Some South African Rubiaceae tree leaf extracts have antimycobacterial activity against pathogenic and non-pathogenic *Mycobacterium* species

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ABSTRACT

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* remains an ongoing threat to human health. Many plant species contain antimycobacterial compounds which may serve as template molecules for new anti-TB drugs. The Rubiaceae family is the largest family of trees in southern Africa and preliminary evidence revealed antimycobacterial activity in several species of the genus, motivating further studies. Leaf extracts of 15 tree species from the Rubiaceae family were screened for antimycobacterial activity against pathogenic *M. tuberculosis* and non-pathogenic *M. smegmatis, M. aurum* and *M. bovis* BCG using a

two-fold serial microdilution assay. Cytotoxicity was determined using a tetrazolium-based colorimetric assay against C3A liver cells and Vero kidney cells. MIC values as low as 0.04 mg/mL against *M. smegmatis* and *M. tuberculosis* were recorded. Activity against *M. aurum* was the best predictor of activity against pathogenic *M. tuberculosis* (correlation coefficient = 0.9). Bioautography indicated at least 40 different antimycobacterial compounds in the extracts. Cytotoxicity of the extracts varied and *Oxyanthus speciosus* had the most promising selectivity index values.

Keywords

Rubiaceae, antimycobacterial, tuberculosis, selectivity, cytotoxicity.

INTRODUCTION

Mycobacterium tuberculosis, a facultative intracellular pathogen belonging to the *Mycobacterium tuberculosis* complex is the causative agent of tuberculosis (TB) in humans. It was responsible for the mortality of 1.4 million of the world's population in 2011, causing morbidity in an estimated 8.7 million cases (World Health Organisation, 2011). TB has become a global health problem with one-third of the world's population latently infected with the disease and one in ten people developing active disease in their lifetime (Bishai *et al.*, 2010). Other than *M. tuberculosis*, other members of the *Mycobacterium tuberculosis* complex such as *M. bovis*, *M. africanum*, *M. microti* and *M. canetti* have the ability to infect humans with TB (McGaw *et al.*, 2008a). Current TB chemotherapy is effective, but the disease is a global challenge due to infections concurrent with the pandemic of HIV/AIDS, with 80% of cases occurring in Asia and Africa due to poverty and poor health systems (LoBue *et al*., 2010; Zager and McNerney, 2008).

The implementation of directly observed treatment, short-course (DOTS) by the World Health Organisation (WHO) involves the administration of first line anti-TB drugs, which are combinations of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB) for 2 months in order to kill the rapidly growing bacteria. The treatment is continued in the next 4 months with the combination of INH and RIF due to their sterilizing activity to eliminate the bacilli which are dormant in the macrophages, or slow growers (Rivers and Mancera, 2008). Toxicity associated with these first line drugs and the long duration of treatment has led to the failure of patient compliance, giving rise to drug-resistant strains. Multi-drug resistant mycobacteria are resistant to INH and RIF, while extremely drug-resistant mycobacteria are also resistant to second line drugs such as fluoroquinolones and at least one injectable drug in addition to INH and RIF. Hence, there is a need to identify new targets for novel anti-TB drugs (McGaw *et al.*, 2008a).

From antiquity, humankind has sought to source drugs from plants. Natural products and their derivatives play a substantial role in the drug discovery and development process, serving as leads for the invention of new drugs (Newman and Cragg, 2007). The use of medicinal plants is an alternative to the use of synthetic drugs as a curative agent for infectious disease, arousing the interest of researchers investigating substitutes to modern medicine (Al-Saghir *et al.*, 2009). Many plant species have antimycobacterial compounds which may serve as template molecules for the development of new anti-TB drugs (Pauli *et al.*, 2005; Garćia *et al.*, 2012). Medicinal plants indigenous to southern Africa have been employed in the treatment of TB-related symptoms such as coughing, chest pain, respiratory discomfort and fever (McGaw *et al.*, 2008a).

