against *Mycobacterium chelonae* has also been reported (Adeniyi et al., 2000). *E. camaldulensis* and *E. torelliana* are used locally in the treatment of sore throat and other bacterial infections of the respiratory tracts, swelling or inflammation of the mucous membranes (Blumenthal, 1996). The bark and the leaves of *Eucalyptus* species are used for cold and cough and the essential oils of the leaves have been used in the treatment of lung diseases (Farah et al., 2002; Kim et al., 2001). Most previous studies on Eucalyptus species have reported on the antimicrobial and antituberculosis activity of the essential oils (Sartorelli et al., 2007; Sherry and Warnke, 2004). There have been no reports on the effect of *E. camaldulensis* and *E. torelliana* on the nontuberculous mycobacteria species; it is our interest to report the activity of the extracts of these plants against the NTMs (Table 1).

Conclusion

The antimycobacterial activities exhibited by *E. camaldulensis* and *E. torelliana* support their local use in the treatment of diseases associated with the NTM species and indicate that important therapeutic agents can be isolated from the extracts of these plants.

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