Inhibition of mycothione disulphide reductase and mycobacterial biofilm by selected South African plants

Oosthuizen Carel B. [a], Gasa Nomasomi [a], Hamilton Chris J. [b] and Lall Namrita [a*]

[a] *Department of Plant and Soil Sciences, Faculty of Natural and Agricultural Sciences, University of Pretoria, Lynwood, Hatfield, Pretoria, South Africa, 0002*

b School of Pharmacy, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, U.K.

[*] Author for correspondence:
Email: namrita.lall@up.ac.za
Tel: + 27 12 420 2425

Highlights

- Antimycobacterial activity of plants traditionally used for the treatment of tuberculosis and its symptoms.
- Biofilm inhibition of *Mycobacterium* sp. and the selectivity towards biofilm inhibition by *Eucomis vandermwerwei* Verd. and *Faurea saligna* Harv.
- Inhibition of the bacterial mycothiol disulphide reductase by *Faurea saligna* and its selectivity towards the bacterial analogue vs the human analogue.

ABSTRACT

The causative agent of tuberculosis is the bacterium *Mycobacterium tuberculosis*. It is the second leading cause of death worldwide. The development of resistance associated with this bacterium is of great concern, and the ability of the bacteria to form protective biofilms and persist for longer periods of time needs to be considered as a possible target. Plants have been considered as an additive to current treatments as an adjuvant, to aid the body in fighting the disease and as a source for new drug candidates to aid in the development of resistance of mycobacteria to available drugs. Twenty plants were selected based on their traditional usage against tuberculosis and related symptoms. The antimycobacterial activity of the ethanolic extracts from different genera and variable plant parts were evaluated using the microplate alamarBlue assay. A biofilm formation inhibition assay was adapted and used to determine the inhibitory activity against *Mycobacterium smegmatis* biofilm formation. The cytotoxic effect of the plants was determined on U937 human macrophage cells using the XTT (2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) cell proliferation kit. A DTNB-coupled Glutathione/Mycothiol disulfide reductase assay was used to determine the inhibitory activity against the human and bacterial reductases. Of the twenty plant extracts tested, it was found that *Salvia africana-lutea, Ficus sur* and *Sphedamnocarpus pruriens* showed moderate antimycobacterial activity with minimum inhibitory concentration (MIC) values of 31.25, 62.5 and 62.5 µg/ml respectively. *S. pruriens* (BF SI 1.00) and *S. africana-lutea* (BF SI 0.33) showed potential biofilm formation inhibition at concentrations, 62.2 and 95.8 µg/ml respectively when compared to Ciprofloxacin (BF SI 0.32), which inhibited at a concentration of 1.9 µg/ml. Most of the plant extracts showed low to moderate cellular toxicity except *Withania somnifera* that showed a higher toxicity with an IC_{50} of 6.8 µg/ml (Actinomycin D 0.0009 µg/ml). *F. sur* showed the highest selectivity index of 3.36. During mechanistic studies, *S. africana-lutea* showed inhibitory activity against Mycothiol disulfide reductase (Mtr) exhibiting an inhibitory concentration (IC_{50}) of 102.3 µg/ml and showed lower inhibitory activity against Glutathione reductase (Gtr) with an IC_{50} of 224.1 µg/ml. This study indicates that South African plant extracts traditionally used to treat tuberculosis-related symptoms have reasonable antimycobacterial, antibiofilm and enzyme inhibitory activity with relatively low toxicity.

Keywords: Antibiofilm, Antimycobacterial, Medicinal plants, Mycothiol disulfide reductase, *Mycobacterium tuberculosis*

1. Introduction

Tuberculosis (TB) is an infectious disease, mostly affecting the lungs or abdominal area. The causative agent of TB is the bacterium *Mycobacterium tuberculosis*, which is the second largest killer worldwide. In 2016, 10.4 million people were infected and 1.7 million died from the disease (WHO, 2017). Standard anti-TB drugs have been used for decades, which have led to widespread drug tolerance and resistance. In some cases, severe drug resistance can occur. Another issue arising from this disease is the persistence of a small subset of tolerant and resistant bacteria following treatment. The mechanism of persistence is poorly understood, but it is suggested that an *in vivo* biofilm might contribute to this pervasive mechanism of the bacteria (Richards and Ojha, 2014; WHO, 2017).