The Rubiaceae is one of the largest families indigenous to tropical and subtropical regions around the world exhibiting great medicinal value. The family consists of about 611 genera and 13 143 species. Of the 611 genera, 48 of them have been reported to have a broad spectrum of antibacterial activity (Davis *et al.*, 2009; Choudhury *et al.*, 2012). In a preliminary large screening study of tree leaf extracts (Pauw and Eloff, 2014; Pauw, 2014), some species belonging to the Rubiaceae family showed promising activity when tested against a panel of Gram-positive and Gram-negative bacteria and the non-pathogenic *M. smegmatis*. This provided motivation for more in depth evaluation of 15 species from this family for antimycobacterial activity and cytotoxicity. Several *Mycobacterium* species have been used as models for studying activity against pathogenic strains (Shiloh and Champion, 2010). The objective of this study was firstly to investigate extracts from 15 species of the Rubiaceae family for antimycobacterial activity and cytotoxicity. Another objective of this study was to compare activities of the extracts against different *Mycobacterium* species to determine which non-pathogenic *Mycobacterium* species correlates best to the pathogenic *M. tuberculosis* in terms of susceptibility to the plant extracts. Acetone was selected as the extracting solvent for the dried leaf powder owing to its ability to extract a wide range of compounds and its low toxicity in the antimicrobial bioassay (Eloff, 1998a).

MATERIALS AND METHODS

Plant collection

The leaves of 15 plant species were collected in November 2009 from the National Botanical Gardens (NBG) in Pretoria, South Africa in terms of a signed Material Transfer Agreement. The plant materials were identified and labelled. Voucher specimens were kept

Table 1: Minimal inhibitory concentration (MIC in mg/mL) and total activity (TA in mL/g) of some South African Rubiaceae species

Plant species	Voucher number[1]	Extraction yield (%)	Microorganisms							
			Ms[2]		***Ma***		***Mb***		***Mt***	
			MIC± SD	TA	MIC± SD	TA	MIC± SD	TA	MIC± SD	TA
Cephalanthus natalensis Oliv.	PRU 120872	2.43	0.17±0.19	139.26	0.10±0.08	249.23	0.47±0.39	51.98	0.04±0.08	607.50
Cremaspora triflora (Thonn.) K.Schum.	114710	1.1	0.23±0.11	56.22	0.10±0.08	134.36	0.19±0.22	67.53	0.16±0.08	81.88
Feretia aeruginescens Stapf	114680	11.32	0.23±0.11	485.84	0.47±0.22	242.14	0.31±0.22	365.16	0.35±0.11	323.43
Hymenodictyon parvifolium Oliv	114762	7.05	0.47±0.22	150.80	0.19±0.16	363.40	0.35±0.00	200.57	0.63±0.16	111.90
Hyperacanthus sp. E. May. ex	PRU 120869	3.42	0.47±0.22	73.16	0.31±0.00	110.32	0.94±0.16	36.48	0.38±0.22	427.50
Keetia gueinzii (Sond.) Bridson	114723	2.54	0.23±0.11	109.01	0.16±0.00	162.82	0.43±0.00	59.07	0.31±0.11	81.94
Keetia sp. E. Phillips	114724	1.48	0.06±0.03	252.99	0.35±0.39	42.11	1.41±1.55	10.53	0.04±0.03	370.00
Kraussia floribunda Harv.	114763	3.23	0.31±0.00	104.19	0.23±0.11	138.63	0.19±1.33	166.49	0.16±0.11	201.88
Mussaenda arcuata Poir.	PRU 120870	4.84	0.08±0.00	620.51	0.39±0.33	123.94	1.56±0.16	30.98	0.31±0.33	156.13
Oxyanthus speciosus DC.	PRU 120873	2.99	0.08±0.00	383.33	0.06±0.03	511.11	0.12±0.06	255.56	0.17±0.22	63.62
Pavetta lanceolata Eckl.	PRU 120874	2.31	0.12±0.06	197.44	0.10±0.08	236.92	0.17±0.19	132.38	0.12±0.00	192.50
Pavetta schumanniana F.Hoffm.ex K.Schum.	PRU 120868	3.2	0.31±0.00	106.13	0.16±0.00	210.90	0.78±0.66	42.18	0.23±0.03	205.63
Psychotria capensis (Eckl.) Vatke	PRU 120875	2.3	0.06±0.03	393.16	0.12±0.06	196.58	0.19±0.16	118.56	0.63±0.39	36.51
Psychotria zombamontana (Kuntze) E.M.A.Petit	PRU 120867	4.2	0.04±0.00	1076.92	0.08±0.00	538.46	0.16±0.00	269.23	0.16±0.00	182.61
Vangueria infausta Burch.	PRU 120871	0.96	0.63±0.00	15.36	0.23±0.11	41.20	0.47±0.44	20.53	0.63±0.00	15.24
Ciprofloxacin (µg/mL)			0.58±0.28	na	2.33±1.09	na	na	na	9.37±4.42	na
Isoniazid (µg/mL)			>100	na	>100	na	9.37±4.42	na	>100	na
Streptomycin (µg/mL)			na	na	2.34±1.10	na	>100	na	37.5±17.68	na
Rifampicin (µg/mL)			12.89±17.13	na	0.78±0.00	na	1.17±0.55	na	9.375±4.42	na

