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SHORT COMMUNICATION

Activity against *Mycobacterium smegmatis* and *M. tuberculosis* by Extract of South African Medicinal Plants

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Seven ethnobotanically selected medicinal plants were screened for their antimycobacterial activity. The minimum inhibitory concentration (MIC) of four plants namely *Artemisia afra*, *Dodonea angustifolia*, *Drosera capensis* and *Galenia africana* ranged from 0.781 to 6.25 mg/mL against *Mycobacterium smegmatis*. *G. africana* showed the best activity exhibiting an MIC of 0.78 mg/mL and a minimum bactericidal concentration (MBC) of 1.56 mg/mL. The MICs of ethanol extracts of *D. angustifolia* and *G. africana* against *M. tuberculosis* were found to be 5.0 and 1.2 mg/mL respectively. The mammalian cytotoxicity IC_{50} value of the most active antimycobacterial extract, from *G. africana*, was found to be 101.3 μg/mL against monkey kidney Vero cells. Since the ethanol *G. africana* displayed the best antimycobacterial activity, it was subjected to fractionation which led to the isolation of a flavone, 5,7,2′-trihydroxyflavone. The MIC of this compound was found to be 0.031 mg/mL against *M. smegmatis* and 0.10 mg/mL against *M. tuberculosis*. This study gives some scientific basis to the traditional use of these plants for TB-related symptoms. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: antimycobacterial activity; crude ethanol extracts; cytotoxicity; *Mycobacterium tuberculosis*; *Mycobacterium smegmatis*.

INTRODUCTION

Each year 8 million people are infected by *Mycobacterium tuberculosis*, and 2–3 million patients die from the disease which it causes, tuberculosis (TB). It is estimated that between 2000 and 2020, nearly 1 billion people will become infected, 200 million will acquire the disease and 35 million will die from TB (World Health Organization, 2007), in contrast to the 1.6 million deaths resulting from TB in 2005. Both the highest number of deaths and the highest mortality rate are in the Africa Region. The TB epidemic in Africa grew rapidly during the 1990s, but this growth has been slowing each year, and incidence rates now appear to have stabilized or begun to fall (World Health Organization, 2007). Many infectious diseases are known to be treated with herbal remedies and, even today, plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries. Traditional medicine has served as a source of alternative medicine, new pharmaceuticals, and healthcare products. Medicinal plants are important for pharmacological research and drug development, not only when plant constituents are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds and the screening of plant extracts is of great interest to scientists for the possible treatment of many diseases (Dimayuga and Garcia, 1991).

The aim of the present study was to evaluate selected medicinal plants used traditionally in South Africa to treat tuberculosis or related symptoms such as cough, fever, chest pains, lung infection and other respiratory tract diseases against *Mycobacterium smegmatis* and *M. tuberculosis*. *M. smegmatis* is used as a test model organism in the initial screening process due to its similarity to *M. tuberculosis* genetically but lack of virulence as an infectious organism (Mitscher and Baker, 1998). *In vitro* screening assay using the method of Newton *et al.* (2002) was employed in the present study and the mammalian cytotoxicity of all the extracts was also determined in order to determine selectivity, a necessary aspect of any compound if it is to be viewed as a possible lead for drug development.

MATERIALS AND METHODS

Plant material. Plant species were selected based on their traditional use for tuberculosis (Table 1). Selection of plant species for this study was based on the information culled from published sources and traditional

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Table 1. Traditional use of selected medicinal plants for the present study

Plant species (Family)	Common name	Parts used	Usage[a]	Voucher specimen No.
Artemisia afra Jacq. Ex Willd (Asteraceae)	Wild wormwood	Leaves	C, F	92824
Dodonaea angustifolia L. f. (Sapindaceae)	Sand olive	Leaves	C, F, T	93724
Drosera capensis L. (Droseraceae)	Cape sundew	Leaves	A, B, F	84924
Galenia africana L. (Aizoaceae)	Yellow bush	Leaves	A, C, T	93723
Prunus africana Hook. f. (Rosaceae)	Red stinkwood	Bark	C, F, M	S 71357
Syzygium cordatum Hochst. Ex Krauss. (Myrtaceae)	Water berry	Bark	C, D, T	95547
Ziziphus mucronata Willd (Rhamnaceae)	Buffalo thorn	Bark	B, C, F	94270

[a] Traditional usage: A, asthma; B, bronchitis; C, chest pains; D, diarrhoea; F, fever; M, malaria; S, stomachache; T, tuberculosis.

healers. Voucher specimens were deposited and identified at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria, South Africa. Different plant parts (roots and leaves) of seven plant species representing different families were collected in autumn from different areas in South Africa. Approximately 3 kg of fresh plant material of each species was air-dried and ground.