In nature *Mycobacteria* spp. are known to form aggregated complex microcolonies encased in an extracellular matrix, called a biofilm. It has been shown that biofilms increase drug tolerance to a subset of bacteria within the heterogeneous biofilms (Kulka et al., 2012). This intrinsic mechanism of survival of the bacteria can explain the persistence in treating TB (Islam et al., 2012). Although the precise mechanism of biofilm formation is not fully understood, it should at least be considered as a possible target. In a study done by Islam and colleagues (2012) some genes have been identified that are upregulated during biofilm formation (Islam et al., 2012). One enzyme mycothiol disulfide reductase, an oxidoreductase, is upregulated and it also supports the drug tolerance of the bacteria to isoniazid and ethionamide (Vilchèze et al., 2011).

Traditional medicine has been used for centuries to treat TB related symptoms such as chest pains, cough, fever and night sweats. The ethnobotanical approach has been found beneficial as the plants have been used for various ailments for generations. This could lead to obtaining medicinal plants and natural products with beneficial use (Nielsen et al., 2012). According to Mahomoodally, (2013), traditional African medicine is one of the oldest medical systems and it is regarded as one of the best sources of new potential leads as drugs. Due to the diverse tropical, subtropical and desert climates, African plants are subjected to a number of different ecophysiological factors that enhances the secondary metabolite profile. The severe environment causes plants, grown in Africa, to accumulate more chemopreventative agents when compared to their northern hemisphere counterparts. This was demonstrated by the higher biological activity seen in the African species *Dorstenia mannii* Hook.f., when compared to the related European species (Abegaz et al., 2004).

Many South African plants have been studied for antimycobacterial activity against *M. tuberculosis* in a number of inspiring publications. These included, *Berchemia discolour* (Klotzsch) Hemsl., *Bridelia micrantha* (Hochst.) Baill., *Croton pseudopulchellus* Pax, *Cryptocarya latifolia* Sond., *Dodonaea angustifolia* L.f., *Ekebergia capensis* Sparrm., *Euclea natalensis* A.DC, *Flueggea virosa* (Roxb. ex Willd.) Royle, *Galenia Africana* L., *Helichrysum melanacme* DC., *Nidorella anomala* Steetz, *Polygala myrtifolia* L., *Rapanea melanophloeos* (L.) Mez., *Terminalia sericea* Burch. Ex DC., *Thymus vulgaris* L. and *Ziziphus mucronata* Willd., to name but a few. These plants exhibited minimum inhibitory concentrations (MIC) less than 200 µg/ml (Dzoyem et al., 2016; Eldeen and Van Staden, 2007; Green et al., 2010; Lall et al., 2016; Lall and Meyer, 1999).

The present study indicates the selectivity of South African medicinal plants, selected based on traditional usage for tuberculosis and TB related symptoms, by comparing their antimycobacterial, biofilm inhibitory and cytotoxic activities. Followed by describing the mode of action by investigating the inhibition of mycothiol disulfide reductase, using glutathione reductase, the human analogue, as the reference.

2. Material and methods

2.1. Plant collection and identification

The fresh leaves of the selected plant species were collected from the Manie van der Schijff Botanical Gardens at the University of Pretoria. The plant material was dried at room temperature for 10 days. Subsequent to drying, the plant materials were ground into a homogeneous powder of 2.0 mm particle size. Identification and authentication were done at the H.G.W.J. Schweickerdt Herbarium, University of Pretoria. Herbarium voucher specimen numbers (PRU voucher numbers) are provided in Table 1.

2.2. Preparation of crude extracts for biological assays

Ground leaf powder was extracted in rectified ethanol (96%)(1:10 W/V) and shaken for 48 hours at 170 rpm using a Labcon 3086U shaker. The mixture was filtered and dried using rotary evaporation (Buchi R-200) in order to obtain a crude dried plant extract. The yield was calculated and the plant extracts were stored in glass polytops at 4ºC (Lall et al., 2016). Ethanol was selected as the extractant as a medium polarity solvent, to increase the yield and quality of the extract. Additionally, ethanol is a class 3 solvent, implying that residual solvent will have the lowest toxic effect compared to other organic solvents (Eloff, 1998).