[1] PRU numbers indicate deposition of voucher specimens at the HGWJ Schweickerdt Herbarium at the University of Pretoria*;* other numbers indicate deposition at the Herbarium of the Lowveld National Botanical Gardens (Nelspruit); [2]*Ms = Mycobacterium smegmatis*, *Ma = Mycobacterium aurum*, *Mb = Mycobacterium bovis* BCG, *Mt = Mycobacterium tuberculosis*

in the Lowveld National Botanical Gardens (Nelspruit) and the HGWJ Schweickerdt Herbarium of the University of Pretoria (PRU) (Table 1). The leaves were air dried at room temperature, ground to a fine powder in a Macsalab mill (model 2000 LAB Eriez) and stored in closed glass containers in the dark until needed.

Plant extraction

One gram of powdered leaf material from each species was extracted with 10 ml of acetone (technical grade, Merck) in a polyester centrifuge tube which was vigorously shaken on an orbital shaker for 30 min (Eloff, 1998b). It was centrifuged at 4000 x g for 10 min and the supernatant was filtered through Whatman No. 1 filter paper into a pre-weighed glass vial. The extraction was repeated twice and the solvent was removed under a stream of air in a fume hood at room temperature to produce dried extracts. Extracts were made up to a concentration of 10 mg/mL in acetone.

Chromatographic analysis

Qualitative screening of crude acetone extracts was performed to obtain thin layer chromatography (TLC) fingerprints of each investigated extract. Ten µl of each extract (containing 100 µg of extract) was loaded on aluminium-backed TLC plates (Merck, Silica gel F_{254}) in lines of about 1 cm wide. The TLC plates were developed in three different mobile solvent systems, namely ethyl acetate/methanol/water (EMW) 10:1.35:1, chloroform/ethyl acetate/formic acid (CEF) 10:8:2 and benzene/ethanol/ammonia (BEA) 18:2:0.2 (Kotze and Eloff, 2002). Chromatograms were examined under ultraviolet light at wavelengths of 254 and 365 nm after which TLC plates were sprayed with vanillin in

methanol and sulphuric acid (vanillin-sulphuric acid) and heated at 110^{o}C to optimal colour development.

Bioautographic investigations

Duplicate chromatograms prepared as above were left uncovered in a dark place under a stream of cold air for one day to allow evaporation of the solvent before being sprayed with an actively growing suspension of *M. smegmatis* (cultured for 18 - 24 h at 37°C) using a spray gun in a fume cupboard. The moist plates were incubated at 37^{o}C in a closed plastic container for 24 h to allow the mycobacteria to grow on the plates. The plates were then sprayed with 2 mg/mL of *p*-iodonitrotetrazolium violet (INT) (Sigma) in distilled water and incubated for a further 30 min to 1 h for the development of clear zones against a purple-red background. Clear zones against the purple-red background were indicative of antimycobacterial activity of compounds separated on the chromatograms (Begue and Kline, 1972).

Antimycobacterial activity

Mycobacterial culture

Antimycobacterial activity was tested against 3 non-tuberculous mycobacteria (NTM): *M. smegmatis* (ATCC 1441), *M. aurum* (NCTC 10437) and *M. bovis* BCG (Pasteur strain P1172) as well as against a *M. tuberculosis* field strain (TB 8104). The mycobacterial species were cultured on Löwenstein–Jensen agar slants, supplemented with glycerol, or pyruvate in the case of the *M. bovis* BCG cultures. Sterile plastic loops were used to scrape cells off the slants prior to each assay 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 4×10^7 cfu/ml), and then diluted with freshly prepared Middlebrook 7H9 broth supplemented with 10% OADC medium to obtain a final inoculum density of approximately 5×10^5 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.

