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Anti-mycobacterial activity of five plant species used as traditional medicines in the Western Cape Province (South Africa)

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Abstract

Ethnopharmacological relevance: Five plants used in traditional medicine in the Western Cape Province of South Africa, have been investigated for anti-mycobacterial activity: *Olea capensis, Tulbaghia alliacea, Dittrichia graveolens, Leysera gnaphalodes and Buddleja saligna*.
Aim of the Study: The aim was to assess antimycobacterial activity in plants used in treatment of symptoms of TB, and through activity-guided fractionation of extracts to isolate compounds or mixtures with potential as anti-TB drug leads.
Materials and Methods: Extracts and derived fractions were assayed against strains of *Escherichia coli*, *Staphylococcus aureus*, and *Mycobacterium aurum* A+. Isolated pure compounds were further tested against *Mycobacterium* species *M. avium* ATCC 25291, *M. scrofulaceum* ATCC 19981, *M. microti* ATCC 19422 and *Mtb* H37Rv, and for cytotoxicity against Chinese hamster ovarian cells.
Results: Extracts of *B. saligna* and *L. gnaphaloides* exhibited significant anti-mycobacterial activity, primarily associated with the presence of non-cytotoxic triterpenoids oleanolic acid in *B. saligna* and both oleanolic and ursolic acids in *L. gnaphaloides*.
Conclusions: Anti-mycobacterial activity of extracts of selected plants is consistent with their traditional use. The identification of oleanolic and ursolic acids in these plants, and verification of their activity, underlines the potential for exploring structure-activity relationships of derivatives of these ubiquitous triterpenoids.
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Keywords: Anti-mycobacterial activity; Medicinal plants; Western Cape Province

1. Introduction

The tuberculosis (TB) pandemic was thought to have been eradicated in the early second half of the past century. However, during the last two decades this old disease has resurged with an alarming speed, both in developing and industrialized countries. Today, TB is the most common cause of adult mortality among infectious diseases, accounting for one third (Grange, 1999), killing more people than AIDS, malaria and other tropical diseases put together. This is equivalent to 26% of all preventable deaths (Zumla et al., 1999). The association of TB and HIV has worsened these trends and contributed to the increase in drug resistant (DR-TB) and multi-drug TB (MDR-TB). Between 1990 and 2003, TB incidence rates tripled in African countries with high HIV prevalence (Corbett et al., 2003). In the Western Cape Province of South Africa the TB incidence has reached alarming proportion of 589/100,000 populations, and a new MDR-TB rate of 0.94% (Schaaf et al., 2003). The current TB chemotherapy is just too long, and it has been identified as an important co-factor in the increasing rate of DR-TB and MDR-TB development. Thus, novel anti-TB drugs are urgently needed to help to control MDR *Mtb*.

Several approaches currently exist for the search for new anti-TB drugs including the screening of plant materials for bioactive compounds (Telenti and Iseman, 2000). Our choice for investigation of traditional plant materials was justified by the fact that South Africa is home to about 10% of higher plant species on the planet, yet less than 5% of these species have been sci-

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entifically assessed for pharmacological activity. Furthermore, it is estimated that 70% of all South Africans use traditional medicines derived from plant species indigenous to the region (Scott et al., 2004).

The objective of this study was to assess the anti-mycobacterial activity of plants with documented use in the treatment of symptoms associated with TB, and to isolate compounds which may account for this activity, and which may therefore be promising leads in the search for new anti-TB drugs. This led us to identification and collection of specimens of five medicinal plant species found in the Western Cape Province of South Africa, *Olea capensis* L. (Oleaceae), *Tulbaghia alliacea* L. (Alliaceae), *Dittrichia graveolens* L. (Astereacea), *Leyssera gnaphaloides* L. (Fabaceae), and *Buddleja saligna* L. (Buddlejacea). The selection of these plants was guided by reports in the literature of their use in traditional medicine to treat various ailments including bronchitis, cough, diarrhoea, fever, and even tuberculosis, which suggested the presence of antimicrobial constituents.

Olea capensis, also called bastard black ironwood, is a shrub that can reach the size of a medium tree of 15–20 m (Goldblatt and Manning, 2000). The plant has reportedly been used in traditional medicine to treat higher upper respiratory tract infections: an infusion of leaves is used as a tea to treat sore throat, back pain and kidney pains (Van Wyk et al., 1997; Anon, 1998). *Dittrichia graveolens* (L.) (Asteraceae), known as Kaapse Kakiebos in the Western Cape Province of South Africa, is an annual herb of about 1 m tall. It is believed that *Dittrichia graveolens* was originally from the Mediterranean region of Europe and was introduced in the Cape region, South Africa about three centuries ago (Bromilow, 1995).