Table 1
The medicinal usage and yield of selected plant species used in the present study

Plant extract	Family name	Plant part	PRU voucher number	Common names	Medicinal uses	Ref	Yield (%)
Alectra sessiliflora (Vahl) Kuntze	Orobanchaceae	Roots	BC 52	Yellow witchweed	Diarrhoea	(Burkill et al., 1985)	2.4
Aloe plicatilis (L.) Mill.	Asphodelaceae	Leaves	119553	Fan Aloe	Laxative	(Grace et al., 2008)	3.5
Cassinopsis ilicifolia (Hochst.) Sleumer	Icacinaceae	Leaves and stems	119552	Lemon thorn	Stomach ailments	(Okem et al., 2012)	6.0
Dracaena aletriformis (Haw.) Bos	Dracaenaceae	Leaves	119554	Dragon tree	Chest pains	(Viljoen, 2003)	4.2
Dracaena draco (L.) L.	Dracaenaceae	Leaves	119555	Dragon tree	Fever, respiratory ailments	(Gupta et al., 2008)	1.9
Eucomis autumnalis (Mill.) Chitt.	Hyacinthaceae	Leaves and flowers	119557	Pineapple flower	Backache, stomach ache, fever, coughs and respiratory ailments	(van Wyk et al., 1997)	2.1
Eucomis humilis Baker	Hyacinthaceae	Leaves	119558	Dwarf pineapple flower	Anti-inflammatory	(Du Plessis et al., 1989)	2.1
Eucomis vandermerwei Verd.	Hyacinthaceae	Leaves	119560	Spotted-leaf eucomis	Anti-inflammatory	(Duncan, 2012)	2.0
Euphorbia tirucalli L.	Euphorbiaceae	Stems	119561	Rubber-hedge euphorbia	Cough	(Gupta et al., 2013)	3.4
Faurea saligna Harv.	Proteaceae	Leaves	BC 37	Willow beechwood	Diarrhoea	(Hutchings et al., 1996)	36.3
Ficus sur Forssk.	Moraceae	Fruits, leaves and stems	119566	Broom cluster fig	Tuberculosis, influenza	(Watt and Breyer-Brandwijk, 1962)	3.5
Ficus sycomorus L.	Moraceae	Fruits, leaves and stems	119570	Sycamore fig	Cough and chest inflammation	(Lansky et al., 2008)	3.2
Leonotis leonurus (L.) R.Br.	Lamiaceae	Leaves and stems	119569	Wild dagga	Fever, headache and cough	(Turner et al., 2001)	10.3
Merwilla plumbea (Lindl.) Speta	Hyacinthaceae	Leaves	119563	Wild squill	Chest pains and lung infection	(Notten, 2001)	12.1
Salvia africana-lutea L.	Lamiaceae	Leaves and stems	119562	Beach salvia	Cough, colds and bronchitis	(Viljoen and Notten, 2002)	3.1
Sphedamnocarpus pruriens (A.Juss) Szyszyl	Malpighiaceae	Seeds and roots	BC 57	Canary nettle	Snake bites	(Watt and Breyer-Brandwijk, 1962)	8.8
Tarchonanthus camphoratus L.	Asteraceae	Leaves and stems	119564	Camphor bush	Headache, cough, abdominal pain and bronchitis	(Letsela and Hankey, 2002)	5.2
Typha capensis (Rohrb.) N.E.Br.	Typhaceae	Leaves and roots	119565	Bulrush	Diarrhoea	(Voigt, 2007)	2.6
Typha minima Funck	Typhaceae	Leaves and roots	119567	Dwarf bulrush	Anticoagulant	(Bown, 1995)	2.9
Withania somnifera (L.) Dunal	Solanaceae	Leaves and stems	119568	Winter cherry	Fever and anti-inflammatory	(Welman, 2011)	7.4

Antimycobacterial screening using Microtitre AlamarBlue Assay (MABA)

The MABA assay as described by Franzblau et al., (1998) with slight modifications was used to assess the antimycobacterial activity of the plant extracts (Lall et al., 2013). Briefly, to the outer wells, 200 µl of sterile distilled water was added to compensate for evaporation. *M. tuberculosis*, H37Rv (ATCC 27264) was maintained on Löwenstein-Jensen medium and allowed to grow for 3–4 weeks at 37°C. A single bacterial colony was transferred into fresh 7H9 media supplemented with 10% OADC and 2% PANTA and incubated for another 3 weeks. The samples were tested at six concentrations (31.25 – 1000 µg/ml). Isoniazid and rifampicin, at a concentration ranging from 0.07 to 2.50 µg/ml were used as the positive controls. Control wells without the tested plant extracts and a solvent control, DMSO at a final concentration of 2.5%, were included in the assays. One hundred microliters of the *M. tuberculosis* inoculum ($1.5x10^6$ CFU/ml) were added to all the test wells to produce a final assay volume of 200 µl. The plates were incubated at 37°C for 5-7 days. After incubation, 20 µl of alamarBlue was added to all the wells. The MIC was defined as the lowest concentration where no colour change from blue to pink could be observed. The extracts were tested in triplicates in three independent assays.