Molecular characterization of *M. tuberculosis* strain

Mycobacterium tuberculosis strain (TB 8104) isolated from a waterbuck was genetically characterized using variable number of tandem repeat typing based on a 13-locus VNTR panel (Hlokwe *et al*., 2013).

Antimycobacterial assay

The modified two-fold serial dilution microplate method was used to determine the minimum inhibitory concentration (MIC) values of the plant extracts (Eloff, 1998b; McGaw *et al*., 2008b). Briefly, aliquots (100 µl) of 10 mg/mL solutions dissolved in acetone of the crude extracts were serially diluted with OADC-supplemented Middlebrook 7H9 broth in 96-well microtitre plates before mycobacterial culture (100 µl) was added to each well. The anti-TB drugs isoniazid, rifampicin and streptomycin represented the positive controls, 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 covered with sterile plastic lids, 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 *M. smegmatis* was incubated at 37°C overnight, while *M. aurum* and *M. bovis* BCG cultures were incubated for 5 to 7 days. *M. tuberculosis* was incubated at 37°C for 10 days. MIC values were detected using a tetrazolium violet (INT) indicator. The colour reaction after addition of INT generally occurred after 30 min to 1 h incubation for the non-pathogenic strains while the corresponding incubation time was 2-4 h for the pathogenic *M. tuberculosis*. MIC values were noted as the lowest concentration of plant extracts that inhibited the growth of the mycobacteria, resulting in a visible decrease in production of the red formazan. The total activity of the extracts was calculated as the total mass (mg) of the extract divided by the MIC value (mg/mL). Correlation between the MIC values of the four *Mycobacterium* strains used was calculated using Microsoft Excel 2010 software. Total activity values indicate the volume to which the extract derived from 1 g of plant material can be diluted and still inhibit the growth of the microorganism.

Cytotoxicity assay

The cytotoxicity of the crude plant extracts against C3A human liver cells (purchased from ATCC, CRL-10741) and Vero African green monkey kidney cells (sourced from the collection of the Department of Veterinary Tropical Diseases, University of Pretoria) was determined using the 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983) with slight modifications (McGaw *et al.*, 2007). The cells were maintained in minimal essential medium (MEM, Highveld Biological, South Africa) supplemented with 10% foetal calf serum (Adcock-Ingram) and sodium pyruvate for C3A

liver cells while Vero cells were supplemented with 5% foetal calf serum and 0.1% gentamicin (Virbac). Cell suspensions were prepared from confluent monolayer cultures and plated at a density of 5×10^4 cells into each well of 96-well microtitre plates. For cell attachment, plates were incubated for 24 h at 37^{o}C in a 5% CO_2 incubator prior to the exposure. The crude plant extracts were dissolved in acetone (100 mg/mL), and appropriate dilutions were prepared in MEM and added to the wells. Cells were exposed to the various concentrations of plant extract for 48 h. Doxorubicin (Pfizer Laboratories) was used as a positive control while acetone was the negative control. After incubation for 48 h, the wells were rinsed with 150 µl of phosphate buffered saline (PBS, Sigma) and 200 µl of fresh medium was dispensed into the wells. MTT (Sigma) dissolved in PBS (30 µl) was added to each well and incubated for 4h at 37^{o}C. The medium was removed and MTT formazan crystals were dissolved in 50 µl DMSO. Absorbance was measured on a microplate reader (BioTek Synergy) at a wavelength of 570 nm. Each extract concentration was tested in quadruplicate and the assay was repeated three times. Selectivity index (SI) values were calculated by dividing cytotoxicity LC_{50} values by the MIC values (LC_{50}/MIC).

RESULTS

Plant extract yield

Powdered leaf materials were extracted with acetone. The percentage yield of the 16 crude extracts (Table 1) ranged from 0.96 to 11.32% with *Feretia aeruginescens* having the highest percentage yield of 11.32% followed by *Hymenodictyon parvifolium* (7.05%). The lowest yield obtained was from *Vangueria infausta* (0.96%).

