Purified Compounds and Extracts from *Euclea* Species with Antimycobacterial Activity against *Mycobacterium bovis* and Fast-Growing Mycobacteria

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Naphthoquinones and other compounds with antimycobacterial activity against *Mycobacterium tuberculosis* have previously been isolated from *Euclea* species. In this study, several constituents of *Euclea natalensis* and *E. undulata*, as well as organic extracts of the leaves, were assessed for efficacy against the zoonotic pathogen, *Mycobacterium bovis*. Also included in the battery of test organisms were *M. bovis* BCG and the fast-growing species *M. smegmatis* and *M. fortuitum*. The acetone extract of *E. natalensis* had potent activity against *M. bovis* (MIC = 26 μg/ml). The naphthoquinone 7-methyljuglone was the most active compound, with an MIC as low as 1.55 μg/ml against pathogenic *M. bovis*. *M. bovis* BCG was not as susceptible to the test compounds as the pathogenic strain, but similar patterns of activity were observed between all the strains tested. *M. smegmatis* appeared to be a better predictor of antimycobacterial activity against pathogenic *M. bovis* (and *M. tuberculosis*), while MIC values obtained using *M. fortuitum* correlated well with those of *M. bovis* BCG.

**Key words** antimycobacterial; *Euclea*; *Mycobacterium bovis*; zoonotic tuberculosis; naphthoquinone; Ebenaceae

Tuberculosis (TB) in humans, caused by *Mycobacterium tuberculosis*, is a chronic infectious disease, with approximately one-third of the world’s population estimated by the WHO to be infected. In 2003, 1.7 million people died of TB and of these, 99% of cases were reported in developing countries. Other organisms belonging to the *M. tuberculosis* complex include *M. bovis*, *M. africanum* and *M. microti*.

Bovine TB (causal organism *Mycobacterium bovis*) is an important zoonotic disease that can spread to humans by inhalation of infectious droplet nuclei and by ingestion of milk which has not been pasteurized or boiled. People regularly exposed to either livestock infected with bovine TB or infected products, such as poorly heat-treated meat and unpasteurised milk, should be considered at risk of contracting zoonotic infections. This risk increases considerably in HIV-infected individuals. The occurrence of *M. bovis* disease by reactivation or primary infection in HIV-infected patients, and the transmission from patients with infectious pulmonary *M. bovis* disease to immune-competent individuals is a health concern.

In developed countries, the prevalence of bovine TB has been significantly reduced by control (test and slaughter) programmes, but complete eradication of the disease is made difficult by reservoirs in wildlife. In the developing world, the combination of the HIV epidemic, poor living conditions and a high burden of tuberculosis in animals is a significant obstacle to control of *M. bovis* in several African countries. The zoonotic effects of *M. bovis*, especially with regard to immunocompromised individuals, have not been comprehensively studied and it is anticipated that this may be an increasingly serious problem, particularly in sub-Saharan Africa. Underdiagnosis of *M. bovis* infection is potentially widespread, as many laboratories do not use the specialized diagnostic techniques required to distinguish between the genetically similar *M. bovis* and *M. tuberculosis*. *M. bovis* is believed to account for up to 10% of cases of human TB worldwide. In developing countries with no active bovine TB control programmes, a serious threat to human health is posed. More than 94% of the world’s population occurs in countries with no strategies in place to control *M. bovis* infections. In terms of public health, as well as economics, bovine TB control or eradication programmes should be a major target of affected countries.

With the rising incidence of drug resistance to existing TB drugs, as demonstrated by the occurrence of multi-drug resistant (MDR) strains of *M. tuberculosis* and the recent outbreak in South Africa of extreme drug resistant TB (XDR-TB), the search for new anti-TB drug leads is achieving new urgency. Although MDR strains of *M. bovis* have been identified, case reports show that anti-TB drugs routinely used to treat *M. tuberculosis*-infected patients are effective when properly administered. It is, however, likely that resistance to currently used drugs will develop if the incidence of *M. bovis* infections and subsequent treatment in humans persists. Plant-derived compounds are a potential source for investigation of alternative lead chemical structures for drug development.