2.3. Biofilm formation inhibition assay

One hundred microliters of a $1.5x10^6$ CFU/ml *Mycobacterium smegmatis* (MC^2 155) inoculum were added into the wells of a polystyrene 96-well microplate (7H9 broth media supplemented with 2% glycerol omitting Tween80). Serially diluted samples (100 µl) ranging from 7.8 to 1000 µg/ml were added to yield a final assay volume of 200 µl. Ciprofloxacin (10 µg/ml) was used as the positive control. Control wells without plant extracts as well as a solvent control (DMSO, 2.5%) was added. The plates were incubated at 30ºC for 4 days under stationary conditions which induce biofilm formation. (Cady et al., 2012; Ishida et al., 2011). The biofilm inhibition activity of the extracts was quantitatively analysed by utilising 0.01% crystal violet, to determine the 50% biofilm inhibition effective concentrations (EC_{50}). Briefly, after the visual analysis, the plates were dried in a desiccating oven to remove all the liquid media. The biofilms were fixed to the surface of the plates by the addition of 200 ul of methanol, followed by three washes with sterile distilled water to remove planktonic cells and any broth residue. The washed plates were left to air dry and 200 µl of a 0.01% crystal violet solution was added to each well as a biofilm biomass indicator. The plates were left at room temperature on an orbital shaker (170 rpm) for 15 min followed by three wash steps as mentioned above. The bound crystal violet was extracted using 96% ethanol and transferred

to a new microtitre plate. The amount of crystal violet was quantified by measuring the absorbance at OD600 nm using a Biotek ELISA plate reader. GraphPad Prism 4.0 software was used to calculate the EC_{50}.

2.4. XTT Cytotoxicity Assay

The cytotoxic effect of the extracts was investigated on U937 monocytes by using the XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) based colorimetric assay Cell Proliferation Kit II (Boehringer-Mannheim). The cells were cultured and maintained in RPMI (10% FBS) media until 90% confluency was reached. The cells were plated on 96-well microplates at a density of $1x10^6$ cells/ml and left for 24 hours to attach. The extracts were tested at a concentration range of 3.12-400 µg/ml for 72 hours. Actinomycin D was used as the positive control. The inhibitory concentration where 50% of the cells were inhibited, were calculated using Graph Pad Prism 4 programme (Seebaluck-Sandoram et al., 2018).

2.5. Glutathione and Mycothiol disulfide reductase DTNB-coupled inhibition assay

The experimental procedure as described by Hamilton et al., (2009) with slight modifications was used. Briefly, enzyme inhibition assays with glutathione reductase (Gtr) and mycothiol disulfide reductase (Mtr) was carried out. The assay containing 50 mM Hepes (pH 7.6), 0.1 mM EDTA, 140 uM NADPH, 100 uM Elman's reagent (DTNB) Gtr or Mtr, GSSG or MSSM (5 uM) and varying concentrations of the plant extract (15.62-1000 ug/ml) were carried out at 30°C in a 96-well plate format with a final assay volume of 200 µl. The extracts were dissolved in DMSO, and the DMSO concentration was kept constant (5% (v/v)). The enzyme, NADPH, DTNB and the extracts were pre-incubated at 30°C for 10-15 min, and the reaction was initiated by the addition of the substrate. Relative rates were measured by the increase in absorbance at 405 nm, due to the formation of TNB (5′-mercapto-2-nitrobenzoic acid) via the continuous DTNB-coupled assay, for 30 min and a linear regression was used to calculate the initial rates. KC junior™ was used for data capturing and GraphPad Prism 4 for analysis. Inhibitory concentrations, where 50% of the enzyme activity was inhibited, were determined by fitting a 4-parameter nonlinear regression.

2.6. Statistical analysis

All results are presented as the mean of three independent triplicate experiments. Differences between test extracts in these experiments were assessed for significance using analysis of variance (one-way ANOVA).