Phytochemical analysis

Compounds of varying polarities were visualized on the TLC plates when sprayed with vanillin reagent. More than 40 compounds were separated by TLC using the BEA and CEF solvent system but CEF was the best system with more than 20 compounds of intermediate polarities separated (Figure 1A).

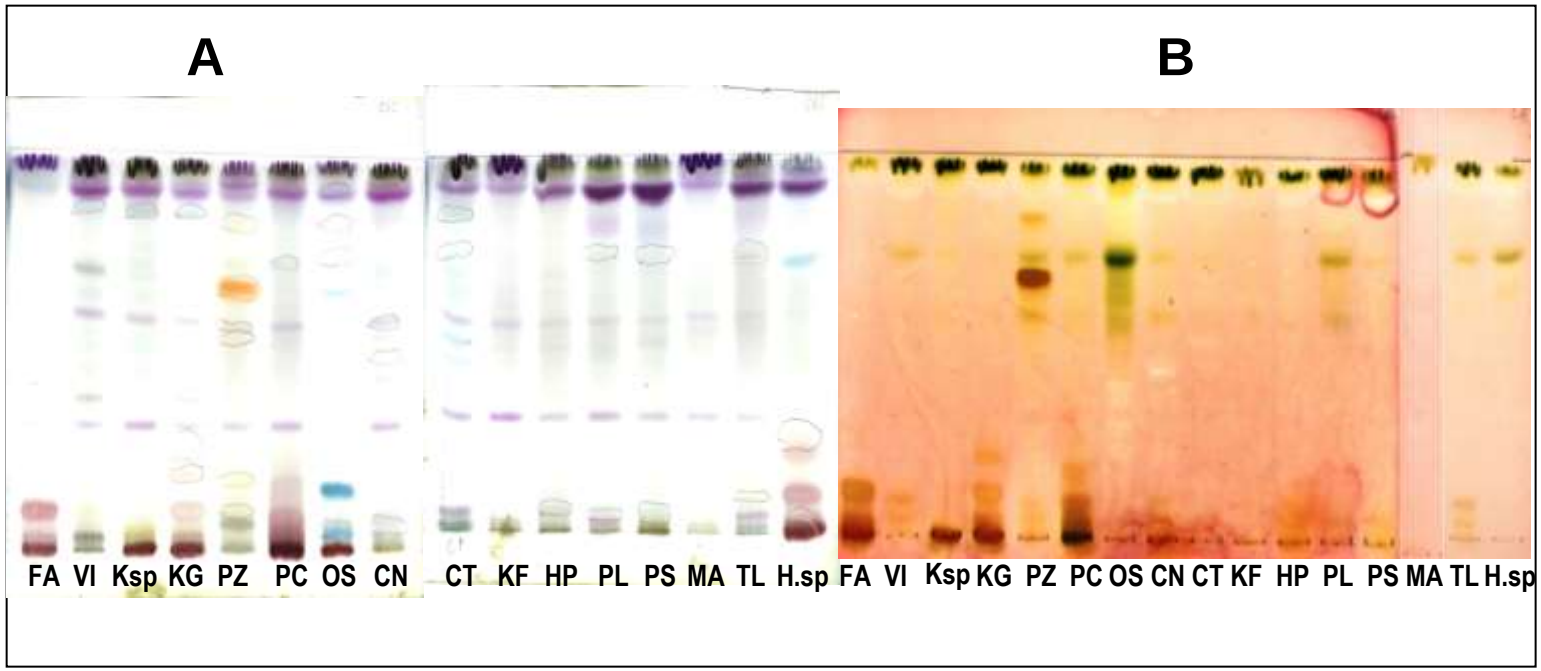
Bioautography

All the crude plant extracts showed at least 1 band of growth inhibition against *M. smegmatis* with the 3 solvent systems used except for *Kraussia floribunda* and *Feretia aeruginescens*. The BEA system gave the best separation of compounds, indicating that most of the antimycobacterial compounds are non-polar. An active compound of Rf value of 0.79 was present in all the extracts for BEA chromatograms. In the BEA and CEF (Figure 1B) chromatograms, *Oxyanthus speciosus* and *Cremaspora triflora* showed 4 active bands each. In the EMW chromatograms, *Cephalanthus natalensis* had 6 active compounds while *Oxyanthus speciosus* had 5 compounds.