Medicinal plants are used in many parts of southern Africa to treat TB-related symptoms including chest complaints and coughing. Several recent reviews emphasize the potential of plant species and natural products as sources of antimycobacterial extracts and chemicals. The structural diversity of plant-derived antimycobacterial compounds is highlighted by the fact that the classes to which these compounds belong include alkaloids, terpenoids, coumarins/chromones, peptides and phenolics.

The Ebenaceae is a medium sized family comprising about 500 species but only two genera, *Euclea* and *Diospyros*, are

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found in the tropics and subtropics. The antimycobacterial activity against *M. tuberculosis* by extracts of *Euclea natalensis* roots has been previously reported. Two new compounds, octahydroeuclein and 20(29)-lupene-3β-isofurate, in addition to the known compounds shinanolone, lupeol and betulin were isolated from an ethanol extract of *E. natalensis*. Of these compounds, only shinanolone exhibited antimycobacterial activity against *M. tuberculosis* (MIC = 100 µg/ml), and antibacterial activity against a variety of test organisms. In further studies of the intracellular activity of naphthoquinones and triterpenes isolated from *E. natalensis*, it was established that the naphthoquinone 7-methyljuglone has superior intracellular (in a macrophage cell line) and extracellular inhibition of *M. tuberculosis* relative to the anti-TB drugs streptomycin and ethambutol. The values of diospyrin, isodiospyrin, 7-methyljuglone and neodiospyrin were 8.0, 10.0, 0.5 and 10.0 µg/ml respectively. The roots of *Euclea* species are used in southern African traditional medicinal preparations to treat chest complaints, chronic asthma, leprosy and infections, among other ailments.

The first objective of the present work was to determine the activity of *E. natalensis* and *E. undulata* root extracts, and compounds isolated from the plants, against the pathogenic bovine TB organism, *M. bovis*, to enable a comparison of activity against human and animal pathogenic mycobacteria of these natural products. The naphthoquinones diospyrin and 7-methyljuglone are major constituents of *E. undulata* roots. Extracts or pure compounds can be used for further studies in pre-clinical trials, and it was therefore decided to compare the antimycobacterial activity of *E. undulata* with that of *E. natalensis*. Close correlations between activity against the pathogenic and slow-growing *M. bovis* and *M. tuberculosis* could reflect the close genetic similarity of the organisms, and provide useful information for screening studies. Secondly, the usefulness of the non-pathogenic vaccine strain, *M. bovis* BCG as a model for detection of activity against slow-growing pathogenic *Mycobacterium* species was evaluated. In a third component, two fast-growing saprophytic species, *M. smegmatis* and *M. fortuitum*, were included as test organisms to observe the degree to which activity against these species reflects activity against the slow-growing pathogenic *M. bovis* and *M. tuberculosis*. If fast-growing species give the same activity profile as slow-growing mycobacteria, this could strongly reinforce the rationale behind using the former as test organisms in screening programmes for antimycobacterial compounds. While some work has been done in this regard, further data are required to support the use of preliminary antimycobacterial screening methods using fast-growing saprophytic mycobacteria, or slower-growing species with decreased safety concerns to the operator such as *M. bovis* BCG.

### MATERIALS AND METHODS

#### Preparation of Extracts

*Euclea natalensis* A.DC. (Ebenaceae) root material was collected from the KwaZulu-Natal province in South Africa. A voucher specimen (N.L.22) was deposited at the H.G.W. Schweickerdt herbarium at the University of Pretoria. The dried roots were ground to a powder using an IKA® Werke MF 10 grinder (Merck), which incorporated a sieve with holes of 1 mm to ensure uniform particle size. Extracts of *E. natalensis* roots were prepared using chloroform, acetone and methanol. An acetone extract of *E. undulata* roots was also prepared. Powdered plant material was combined in a 1 : 10 ratio with solvent and shaken vigorously for 30 min using a mechanical shaker (Labotec) before being filtered through Whatman No. 1 filter paper. The solvent was removed in a stream of air and the residues redissolved in DMSO to a concentration of 200 mg/ml. The naphthoquinones diospyrin, neodiospyrin, shinanolone, and 7-methyljuglone, as well as the triterpenoid lupeol were isolated from *E. natalensis* roots as previously described. Stock solutions of the pure compounds were prepared in DMSO to a concentration of 20 mg/ml.