3. Results and discussion

3.1. Antimycobacterial activity

Only 7 extracts showed significant activity when compared to the rest against *M. tuberculosis* (Table 2). The positive controls, Isoniazid and Rifampicin both showed an MIC of 0.15 µg/ml. *Salvia africana-lutea* exhibited the highest MIC of 31.25 µg/ml. The other plant extracts that were also found to be effective against *M. tuberculosis* were *Ficus sur* and *Sphedamnocarpus pruriens* both with a MIC of 62.5 µg/ml. This was followed by *Electra sessiliflora*, *Euphorbia tirucalli*, *Leonotis leonurus* and *Withania somnifera* showing an MIC of 125 µg/ml. The extracted secondary metabolites in the above-mentioned extracts contained specific bioactive phytochemicals or a combination of compounds that have been reported earlier to have bacteriostatic and/or bactericidal activities. In a study by Madikizela et al., 2014, the ethanolic root extract of *Ficus sur,* traditionally used to treat pulmonary tuberculosis, showed an antimycobacterial activity with an MIC of 780 µg/ml which is >10 times less active than the ethanolic leaf extract that was observed in the current study (Lawal et al., 2014; Madikizela et al., 2014; McGaw et al., 2008). *F. sur* showed the highest selectivity towards antimycobacterial activity when compared to the cellular toxicity, with a selective index (SI) of 3.36. It was also shown that the ethanolic leaf extract of *F. sur* showed a MIC of 3120 µg/ml in another study (Eldeen et al., 2005). Many reasons can lead to the differences observed in the activity of this plant extract. In the present study, the leaves and shoots were utilised in the extract preparation, whereas in the study by Madikizela et al., 2014, a root extract of the plant was tested (Madikizela et al., 2014). The environmental conditions and seasonal variation might also explain the differences in activity observed when comparing these three values.

S. africana-lutea from the Lamiaceae family showed high activity with a MIC of 31.25µg/ml in the present study. In previous studies the antimycobacterial efficiency of the methanol: chloroform (1:1) and the acetone extract of *S. africana-lutea* was shown to have an MIC of 500 and 312.5 µg/ml, respectively (Kamatou et al., 2007; Nielsen et al., 2012). It has

Table 2
The antimycobacterial activity, cytotoxicity, selective index and inhibition on mycothiol disulfide- and glutathione reductases of plant extracts.

Medicinal Plants	Biological activity (µg/ml)					Enzymology (µg/ml)	
	MIC[a]	U937 IC_{50}[b]	SI[c]	Biofilm EC_{50}[d]	BF SI[e]	Gtr IC_{50}[f]	Mtr IC_{50}[g]
Alectra sessiliflora	125	110.2±7.8	0.88	221.7±23	0.56	325.1±37.4	167.8±29.4
Aloe plicatilis	1000	38.3±6.8	0.04	599.5±41	1.67	na[h]	993.4±12.4
Cassinopsis ilicifolia	500	141±4.0	0.28	317.6±23	1.57	551.1±11.4	na
Dracaena aletriformis	500	90.7±8.3	0.18	338.9±22	1.48	na	na
Dracaena draco	500	20.8±10.3	0.04	343.3±16	1.46	na	na
Eucomis autumnalis ssp. clavata	1000	68.5±8.7	0.07	Na	-	na	na
Eucomis humilis	1000	57.2±8.2	0.06	Na	-	na	320.6±27.2
Eucomis vandermerwei	1000	51.4±6.5	0.05	266.7±14	3.75	na	134.7±24.4
Euphorbia tirrucalli	125	14.8±5.8	0.12	280.6±39	0.45	157.3±16.9	239.1±31.8
Faurea saligna	>1000	202.4±6.6	0.20	276.4±22	3.62	>1000	42.0±10.3
Ficus sur	62.5	210.2±12.0	3.36	470.7±18	0.13	44.5±6.0	na
Ficus sycomorus	1000	30.4±2.1	0.03	394.5±45	2.53	765.4±8.2	na
Leonotis leonurus	125	40.0±9.1	0.32	50.2±10	2.49	na	na
Merwillia plumbea	500	22.5±7.6	0.05	353.2±40	1.42	382.3±9.0	236.1±22.6
Salvia africana-lutea	31.25	83.9±9.1	2.68	95.8±21	0.33	224.1±8.9	102.3±15.0
Sphedamnocarpus pruriens	62.5	125.9±7.6	2.01	62.2±12	1.00	54.3±10.5	61.5±11.3
Tarchonanthus camphorates	500	25.2±6.4	0.05	548.3±31	0.91	na	132.9±24.3
Typha capensis	1000	24.5±5.8	0.02	422.7±21	2.37	65.5±9.4	53.8±16.4
Typha minima	500	198.2±8.2	0.40	504.5±37	0.99	188.4±33.2	47.9±24.6
Withania somnifera	125	6.8±4.3	0.05	212.2±18	0.59	221.2±19.1	162.4±21.8
Isoniazid[i]	0.156	-	-	-	-	-	-
Rifampicin	0.156	-	-	-	-	-	-
Ciprofloxacin	0.625	-	-	1.9±0.08	0.32	-	-
Actinomycin D[j]	-	0.009±0.0001	-	-	-	-	-