Preparation of plant extracts. Fifty grams of each plant part was extracted with two successive 500 mL portions of ethanol for 24 h at room temperature. The extracts were concentrated to dryness at reduced pressure with a rotary evaporator at 40 °C. Percentage yield (%w/w) of each ethanol extracts were: *A. afra* (12.4 dry wt), *D. angustifolia* (18.7 dry wt), *D. capensis* (10.4 dry wt), *G. africana* (24.8 dry wt), *P. africana* (21.4 dry wt), *S. cordatum* (32.4 dry wt) and *Z. mucronata* (27.3 dry wt).

Mycobacterium species. The microorganisms, *Mycobacterium smegmatis* (MC^2 155) and a drug-susceptible strain of *M. tuberculosis*, H37Rv (ATCC 27264) were obtained from American Type, MD, USA Culture Collection. *M. smegmatis* was cultured onto Middlebrook 7H11 agar base (7H11) and allowed to grow for 24 h at 37 °C. *M. tuberculosis* was plated onto Löwenstein-Jensen medium and allowed to grow for 3–4 weeks at 37 °C.

Antimycobacterial bioassay against *M. smegmatis* and *M. tuberculosis*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of extracts were determined according to the methods as mentioned earlier (Mativandlela *et al*., 2007) The ethanol extracts were dissolved in 10% DMSO in sterile Middlebrook 7H9 broth base to obtain a stock concentration of 100.0 mg/mL. Serial two-fold dilutions of each sample to be evaluated were made with 7H9 broth to yield volumes of 100 µL/well with final concentrations ranging from 25.0 mg/mL to 0.390 mg/mL. Ciprofloxacin at a final concentration of 0.156 mg/mL, served as the positive drug control.

The radiometric respiratory techniques using the BACTEC 460 system (Becton Dickinson Diagnostic Instrument, Sparks, MD) was used for susceptibility testing against *M. tuberculosis* as described previously (Mativandlela *et al*., 2007). Isoniazid (INH) (Sigma-Aldrich, South Africa) at final concentration of 0.2 µg/mL served as drug-control in our bioassay. All the extracts were tested at concentrations ranging from 5.0 to 0.1 mg/mL. The bactericidal effect, (minimum bactericidal concentration, MBC) of extracts was assessed by plating the bacterial suspensions from BACTEC vials which exhibited MIC, at the end of the experiment on 7H11 agar medium for viable count enumeration. A total of 0.1 mL of *M. tuberculosis* from BACTEC vials was successively diluted 10-fold in sterile double-distilled water to give dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. A total of 0.1 mL of 10^{-2} and 10^{-4} was plated onto 7H11 agar medium, and the resulting bacterial counts were enumerated after 20 days of incubation at 37 °C. The MBC was defined as the minimal concentration which effectively reduced by at least 99% the viable counts in the extract or compound-containing sample compared with those in control vials (extract or compound free vials).