#### Experimental Procedures. Mycobacterial Cultures

Antimycobacterial activity was tested against *M. bovis* ATCC 19210, *M. bovis* BCG (Pasteur strain P1172), *M. fortuitum* (ATCC 6841) and *M. smegmatis* (ATCC 1441). The mycobacterial species were cultured on Löwenstein–Jensen agar slants, supplemented with glycerol, or pyruvate in the case of the *M. bovis* cultures. Prior to each assay, sterile plastic loops were used to scrape cells off the slants and these were carefully suspended in a small volume of sterile distilled water to avoid formation of clumps. These suspensions were diluted with sterile water to render a concentration of cells equal to a MacFarland No. 1 standard solution (approximately 4×10⁷ cfu/ml), and then diluted with freshly prepared Middlebrook 7H9 broth supplemented with 10% OADC medium to obtain a final inoculum density of approximately 5×10⁵ cfu/ml. This was confirmed by spreading 100 µl volumes of 10-fold serial dilutions of each culture suspension onto agar plates using a glass spreader and counting colonies growing after incubation at 37°C.

#### Antimycobacterial Assay

A modified two-fold serial dilution assay in 96-well microtitre plates was used to detect antimycobacterial activity. Extracts and isolated compounds were serially diluted (100 µl) with OADC supplemented Middlebrook 7H9 broth in wells of microtitre plates before mycobacterial culture (100 µl) was added to each well. The anti-TB drug isoniazid represented the positive control, and solvent controls were included. Doses were tested at least in triplicate and the entire experiments were repeated, providing a minimum of six data sets per dilution. The microplates were sealed with parafilm and placed in a stainless steel chamber, the base of which was lined with paper towel saturated with sterile water to maintain humidity. The fast-growing mycobacteria, *M. smegmatis* and *M. fortuitum*, were incubated at 37°C for 1 and 2 d, respectively and the slow-growing *M. bovis* and *M. bovis* BCG cultures were incubated for 7 to 9 d. MIC values were detected using a tetrazolium salt indicator, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), which is reduced to a blue-purple colour by viable bacteria. In the MIC assay of
antimycobacterial activity of leaf extracts on \textit{M. smegmatis},
tetrazolium violet (INT) worked well (unpublished results). However, because some root extracts have a reddish colour
making it difficult to see the red colour changes with INT we used another tetrazolium salt as previously suggested\(^\text{39}\)
which produces a tetrazolium salt more blue in colour. An aliquot (40 \(\mu\)l of a 0.2 mg/ml solution) of MTT was pipetted
into each well and once growth control wells revealed a purple
colour following incubation at 37 \(^\circ\)C, results were recorded. This colour reaction after addition of MTT gener-
ally occurred after a 1 h incubation period for the fast-grow-
ners, and after approximately 6 h for the slow-growers.

Minimal inhibitory concentration (MIC) values were read as
those concentrations where a marked reduction in colour for-
mation was noted. Aliquots (100 \(\mu\)l) from the MIC wells and
those of increasing concentrations were spread onto agar
plates to detect minimum bactericidal concentration (MBC)
values for the active substances. The MBC was defined as the
concentration resulting in a sharp reduction (>99%) in the
growth of mycobacteria after incubation.

RESULTS AND DISCUSSION

The results of the MIC and MBC determinations are pre-
"owed in Table 1. As a comparison, previously published
data of MIC values against \textit{M. tuberculosis} where available
are also included in Table 1. \textit{E. natalensis} acetone and chlo-
roform extracts were in general the most active of the plant
extracts against the \textit{Mycobacterium} species tested. The chlo-
roform extract of \textit{E. natalensis} has been reported to be active
against \textit{M. tuberculosis} with an MIC of 8.0 \(\mu\)g/ml.\(^\text{19}\) A similar
MIC value of 7.33 \(\mu\)g/ml against \textit{M. smegmatis} is re-
ported in this study, but \textit{M. fortuitum} and the \textit{M. bovis} strains
were less susceptible to the extract. The acetone extract of \textit{E.
undulata} was far less active against all the test organisms
than the \textit{E. natalensis} acetone extract, with an average MIC
almost five times higher than that of \textit{E. natalensis}.