[a] Minimum inhibitory concentration on *M. tuberculosis* H37Rv, [b] Inhibition concentration where 50% of the U937 cell growth was inhibited, [c] Selective index [SI=IC_{50}(U937)/MIC] – Indication of plant extract selectivity towards antimycobacterial activity, [d] Effective concentration where 50% biofilm formation was inhibited, [e] Selective index [SI=MIC/BF IC_{50}] – Indication of plant extract selectivity towards biofilm inhibition, [f] Glutathione reductase IC_{50}, [g] Mycothiol disulfide reductase IC_{50}, [h] Non active (IC_{50}>1000 µg/ml) , [i] Positive control for antimycobacterial assay, [j] Positive control for cytotoxicity assay

been reported by Seaman, (2006) that *S. africana-lutea* methanol plant extract has antimycobacterial activity with an MIC of 1000 µg/ml using the BACTEC 460 method. In a paper by Hussein et al., (2007) three diterpenoid compounds namely, rosmadial, carnosol, and carnosic acid were isolated from the ethanolic extract of *S. africana-lutea* and evaluated for their antimycobacterial activity. The latter two compounds showed activity at 157 and 28 µM, which might explain the activity observed. The present study indicates the significant antimycobacterial activity as compared to the previously reported result. This could be due to ethanol being used as the extractant, potentially contributing to the high concentration of diterpenoids in the extract. No literature could be found to describe the antimycobacterial activity observed on *S. pruriens*.

A. sessiliflora is one of the medicinal plants used for tuberculosis management in Nigeria (Ogbole and Ajaiyeoba, 2010). The MIC of this plant in the present study was found to be 125 µg/ml, which indicates a strong relationship between traditional usage and scientific evaluation. This is the first indication of antimycobacterial activity of *A. sessiliflora*. The plant exhibited antibacterial activity in previous studies against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella dysenteriae* and *Bacillus pumilus* at an MIC between 3.13 and 25 mg/ml. In a study by Mariita et al., (2010), the antimycobacterial activity of *E. tirucalli* was reported to have an MIC of 2 mg/ml using the BACTEC MGIT 960 system. This activity could be attributed due to its high tannin, flavonoid and terpenoid content, especially diterpenoids and triterpenoids found in the Euphorbiaceae family (Oliveira et al., 2014; Seebaluck-Sandoram et al., 2018; Zengin et al., 2017).

Another plant from the Lamiaceae family; *Leonotis leonurus* L. is traditionally used for coughs, influenza and respiratory ailments (Makunga et al., 2008). Biochemical analysis of the plant extract indicated potential diterpenoid bioactive compounds, which could contribute to the antimycobacterial activity observed in this study (Naidoo et al., 2011). *Withania somnifera* L. Dunal (Solanaceae) aqueous extract showed antimycobacterial activity exhibiting an MIC of 1000 µg/ml (Adaikkappan et al., 2012; Gautam et al., 2004). The ethanol extract in this study showed an MIC of 125 µg/ml, which is significantly lower as compared to the previously reported MIC. The difference in the use of solvents could possibly extricate different compounds based on the polarity and can indicate the contrast in activity.

3.2. Biofilm formation inhibition of M. smegmatis

Biofilm formation inhibition assays were conducted to determine the effect of the plant extracts on biofilm formation against *Mycobacterium smegmatis* MC^2 155. The results were analysed by using the crystal violet method. *M. smegmatis* was selected as it has the fastest biofilm formation characteristic as compared to other *Mycobacterium* species (Bonkat et al., 2012). The values in Table 2 depicts the effective concentration of the plant extracts at which 50% biofilm formation is inhibited (EC_{50}).