Antimycobacterial activity

The results of the antimycobacterial activity assay are presented in Table 1. Extracts of *Psychotria zombamontana*, *Cephalanthus natalensis* and *Keetia* sp. had the best MIC value of 0.04 mg/mL followed by *Oxyanthus speciosus* and *Psychotria capensis* (0.06 mg/mL). *Mussaenda arcuata* and *Keetia* sp. extracts had weak activity against the non-pathogenic *M. bovis* BCG with MIC values of 1.56 mg/mL and 1.41 mg/mL respectively. Plant extracts with MIC values lower than 0.1 mg/mL are regarded as being significantly active;

Figure 1: **(A)** TLC plates developed in CEF solvent system, sprayed with vanillin sulphuric acid showing varied chemical constituents of the Rubiaceae plant extracts screened **(B)** Bioautography of the screened plant extracts against *Mycobacterium smegmatis* eluted using CEF solvent system



FA: *Feretia aeruginescens*, VI: *Vangueria infausta*, Ksp: *Keetia* sp., KG: *Keetia gueinzii*, PZ: *Psychotria zombamontana*, PC: *Psychotria capensis*, OS*: Oxyanthus speciosus*, CN: *Cephalanthus natalensis*, CT: *Cremaspora triflora*, KF: *Kraussia floribunda*, HP: *Hymenodictyon parvifolium*, PL: *Pavetta lanceolata*, PS: *Pavetta schumanniana,* MA: *Mussaenda arcuata*, TL: *Tricalysia lanceolata,* Hsp: *Hyperacanthus* sp.

those with MIC values > 0.1 to 0.625 mg/mL are considered moderately active, and if the MIC is > 0.625 mg/mL the activity is weak (Eloff, 1998b; Sánchez and Kouznetsov, 2010).

Good correlations were obtained between the activity of the non-pathogenic strain and the pathogenic isolate *M. tuberculosis* with the (*r*) coefficient correlation values of 0.9, 0.8 and 0.4 for *M. aurum*, *M. bovis* BCG and *M. smegmatis* respectively. The plant extract of *P. zombamontana* showed the highest total activity of 1 077 mL/g against *M. smegmatis*. The value of 1 077 mL/g means that if 1 g of the acetone extract is diluted to 1077 mL, it will still inhibit the growth of the specific mycobacterial species (Eloff, 2000). The extract of *Oxyanthus speciosus* was the second best with values of 511 mL/g and 438 mL/g against *M. aurum* and *M. bovis* BCG respectively.

Cytotoxicity and Selectivity Index (SI)

The crude extract of *Vangueria infausta* had the highest LC_{50} value (lowest toxicity) of 0.76 mg/mL while the extract of *Psychotria capensis* was the most toxic (LC_{50} = 0.015 mg/mL) against C3A liver cells (Table 3). The extract of *Feretia aeruginescens* was the least toxic (LC_{50} = 0.24 mg/mL) while *Psychotria capensis* (LC_{50} = 0.034 mg/mL), *Psychotria zombamontana* (LC_{50} = 0.032 mg/mL) and *Hymenodictyon parvifolium* (LC_{50} = 0.037 mg/mL) were the most toxic extracts against Vero kidney cells. Selectivity index (SI) values of the extracts were calculated by dividing the LC_{50} (mg/mL) values by MIC (mg/mL) values (Table 2). The higher the SI value, the safer the extract. The average SI value for all the crude extracts ranged from 1.5-1.7 and 0.7-0.9 for C3A liver cells and Vero kidney cells respectively. The extract of *Oxyanthus speciosus* had the best SI values (4.91,

Table 2: Pearson's correlation coefficients (*r*) between MIC values of the four *Mycobacterium* strains used

	Ms	*Ma*	*Mb*	*Mt*
Ms	1			
Ma	0.305342	1		
Mb	0.79139	0.797694	1	
Mt	**0.439961**	**0.909805**	**0.875851**	1

Ms = Mycobacterium smegmatis, *Ma = Mycobacterium aurum*, *Mb = Mycobacterium bovis* BCG, *Mt = Mycobacterium tuberculosis*
Values in bold indicate the correlation between the fast growing *Mycobacterium* strains and with pathogenic *M. tuberculosis* isolate.