All the naphthoquinones displayed antimycobacterial ac-
tivity, with 7-methyljuglone the most active against all
species, but the triterpenoid lupeol lacked activity, as with \textit{M.
tuberculosis}.\(^\text{19}\) \textit{M. smegmatis} and the ATCC strain of \textit{M.
bovis} were the most susceptible to the extracts and com-
pounds, while \textit{M. fortuitum} was the most resistant species.
Overall, the \textit{M. bovis} BCG strain was not as susceptible as
the pathogenic \textit{M. bovis} strain to the test substances in this
study, but similar patterns of activity were observed between
all the strains tested. In Fig. 1, it is apparent that, particularly
in the case of the isolated compounds, the activity against \textit{M.
smegmatis} correlates to a larger extent with that against the
\textit{M. bovis} ATCC strain, while results obtained with \textit{M. fortui-
tum} are closer to those with \textit{M. bovis} BCG. This is interest-
ing to note because it would be expected that activity against
the fast-growing species would be closely associated and that
activity against the two \textit{M. bovis} strains would be more simi-
lar. Comparison with previously published data\(^\text{11}\) reveals that
in the case of diospyrin, neodiospyrin and 7-methyljuglone,
M. smegmatis and M. bovis ATCC 19210 show a greater similarity in MIC values to those against M. tuberculosis than the other organisms used in this study. The structures of the isolated naphthoquinone compounds are shown in Fig. 2. The MBC values (Table 1) were relatively high with regard to the crude extracts of Euclea species. The lowest MBC of 15.63 μg/ml was shown by 7-methyljuglone against M. smegmatis.

In vitro screening techniques for detecting antimycobacterial activity in plant extracts and isolated compounds are varied, and include the radiometric BACTEC 460, agar incorporation and broth dilution methods. Agar-based techniques introduce concerns about compound stability or inactivation by agar medium constituents, and are therefore not ideal. Although the BACTEC method as a rapid broth dilution technique has many advantages, its drawbacks include expense, equipment needs and high sample volume requirements. Broth dilution techniques offer benefits such as ease of operation, no expensive equipment needs and, in a microplate format, low sample volumes. Additionally, in liquid medium there is increased cell-to-drug contact and the shorter incubation time than is required for agar methods lowers the likelihood of breakdown of the test compounds.37

Many researchers have made use of the 96-well microplate format to screen test substances for antimycobacterial activity against M. tuberculosis, for example the Microplate Alamar Blue Assay or MABA.31 Results obtained using the MABA assay correlated favourably with those obtained using the BACTEC 460 system, and it is additionally faster and less expensive.32 Alamar Blue is a resazurin-based oxidation-reduction indicator, and other oxidation-reduction dyes, for example tetrazoliums, have also been used to obtain drug susceptibility measurements for mycobacteria.38 The tetrazolium salt is converted to a coloured formazan salt in the presence of actively dividing cells. Bearing in mind considerations of rapidity, low technology requirements and low cost, microplate assays that use Alamar Blue or tetrazolium-type compounds have the potential of becoming the methods of choice for drug susceptibility testing of M. tuberculosis in places where TB is a major problem.33 Similar considerations would apply for any of the slow-growing pathogenic mycobacteria including M. bovis and M. avium.

M. bovis BCG has been used by several groups as a slow-growing but non-pathogenic model organism for anti-TB screening of plants. In these assays, methods used include the radiometric BACTEC assay,31 and 96-well microtitre plate assays using the MABA assay34 or turbidity as a measure of mycobacterial growth inhibition.35,36 A broth dilution technique making use of 8.5 ml screw-capped culture tubes has also been used, with determination of the reduction in optical density of the culture after incubation with test substance.37 The microtitre plate assay employing the tetrazolium salt indicator used in this study gave reproducible results and is more cost effective than the Bactec and MABA assays. A much smaller quantity of test substance is required for the microplate method than for the culture tube procedure, and the colour change is easier to detect compared to increased turbidity. Optical density readings may also be obscured by precipitation of substances or colours in the different extracts.