The following plants; *L. leonurus, S. pruriens* (Fig 1.) and *S. africana-lutea* indicated significant effect against biofilm formation with an EC_{50} of 50.2, 62.2 and 95.8 µg/ml, respectively. Moderate inhibition was observed by *A. sessiliflora*, *E. vandermerwei* and *W. somnifera* with 50% effective doses of 221.7, 266.7 and 212.2 µg/ml, respectively. Ciprofloxacin (positive control) displayed a biofilm inhibitory activity (EC_{50}) of 1.98 µg/ml, which is higher than the MIC. This indicates the selectivity (SI = 0.32) of ciprofloxacin for antibacterial activity over biofilm inhibition activity. Although *L. leonurus* showed the highest EC_{50}, the plant with the highest selectivity towards biofilm inhibition as compared to antimycobacterial growth inhibition is *E. vandermerwei* with a selectivity of 3.75. This is the first report of aforementioned plants for mycobacterial biofilm inhibition activity.

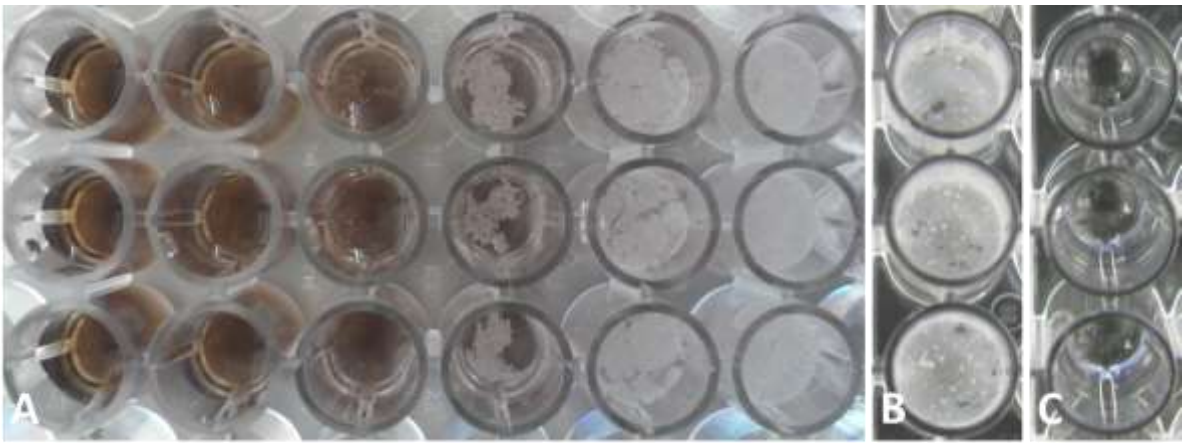

Fig 1. I*n vitro* biofilm formation inhibition by *S. pruriens* (A)(concentrations ranging from 1000 (left) to 31.25 (right) µg/ml), 100% biofilm formation (B), media control (C).

The potential antibiofilm activity of the plant extracts could be due to their chemical constituents. *S. africana-lutea* has been reported to have phenolic compounds, which have the ability to complex with bacterial cell walls and disrupt microbial membranes by inhibiting matrix formation (Kamatou et al., 2007). *L. leonurus* and *S. africana-lutea* are from the

Lamiaceae family which to a certain extent might have similar chemical compositions, therefore, supporting the potential of *L. leonurus* for disrupting microbial membranes. *W. somnifera* has been reported to contain bioactive alkaloids; which have antimicrobial activity (Mishra et al., 2000; Singh and Kumar, 2011). *S. pruriens* (Malpighiaceae) phytochemical composition has not been reported in the literature. Chenthurpandy et al., (2009), reported on the phytochemical composition of chloroform and methanol extracts of *Hiptage benghalensis* from the Malpighiaceae family (Chenthurpandy et al., 2009). Both the extracts showed the presence of flavonoids, which have been reported to bind to and inhibit matrix formation (Abidi et al., 2014). Since *S. pruriens* is from the same family as *H. benghalensis* there might be similarities in their phytochemical composition. Further phytochemical analysis of these plants has to be conducted to identify the active constituents.