Buddleja saligna, also called unGqeba (Xhosa, Western Cape Province, South Africa) is a typically southern African species. The plant is used for traditional medicinal purposes, the roots as a purgative and the leaves to treat coughs, colds and chest pain. Its leaves were reportedly used as soap (Van Wyk et al., 1997; Scott et al., 2004). There is no record of any phytochemical or biological research on *Buddleja saligna*.

2. Material and methods

2.1. General chemical and analytical methods

Gravity column chromatography was done on glass columns packed with Merck silica gel 60 (70–230 mesh). Column fractions were monitored by thin layer chromatography (TLC) on aluminum-backed Merck silica gel 60 F_{254} plates using an ascending technique. The plates were visualized by spraying with ceric ammonium sulphate in 8 M sulphuric acid or a 1:1 solution of 5% *p*-anisaldehyde in ethanol and 10% sulphuric acid in ethanol baking at 150 °C. Melting points were determined using a Reichert-Jung Thermovar hot-stage microscope and are uncorrected. Optical rotations were determined on a PerkinElmer 141 polarimeter in chloroform solutions unless otherwise stated. The concentration *c* refers to gram per 100 mL. Proton nuclear magnetic resonance (^1H NMR) spectra were recorded, unless otherwise specified, as $CDCl_3$ solutions using $(CH_3)_4Si$ as an internal standard on a Varian Mercury 300 MHz or a Varian Unity 400 MHz spectrometer. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded on the same instruments at 75 or 100 MHz using $(CH_3)_4Si$ as an internal standard. Chemical shifts are reported in ppm. Mass spectra were recorded on a VG70 SEQ micromass spectrometer.

2.2. Collection, preparation and extraction of plant material

Olea capensis (leaves), *Dittrichia graveolens* (entire plant), and *Buddleja saligna* (leaves and stems) were collected on 15 March 2001 from Kirstenbosch Garden in Cape Town by K. Bamuamba. *Tulbaghia alliacea* (rhizomes) were collected and identified by Dr. Gillian Scott, botanist at the Department of Botany, University of Cape Town, South Africa. *Leyssera gnaphaloides* (entire plant) was collected by K. Bamuamba and G. Scott in March 2001 from Blaauwberg Farm near Atlantis, about 50 km north-west of Cape Town. At the time the collection took place, the plants bore pinkish flowers, some fresh, some dying.

Olea capensis L. (Oleaceae) K. Bamuamba, sn. (BOL), *Tulbaghia alliacea* L. (Alliaceae) G. Scott sn. (BOL), *Dittrichia graveolens* L. (Astereacea) K. Bamuamba, sn. (BOL), *Leyssera gnaphaloides* L. (Fabaceae) K. Bamuamba, sn. (BOL), and *Buddleja saligna* L. (Buddeljacea) K. Bamuamba, sn. (BOL) as authenticated by Dr. Terry Trinder-Smith, botanist at the Bolus Herbarium, University of Cape Town.

Plant materials were air-dried at 37 °C and ground to a powder using a coffee grinder.

Two methods of extraction were used for each plant species:

(a) *Preparation of aqueous infusion*: dried powdered plant material (100 g) was infused with boiling distilled water (100 mL) in an Erlenmeyer flask and shaken for 10 min. The infusion was left to cool at room temperature for 4 h, then filtered through filter paper (Whatman no. 3) in a large funnel, and the resultant filtrate freeze-dried (Labotec Dry-o-vac freeze-drier).
(b) *Extraction with a mixture of acetone and water (4:1) at room temperature*: dried powdered plant material (500 g) was extracted at room temperature with a mixture of acetone and water (4:1 (v/v), 1000 mL) in a 2.5 L flask. The mixture was shaken for 15–20 min and allowed to stand overnight. The resultant supernatant was filtered through filter paper (Whatman no. 1), and the residue returned to the flask and extracted with a further two portions of acetone/water (4:1 (v/v), 1000 mL) for 6 h each. The combined acetone/water extracts were evaporated under vacuum at 30 °C to obtain a concentrated aqueous suspension, which was then transferred into a separating funnel and extracted sequentially (gently, to avoid emulsification) with hexane, chloroform, and ethyl acetate (3× 100 mL of each). This gave rise, after concentration under vacuum to remove solvents, to the acetone/water, hexane, chloroform and ethyl acetate fractions.

2.3. Activity-guided fractionation and isolation of active principles

Portions of the acetone/water, hexane, chloroform and ethyl acetate fractions were first bio-autographed against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Mycobacterium aurum* A+, the latter as a non-virulent indicator (Chung et al., 1995), before being tested against *Mtb*. The most active fractions (hexane extracts) from three plants (*Buddleja saligna*, *Leyssera gnaphaloides and Inula graveolens*) were concentrated under vacuum and fractionated further on a column of Merck silica gel 60 (70–230 mesh) suspended in *n*-hexane. In each case the column was initially washed with *n*-hexane (500 mL) to remove unwanted fatty substances, then eluted with hexane/ethyl acetate (5:1). The ratio of ethyl acetate was then sequentially increased by 2.5% every 500 mL. Fractions (25 mL) were collected and their composition monitored by TLC.