Table 3: Cytotoxicity (LC_{50} in mg/mL) of extracts and selectivity index against C3A liver cells and Vero kidney cells

Plant species	LC_{50}	SI C3A cells				LC_{50}	SI Vero cells			
		Ms	***Ma***	***Mb* BCG**	***Mt***		***Ms***	***Ma***	***Mb* BCG**	***Mt***
Cephalanthus natalensis Oliv.	0.138	0.593	1.417	0.836	1.177	0.076	0.328	0.784	0.463	0.648
Cremaspora triflora (Thonn.) K.Schum.	0.254	3.257	2.606	2.895	1.628	0.099	1.275	1.02	1.133	0.635
Feretia aeruginescens Stapf	0.174	0.747	0.372	0.497	0.278	0.242	1.038	0.517	0.69	0.387
Hymenodictyon parvifolium Oliv	0.145	0.833	0.749	0.789	3.919	0.037	0.213	0.191	0.201	0.159
Hyperacanthus sp. E. May. ex	0.181	0.289	0.582	0.386	0.386	0.168	0.269	0.542	0.359	0.358
Keetia gueinzii Sond.	0.063	0.161	0.134	0.161	1.615	0.142	0.364	0.303	0.364	3.641
Keetia sp. E. Phillips	0.167	2.848	0.474	0.813	0.539	0.158	2.705	0.45	0.772	0.51
Kraussia floribunda Harv.	0.402	0.859	1.724	1.147	1.144	0.103	0.221	0.444	0.295	0.293
Mussaenda arcuata Poir.	0.404	1.302	1.033	1.152	0.646	0.226	0.729	0.578	0.645	0.362
Oxyanthus speciosus DC.	0.383	4.914	6.552	5.616	4.91	0.203	2.604	3.472	2.976	2.603
Pavetta lanceolata Eckl.	0.277	2.368	2.841	2.583	1.776	0.097	0.831	0.997	0.907	0.622
Pavetta schumanniana F.Hoffm. ex K.Schum.	0.135	0.435	0.864	0.578	0.435	0.135	0.435	0.865	0.579	0.435
Psychotria capensis (Eckl.) Vatke	0.016	0.269	0.134	0.179	0.103	0.035	0.594	0.297	0.396	0.224
Psychotria zombamontana (Kuntze) E.M.A.Petit	0.16	4.106	2.053	2.737	4.103	0.032	0.825	2.425	1.819	0.821
Vangueria infausta Burch.	0.763	3.935	3.319	3.935	1.221	0.074	0.383	0.323	0.383	0.118

Ms = Mycobacterium smegmatis, *Ma = Mycobacterium aurum*, *Mb* BCG = *Mycobacterium bovis* BCG, *Mt = Mycobacterium tuberculosis*

6.55 and 5.62 respectively against *M. smegmatis, M. aurum* and *M. bovis* BCG). *Oxyanthus speciosus* also had the highest SI values of 2.60, 3.47 and 2.97 against *M. smegmatis, M. aurum* and *M. bovis* BCG for Vero kidney cells.

DISCUSSION

The acetone extract of *Oxyanthus speciosus* contained the highest number of chemical constituents active against the tested mycobacteria based on the bioautography results while *Vangueria infausta* showed the least. There appears to be a common compound close to the solvent front producing a zone of inhibition seen in the chromatogram of all the crude extracts and based on the Rf value it can be concluded that there may be a non-polar antimycobacterial compound common to all the tested extracts. *Psychotria zombamontana* had the lowest MIC value but showed only two antimycobacterial compounds in bioautography. It is possible that more antimycobacterial compounds could be present in this active extract, but these may not be stable when separated by TLC. *Oxyanthus speciosus* had the highest number of inhibition bands in all the solvent systems, together with low MIC values. Most of these bands were not visible in the reference TLC plate so they most likely did not react with the vanillin-sulphuric acid spray reagent. Alternatively they may have been present in quantities too low to be seen in the reference plate but were still able to inhibit the growth of the *M. smegmatis*.