3.3. Cytotoxicity on U937 cell line and selectivity

Withania somnifera indicated significant cellular toxicity with an IC_{50} of 6.81 µg/ml on the U937 cells (Table 2). This toxicity can be as a result of a steroid compound; 5, 6-de-epoxy-5-en-7-one-17-hydroxy withaferin A, which was isolated from aerial parts of *W. somnifera* in a previous study. The compound, withaferin A, was found to be very toxic when screened against two cancer cell lines; MCF-7 breast and WRL-68 liver lines with an IC_{50} of 1.0 µg/ml. Moderate toxicity was observed on prostate (PC-3) and Colon (Caco-2) cell lines with an IC_{50} of 7.4 µg/ml and 3.4 µg/ml respectively (Siddique et al., 2014). *Euphorbia tirrucali* has been reported to be toxic due to the latex it contains (Silva et al., 2007).

To further determine the efficacy of the plant extracts against *M. tuberculosis*, the selective index (SI) of each extract was calculated by dividing the MIC's with the 50% inhibition concentration on the cell line and is represented in Table 2.

The selective index value is used to distinguish toxicity that is due to plant extracts and toxicity in general (Adamu et al., 2014; Prince et al., 2012). *Ficus sur* showed the highest selectivity index of 3.363 in this study; which means it is three times more effective against *M .tuberculosis* as compared to human macrophage U937 cells. Plant extracts with high selectivity indexes and low or moderate toxicity; can be considered as good candidates for inclusion in drug discovery and development.

3.4. Inhibitory activities on Glutathione and Mycothiol disulfide reductase

The crude plant extracts were analysed for their inhibitory activity on Glutathione reductase (Gtr, Human analogue) and Mycothiol disulfide reductase (Mtr, *Mycobacterium* analogue) enzymes. Inhibition concentrations at which 50% of the enzymes' activity was inhibited (IC_{50}) were calculated. A plant extract with an IC_{50} greater than 1000 µg/ml was considered to have no activity against a particular enzyme. Ideally, plant extracts with a high affinity for Mtr and low affinity for Gtr would be considered to be good candidates. *Typha minima* had a higher affinity for Mtr as compared to Gtr, having an IC_{50} of 47.9 µg/ml and 188.4 µg/ml, on the two enzymes respectively (Table 2). The exact mechanism of Mtr inhibition is not known but can be attributed to the phytochemical makeup; consisting of phenols, alkaloids, tannins and flavonoids (Londonkar et al., 2013).

S. pruriens had a low but similar affinity for both enzymes, which could indicate similar effect in both humans and bacteria. Both *Eucomis* species had a higher affinity for Mtr and low affinity for Gtr. This can indicate to higher selectivity towards the bacterial analogue Mtr, which can result in increased levels of reactive oxygen species in bacterial cells as a result of the decline of mycothiol. In a previous study, a pure isolated flavone compound, 5, 7, 2`-trihydroxyflavone from *Galenia africana* was tested to determine if it can act on Mtr as a subversive substrate. The results showed that the compound failed to bind as a substrate on Mtr which indicates non-selectivity on Mtr as a target. Flavones could possibly target other flavoprotein oxidoreductases (Mativandlela et al., 2008). It has been reported that naphthoquinones which are widely distributed in plants have pharmacological properties including antibacterial, antiviral, anticancer, antimalarial and antifungal activity and can act on Mtr as a subversive substrate. A naphthoquinone; 7-methyljuglone (5-hydroxy-7-methyl-1, 4-naphthoquinone) was isolated from *Euclea natalensis* and has shown activity against *M. tuberculosis* and as subversive substrates on Mtr (Mahapatra et al., 2007). The inhibition mechanism manifests by inhibiting the normal reduction of the original substrate such as MSSM in mycobacteria, therefore interfering with the normal occurrence of the enzymatic reaction.

4. Conclusion

Sphedamnocarpus pruriens and *Salvia africana-lutea* showed good antimycobacterial and antibiofilm activity with *S. pruriens* being less toxic to U937 cells. *Typha minima* showed good mycothiol disulfide reductase inhibition as it had less affinity for Gtr and higher affinity

for Mtr. *S. pruriens* showed good overall activity when compared to all the plant extracts in this study. This plant is an ideal sample for further evaluation for its consideration as a possible adjuvant for TB-patients as it showed antimycobacterial activity with lower toxicity to human cells, inhibition of mycothiol disulfide reductase, and antibiofilm activity. In other words, the plant extract would be active against planktonic and biofilm forming cells, higher intracellular activity with regards to the inhibition of Mtr.

Conflict of interest

The authors declare that there is no conflict of interest.

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