In this study, experimental microtitre plates were observed at different times for a colour reaction following addition of MTT. It takes in the order of 6 h for slow-growing M. bovis cultures to yield a blue colour at the lowest non-inhibiting concentrations, while for the fast-growing species this colour change occurs in approximately 1 h. Leaving the microtitre plates to incubate for a longer period does not change the MIC value, but only makes differences easier to detect. The 1 and 6 h incubation periods therefore refer to the time required to see the effect on growth of low concentrations of the active compounds. In another study it was reported that MICs of 30 antimicrobial agents for M. tuberculosis H37Rv were available in 9 to 10 d with the BACTEC 460 system, and 7 to 9 d with Alamar Blue.32 Using Alamar Blue as an indicator required at least 12 h of incubation for sufficient reduction by viable M. tuberculosis.32 Growth inhibition of cryptolepine against a range of fast-growing Mycobacterium species following a 72 h incubation period was assessed only 20 min after addition of MTT by Gibbons and co-workers.35 Overall, in comparison to other available methods for antimycobacterial testing, the MTT assay is a rapid and easy test suitable for screening inhibition of both fast- and slow-growing Mycobacterium species by potential antimycobacterial compounds.

Several researchers have utilized non-pathogenic, fast-growing Mycobacterium species in rapid and easy screens for antimycobacterial activity in plant extracts and pure plant-derived compounds.27,30 In one study it was concluded that activity against the rapidly growing, non-pathogenic organism M. aurum is highly predictive of activity against M. tuberculosis, as the two species have similar drug sensitivity profiles.27 It is unknown whether activity against M. aurum is also predictive of activity against M. bovis and this will be the focus of future research. The vaccine strain M. bovis BCG as a test organism for anti-TB drug discovery has been recommended as this is more closely related to pathogenic M. tuberculosis than avirulent rapidly growing species, which have only a limited degree of similarity to M. tuberculosis with respect to drug susceptibility profile and genetic composition.38

The microplate method was well-suited to detecting antimycobacterial activity of Euclea root extracts and pure constituents against both slow-growing and fast-growing species in the present work. Compounds found in the roots of certain plants may have a protective role in a chemical defence system against mycobacteria which are natural components of the soil bacterial population. In the available literature, no reference could be found to the use of infectious M. bovis to
detect antimycobacterial activity in plant extracts or compounds isolated from them, so it is likely that this is the first report of activity of plant-derived material against pathogenic *M. bovis*. The results of the present study indicated that *M. smegmatis* was a better predictor of activity against pathogenic *M. bovis* (and *M. tuberculosis*), while MIC values obtained using *M. fortuitum* correlated well with those of *M. bovis* BCG. However, this situation will possibly vary with different plant extracts and different classes of compounds. Further work is concentrating on testing a variety of plant extracts against a range of mycobacterial species, including pathogenic *M. bovis*, to detect correlations in activity profiles.

CONCLUSIONS

Zoonotic *M. bovis* infections pose a risk to human health, particularly in developing countries. This is exacerbated by insufficient monitoring of the TB status of rural cattle herds, coupled with the high incidence of HIV and AIDS. Many plant-based remedies are used in traditional medicine to treat TB-related symptoms. Following the discovery of plant extracts and compounds isolated from plants with promising activity against *Mycobacterium tuberculosis*, screening of plants against *M. bovis* may also yield good leads for new anti-TB drugs. In this study, compounds isolated from *Eu clea natalensis* revealed strong antimycobacterial activity against pathogenic *M. bovis*. Susceptibility patterns to the plant extracts and isolated compounds were similar between the pathogenic and non-pathogenic (BCG) *M. bovis* strains. Similar patterns in activity were also observed against fast-growing *Mycobacterium* species. It is clear that more research is needed to validate the use of non-pathogenic species such as *M. bovis* BCG, *M. smegmatis* and *M. fortuitum* as models for detecting activity of plant-derived extracts against pathogenic *M. bovis*, as well as against *M. tuberculosis*. This study warrants further investigation of *Euclea*-derived extracts and isolated compounds in preclinical trials.

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