Fractions containing the same single compounds were then combined and concentrated under vacuum.

Chromatographic separation of the hexane extract of *Buddleja saligna* (2.7 (w/w%) of dried plant material) produced the bioactive fractions BS1, BS2, BS2, BS3, BS4, BS5 and BS6 corresponding to elution with 20–40% ethyl acetate in hexane (v/v). Fraction BS1 yielded a whitish amorphous powder (m.p. 280–282 °C), migrating as a single spot on TLC. The ^{1}H and ^{13}C NMR and EI- and FAB-MS spectra (EI MS and FAB), together with the m.p. (lit. (Seebacher et al., 2003) m.p. 282–284 °C) confirmed that BS1 was the hydroxylated pentacyclic triterpenoid, oleanolic acid. The other fractions BS2, BS3, BS4, BS5, and BS6 were found by TLC to be mixtures containing BS1 as the major component.

Chromatographic separation of the hexane extract of *Leyssera gnaphaloides* (17.8% (w/w%) of dried plant material) gave rise to fractions LG1, LG2, LG3 and LG4. Fraction LG1 yielded an off-white, amorphous powder (m.p. 279–280 °C). The HREIMS and ^{13}C NMR spectra of compound LG1 were identical to those reported for ursolic acid (Mahato and Kundu, 1994; Takeoka et al., 2000; Seebacher et al., 2003). The MS and NMR data of LG2 were identical to that of compound BS1, and thus identified as oleanolic acid. Fractions LG3 and LG4 were shown by TLC to be the mixtures of compounds which, from ^{1}H NMR were structurally related to LG1 but with R_f values so close that they could not be separated by chromatography.

2.4. Evaluation of antimicrobial activity

2.4.1. Cultivation of test bacteria

The test bacteria comprised *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Mycobacterium aurum* A+. The cultures were grown overnight in 2YT broth medium with shaking at 37 °C (Sambrook et al., 1989). The mycobacterial species, *Mtb* H37Rv, *Mycobacterium avium* 25291, *Mycobacterium microti* ATCC 19422, and *Mycobacterium scrofulaceum* ATCC 19981 were each grown at 37 °C as standing, 10 mL Middlebrook 7H9 cultures (supplemented with 0.5% bovine serum albumin and 2% glucose) with intermittent agitation. The assays with these mycobacteria were all carried out in the TB laboratory in the Institute of Infectious Diseases and Molecular Medicine, University of Cape Town under Biosafety level 3 conditions. The purity of the cultures was checked by means of the Ziehl-Neelsen stain before use in bioautography.

2.4.2. Evaluation of antimicrobial activity

The evaluation of the antimicrobial properties of the samples was carried out in two separate sets of experiment. Firstly, as part of the bioassay guided fractionation process, total crude extracts and fractions generated from these were tested against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Mycobacterium aurum* A+ using the MTT bioautography method. Secondly, the isolated bioactive compounds/mixtures were tested against non-tuberculous mycobacterium (NTM) species, *Mycobacterium avium* ATCC 25291, *Mycobacterium scrofulaceum* ATCC 19981, *Mycobacterium microti* ATCC 19422 and *Mtb* H37Rv using both the agar-plate disk diffusion method and bioautography.

2.4.2.1. Bioautography of isolated compounds based on MTT colorimetric assay (Mosman, 1983). 10 μl of sample solutions (sample dissolved in dichloromethane at different concentrations: 2 mg/ml; 1 mg/ml; 500 μg/ml; 250 μg/ml; 125 μg/ml; 62.5 μg/ml) were spotted in five replicates each on a TLC plate (silica gel 60 on aluminium sheet, Merck). Rifampicin (Sigma, catalogue no. R3501) was used as a positive control for anti-mycobacterial activity. The spotted plates were left to dry completely. A 3-week-old *Mtb* H37Rv suspension culture was then dabbed, using sterile adsorbent cotton wool, onto the spotted TLC plates The inoculated TLC plates were placed in sealed plastic boxes containing damp paper towel, which served to ensure sufficient humidity to prevent the plates drying out. The boxes were then incubated overnight at 37 °C, after which an aqueous MTT solution (0.25% in sterile water) was dabbed onto the plates using sterile cotton wool, as for the bacterial culture. The plates were incubated again at 37 °C overnight in sealed plastic boxes to allow the formazan blue colour to develop. A clear spot on blue background was indicative of the anti-*Mtb* activity of the compounds present in that spot.