Isolation of antimycobacterial compound from *G. africana*. Since the ethanol extract of *G. africana* showed antituberculosis activity against *M. smegmatis* and *M. tuberculosis*, it was selected for isolation. The ethanol (10 g) extract was dissolved in 2 L of 100% ethanol and subjected to a silica column chromatography (CC, size 70×120 cm) using the gradient system of hexane: ethyl-acetate mixtures of increasing polarity (0 to 100%) as an eluant. Thirty three 50 mL fractions were collected, similar fractions were pooled together based on the thin layer chromatography (TLC) profile and dried, resulting in six main fractions 1–6. For antimycobacterial activity, all fractions were tested against *M. smegmatis* and *M. tuberculosis* at concentrations ranging from 1000 to 125.0 µg/mL. Repeated chromatography on silica column, Sephadex LH-20 and preparative TLC on silica gel eluted using hexane: ethyl-acetate mixtures (5% to 10%) resulted in one pure compound (yield 0.0003%) from fraction 4. The isolated compound was identified as 5,7,2′-trihydroxyflavone (Fig. 1a), mainly using ^{1}H and ^{13}C spectra, which agreed with the reported data (Harborne and Williams, 1997). Direct bioassay on TLC plate was done by applying a small spot of 20 µL of the isolated compound (40 mg/mL) to silica gel 60 F_{254} plate (Merck, Johannesburg, South Africa). The plate was developed in hexane: ethyl acetate (8:2) and dried carefully. The 24 h *M. smegmatis*, in 7H9 broth was centrifuged at 1000 rpm for 15 min. The supernatant was discarded and the pellet was resuspended in fresh 7H9 broth. A fine spray was then used to apply the bacterial suspension onto TLC plate (Meyer and Dilika, 1996). The plate was then incubated at 37 °C for 24 h in humid conditions. After incubating, the plate was sprayed with 2.0 mg/mL INT and an inhibition zone was noted. The compound was also tested against *M. smegmatis* and *M. tuberculosis* at concentrations ranging from 200 to 1.0 µg/mL.

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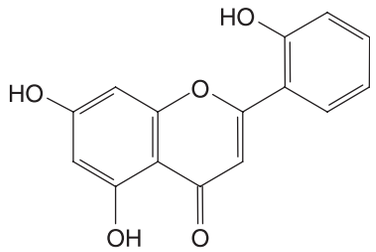

(a)

(b)

Figure 1. (a) Chemical structure of 5,7,2′-trihydroxyflavone (14), isolated from *G. africana* ethanol extract. (b) Zone of inhibition of *M. smegmatis* on TLC plate in a direct assay of the isolated compound. TLC plates developed in hexane: ethyl acetate (8:2). *p*-Iodonitrotetrazolium violet (INT) was used as the detection reagent.

Mycothiol disulfide reductase (Mtr) assays. Recombinant *M. tuberculosis* mycothione reductase was over expressed and purified from an *M. smegmatis* mc^2155 transformant (a generous gift from J. Blanchard) as previously described (Patel and Blanchard, 1999).

Subversive substrate assays of 5,7,2′-trihydroxyflavone with Mtr were carried out at 30 °C in 1 cm^3 of 50 mM HEPES (pH 7.6), 0.1 mM EDTA containing Mtr (30 μg), NADPH (70 μM), and varying concentrations of substrate. Mtr was pre-incubated with NADPH for 5 min at 30 °C before initiating the reaction by addition of a DMSO solution of 5,7,2′-trihydroxyflavone. The final DMSO concentration in the assays was (2% (v/v)). Enzyme activity was monitored by the decrease in absorbance at 340 nm due to NADPH consumption.

Mammalian cytotoxicity assay. Microtitre well plates with Vero cells were used for testing all the ethanol extracts (400.0 to 3.125 μg/mL) for cytotoxicity following the method of Zheng *et al*. (2001). The positive control, zelaralenone, at a final concentration of 1.25 μg/mL was included. The 'GraphPad Prism 4', statistical program was used to analyse the 50% inhibitory concentration (IC_{50}) values.

RESULTS AND DISCUSSION

Antimycobacterial activity against *M. smegmatis* and *M. tuberculosis*

Of the seven selected plants, the ethanol extract of *G. africana* (**7**), was found to be the most effective against *M. smegmatis*, exhibiting a MIC of 0.78 mg/mL. *A. afra* (**1**), was the next best plant which inhibited growth at 1.56 mg/mL. *D. angustifolia* (**2**) and *D. capensis* (**3**) had the same MIC of 3.13 mg/mL. *S. cordatum* (**5**) inhibited *M. smegmatis* at a concentration of 6.25 mg/mL. *P. africana* (**4**) and *Z. mucronata* (**6**) were found not active at the highest concentrations tested. Six fractions (**8**–**13**), obtained from the ethanol extract of **7** were tested against *M. smegmatis*. Fractions two to five (**9**–**12**) showed activity and fraction four (**11**) was the most active fraction, exhibiting an MIC of 0.125 mg/mL (Table 2). In a direct bioassay when the developed TLC plate was sprayed with the suspension of *M. smegmatis*, a zone of inhibition was observed due to 250 μg of purified compound, 5,7,2′-trihydroxyflavone (**14**), which was spotted at the origin of TLC plate (Fig. 1b). The MIC of **14** was found to be 0.031 μg/mL (Table 2).