Of the extracts screened from the Rubiaceae family, seven of them had good antimycobacterial activity against the four mycobacterial test species. Crude extracts with an MIC less than 0.1 mg/mL are usually considered as significantly active (Eloff, 1998b)

and therefore could be regarded as promising candidates for further studies. If the MIC value is > 0.1 to 0.625 mg/mL it is considered moderate, and weak if MIC is > 0.625 mg/mL (Sánchez and Kouznetsov, 2010). Based on these criteria, the acetone extracts of *Psychotria zombamontana*, *Psychotria capensis*, *Keetia* sp., *Oxyanthus speciosus*, *Cephalanthus natalensis*, *Cremaspora triflora* and *Mussaenda arcuata* had good activity against *M. smegmatis, M. aurum* and *M. tuberculosis*. The methanol extracts of *Cephalanthus natalensis* and *Vangueria infausta* have been reported to have good antimalarial activity (Clarkson *et al.,* 2004) but the acetone extracts of *Vangueria infausta* had only moderate antimycobacterial activity in this study with MIC values ranging from 0.23 to 0.63 mg/mL. The extracts of *Cephalanthus natalensis* and *Keetia* sp. had good activity against *M. tuberculosis* with moderate activity against the non-pathogenic mycobacteria. Methanol extracts of *Hymenodictyon parvifolium* have previously been reported to have good antibacterial activity against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) bacteria (Choudhury *et al*., 2012). The results obtained from Pearson's correlation (Table 2) indicated that *M. aurum* was a much better predictor of activity against pathogenic *M. tuberculosis* than *M. smegmatis.* This supports the findings of Chung *et al*. (1995) who reported that the inhibition of growth of *M. aurum* is highly predictive of activity against *M. tuberculosis. M. bovis* BCG also showed a strong correlation with activity against *M. tuberculosis*, supporting the use of this species as a model organism for antimycobacterial activity studies of plant extracts. These correlations may likely vary with different plant extracts and classes of compounds.

Total activity values indicate which species could be the best source of an extract for use by poor communities or for organic production (Eloff, 2000). The crude extract of *Psychotria zombamontana* had the best MIC values and the highest % yield thereby resulting in the highest total activity of 1 077 mL/g. However, *Oxyanthus speciosus* proved to be the best candidate for further study owing to its favourable cytotoxicity profile and the varying polarities of antimycobacterial compounds present in the extract. The other extracts with MIC values < 0.1 mg/ml are also worthy of further investigation but priority should be given to those with low cytotoxicity.

Two different cell lines, C3A liver and Vero kidney, were chosen to be representative of organs targeted in general toxicity, and were expected to have different metabolic activities and uptake capabilities. Hepatic cell lines have metabolic enzymes and also generally show increased sensitivity compared to other cell types (Froscio *et al.*, 2009). Extracts showing sensitivity to cell lines with LC_{50} values > 0.1 mg/mL are considered not cytotoxic (Kuete, 2010). The C3A liver cells were in this study less susceptible than the Vero cells to the tested extracts. Thirteen of the extracts had LC_{50} values > 0.1 mg/mL against C3A cells while eight extracts had LC_{50} values > 0.1 mg/mL against Vero cells. The extracts of *Psychotria capensis* had good antimycobacterial activity but were cytotoxic to both cell lines, meaning that its activity could be due to general metabolic toxins. Plant extracts with SI values less than 1 imply that the extracts are less toxic to bacteria than to the mammalian cells (Eloff, 2000). Therefore, crude extracts with higher SI values are considered relatively safer when used *in vivo* because the extract is more toxic to the pathogen than to the mammalian cells. It should be kept in mind that *in vivo* efficacy and toxicity of extracts or

active compounds upon administration to animals or humans may differ substantially from their *in vitro* properties owing to pharmacokinetic and pharmacodynamic considerations. In this study, the extract of *Oxyanthus speciosus* had the highest SI value against the four mycobacterial strains tested and had the second highest total activity.

CONCLUSION

This is the first report of the antimycobacterial activity of the selected plant extracts. The fast-growing *Mycobacterium* species revealed similar patterns in susceptibility to the extracts. The activity against *M. aurum*, followed closely by activity against *M. bovis* BCG, correlated the best with the activity against *M. tuberculosis* in this study, while activity against *M. smegmatis* did not correlate as well. The extracts of *Oxyanthus speciosus* may contain compounds which can act as leads for the development of new anti-TB drugs. Further work is continuing on isolation and identification of antimycobacterial compounds, particularly from *Oxyanthus speciosus*, and evaluation of their effects in combination with current anti-TB drugs.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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