The antimycobacterial assay of the ethanol extracts against *M. tuberculosis* using the BACTEC radiometric method showed that **7** inhibited *M. tuberculosis* at 1.2 mg/mL. In addition, **2** inhibited the mycobacteria at a concentration of 5.0 mg/mL, whereas the other extracts did not show activity against *M. tuberculosis*. Fractions three (**10**) and five (**12**) from ethanol extracts of **7** showed inhibitory activity at 1.0 mg/mL and fraction four (**11**) at 0.5 mg/mL. The isolated compound (**14**) from **11** inhibited *M. tuberculosis* at 0.1 mg/mL whilst the antituberculosis positive drug, INH, inhibited the growth of *M. tuberculosis* at 0.2 μg/mL (Table 2). The ethanol extract of **1** showed inhibition against *M. smegmatis* at 1.56 mg/mL using the microplate dilution method, but no inhibition of *M. tuberculosis* at the highest concentration (5.0 mg/mL) tested.

The bactericidal effect of various active extracts, fractions, purified compound and the positive drug in the BACTEC system, was compared between the treated and untreated cultures. 100 μL of the bacterial suspensions from BACTEC vials (exhibiting MICs) at the end of the experiment was plated on 7H11 agar medium for viable count enumeration. Only selected results (expressed as mean viable counts ± standard error) in case of treated and untreated vials are illustrated in Fig. 2. Both **2** and **7** resulted in 1 log (90%) killing of the bacterial inoculum at a fixed concentration of 5.0 and 1.2 mg/mL respectively. Fractions three (**10**) and four (**11**) resulted in 90% kill at 0.1 mg/mL. The isolated compound, **14** was more bactericidal than the crude ethanol extract of **7** and resulted in a 2 log (99.5%) killing at 0.1 mg/mL (Fig. 2). The ethanol extract, fractions and 5,7,2′-trihydroxyflavone, the compound isolated from **7**, showed the best inhibitory effects against both *Mycobacterium* species tested using different methods used.

During our investigation on the NADPH oxidase activity of **14** with Mtr it was found that this compound failed to exhibit any NADPH oxidase activity at 800 μM concentrations. Mtr is evidently not the target for the antitubercular activity of **14**. Additional targets could include other flavoprotein oxidoreductases such as LADH and thioredoxin reductase, which unlike Mtr are also found in eukaryotes.

Cytotoxicity activity

The ethanol plant extracts demonstrated moderate cytotoxicity on Vero cells, except for **6** which showed IC_{50}

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Table 2. Antimycobacterial activity of ethanol extracts, fractions and isolated compound against *M. smegmatis*, *M. tuberculosis* and cytotoxicity on Vero cells

Tested sample	*M. smegmatis*		*M. tuberculosis*		Vero cells
	MIC[a] (mg/mL)	MBC[b] (mg/mL)	MIC (mg/mL)	ΔGI[c]	IC_{50}[d] (μg/mL ± SD)
Artemisia afra (**1**)	1.563	6.25	na	938.0 ± 86.2	113.0 ± 2.05
Dodonaea angustifolia (**2**)	3.125	12.5	5.0 (S[e])	0.5 ± 0.7	91.0 ± 1.09
Drosera capensis (**3**)	3.125	na	na	200.5 ± 86.2	141.4 ± 2.14
Prunus africana (**4**)	na	nt	na	971.0 ± 39.5	101.3 ± 2.19
Sygium cordatum (**5**)	6.25	na	na	919.0 ± 113.1	212.0 ± 2.33
Ziziphus mucronata (**6**)	na	nt	na	839.0 ± 226.2	2.7 ± 2.54
Galenia africana (**7**)	0.781	1.563	1.2 (S)	0.0 ± 0.0	118.2 ± 2.36
Fractions of ethanol extract from *G. africana*					
Fraction I (**8**)	na	nt	na	227.5 ± 314.6	nd
Fraction II (**9**)	0.500	na	na	200.5 ± 122.3	nd
Fraction III (**10**)	0.250	na	1.0 (S)	18.5 ± 4.94	nd
Fraction IV (**11**)	0.125	0.0625	0.5 (S)	20.0 ± 1.4	nd
Fraction V (**12**)	0.250	na	1.0 (S)	28.0 ± 5.6	nd
Fraction VI (**13**)	na	nt	1.0 (S)	35.5 ± 6.3	nd
5,7,2′-Trihydroxyflavone (**14**)	0.0312	0.125	0.1 (S)	22.0 ± 3.6	39.87 ± 2.836
Ciprofloxacin (positive drug control for *M. smegmatis*) (**15**)	0.156	0.312	nd	nd	nd
Isoniazid (positive drug control for *M. tuberculosis*) (**16**)	nd	nd	2×10^4	4.6 ± 2.8	nd
Zeralenone (positive drug control for Vero cells) (**17**)	nd	nd	nd	nd	2.318 ± 0.301

na, no activity at highest concentration tested; nd, not determined.
[a] Minimum inhibitory concentration; [b] minimum bactericidal concentration; [c] ΔGI value (mean ± SD) of the control vial was 47.5 ± 19.0 for the sensitive strain; [d] concentration giving 50% inhibition of growth; [e] susceptible.

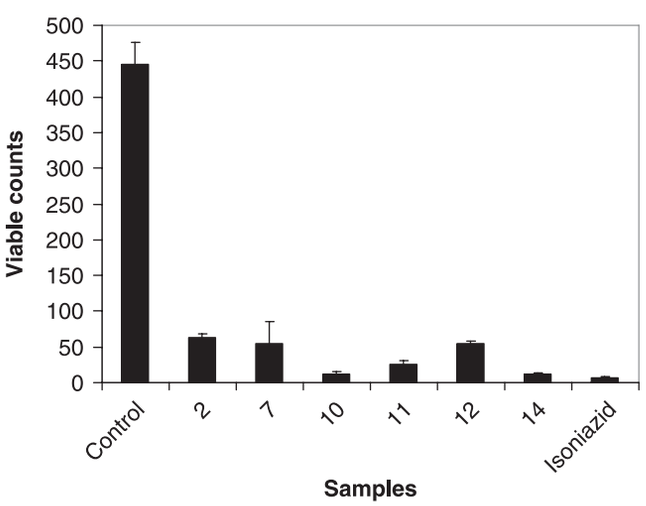

Figure 2. The comparative bactericidal effect of active extracts (**2** and **7**), fractions (**10**–**12**) and compound (**14**) in BACTEC 7H12 vials against the drug-susceptibility strain of *M. tuberculosis*. Results illustrate mean viable counts ± standard error in the presence of selected concentrations at various samples after 21 days of incubation at 37 °C compared with growth in control vials.

of 2.67 μg/mL against the Vero cells. Ethanol extract of **5** had the highest IC_{50} value (212.0 μg/mL) compared with other plant extracts. Ethanol extract of **7** showed moderate toxicity exhibiting IC_{50} of 101.3 μg/mL (Table 2). The isolated compound **14** from **7** also demonstrated moderate toxicity on Vero cells.

Of the different methods employed in this study, the microplate method gave an indication of potency of the selected plant species. Rapid radiometric method using BACTEC-460 system was found to be very reliable for testing the efficacy of plant extracts, fractions and compound against *M. tuberculosis*. The selected plant species in this study have not been tested previously against *M. smegmatis* and *M. tuberculosis*, and this is the first report of their antimycobacterial activity.

In conclusion, our findings indicated some correlation between the activities of the ethanol plant extracts when screened against both *M. smegmatis* and *M. tuberculosis*. Selection of plants by ethnobotanical criteria offers a good probability of finding candidates which contain compounds active against mycobacteria (Lall and Meyer, 1999).

Our research pointed to the purified compound and/or extract of **7** as potential candidates for further investigation in pre-clinical trials for their potential as antimycobacterial agents.

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