2.4.2.2. The disk diffusion method. Sterile filter-paper disks (Whatman 3 mm; 6 mm diameter) were each impregnated with 10 μl of a sample solution (sample dissolved in dichloromethane at different concentrations: 2 mg/ml; 1 mg/ml; 500 μg/ml; 250 μg/ml; 125 μg/ml; 0.625 μg/ml). The impregnated-disks were left to completely dry. A 3-week-old *Mtb* H37Rv culture suspension (100 μl) was spread on the surface of Middlebrook 7H9 agar plates (containing 0.2% glucose) using a sterile glass spreader. After 5 min, the impregnated-disks were placed on the agar. The culture plates were sealed in plastic bags and incubated at 37 °C for 3 weeks, after which the culture-growth was clearly visible on the agar. A clear zone visible all around the impregnated-disk was indicative of the sample's inhibitory effects.

2.5. In vitro cytotoxicity testing

In vitro cytotoxicity assays were performed on Chinese hamster ovarian (CHO) cells using the MTT colorimetric assay (Mosman, 1983). Emetine dihydrochloride (Sigma, catalogue no. E 2375), made up in sterile water and serially diluted in the cell culture medium to the required concentrations was used as a positive control.

3. Results and discussion

Aqueous crude extracts of *Olea capensis* (leaves), *Tulbaghia alliacea* (tubercles), *Buddleja saligna*, *Leyssera gnaphaloides*, and *Inula graveolens* showed no activity (by bioautography) against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Mycobacterium aurum* A+. In contrast, the acetone/water (4:1) crude extracts of *Buddleja saligna*, *Leyssera gnaphaloides* and *Inula graveolens* exhibited significant activity against the test bacteria. These bioactive crude extracts were further each successively extracted with hexane, dichloromethane and ethyl acetate, with the hexane fractions exhibiting the highest antibacterial activity. After further chromatographic separation the hexane extract of *Buddleja saligna* yielded six bioactive fractions (BS1–BS6) while the corresponding hexane extract of *Leyssera gnaphaloides* yielded bioactive fractions LG1–LG4 and that from *Inula graveolens* and the bioactive fractions IG1 and IG2.

Fraction BS1 from *Buddleja saligna* was a single pure compound, identified as the hydroxylated penatacyclic triterpenoid oleanolic acid (**1**) from comparison of its NMR spectra (^{1}H and ^{13}C), MS spectra (EI MS and FAB), and physicochemical properties with published data on this known compound. (Budzikiewicz et al., 1963; Seebacher et al., 2003). Fractions BS2–BS6 from this same plant contained **1** as principle component, contaminated by inseparable and unidentified components. Fraction LG1 from the hexane extract of *Leyssera gnaphaloides* was similarly identified as a single pure compound with HREIMS and ^{13}C NMR spectroscopy and physicochemical properties indicating its identity as the known compound ursolic acid (**2**) (Mahato and Kundu, 1994; Takeoka et al., 2000; Seebacher et al., 2003). Fraction LG2 contained the pure compound oleanolic acid (**1**) with its spectroscopic data and melting point correlating with those of BS1. Fractions LG3 and LG4 were inseparable mixtures, each containing **1** as a major component.

The isolated fractions and compounds were tested against *Mtb* H37Rv by the agar-plate disk diffusion method and bioautography, and against non-tuberculosis mycobacteria, *Mycobacterium microti* ATCC 19422, *Mycobacterium avium* ATCC 25291, and *Mycobacterium scrofulaceum* ATCC 19981 using the bioautography method, in all cases using rifampicin as positive control. The results of these assays (Table 1) showed that all fractions exhibited significant bactericidal activity at a loading of 2.5 μg/spot against *Mtb* H37Rv (except BS2: 5 μg/spot) and *Mycobacterium microti* ATCC 19422, and at a loading of 1.25 μg/spot against *Mycobacterium avium* ATCC 25291 and *Mycobacterium scrofulaceum* ATCC 19981. All the test samples were twice as active against *Mycobacterium avium* ATCC 25291 as the control, rifampicin.

Because of their hydrophobic nature, the isolated bioactive compounds diffused poorly on agar medium, showing inhibitory activity at higher loadings (5 μg/spot) using the disk diffusion method than by bioautography (2.5 μg/spot). It is worth noting that the inhibitory effects of the samples are easier to notice on bioautographic plates than on agar plates because of the contrast of white spots on a blue background, especially in the case of slow-growing micro-organisms such as *Mtb*. Another important advantage of bioautography is that the duration of the anti-*Mtb* assay is considerably reduced to a maximum of 48 h, compared to 3–6 weeks when using the disk diffusion method. The anti-*Mycobacterium* activity of bioactive compounds isolated from *Buddleja saligna* was comparable to rifampicin.

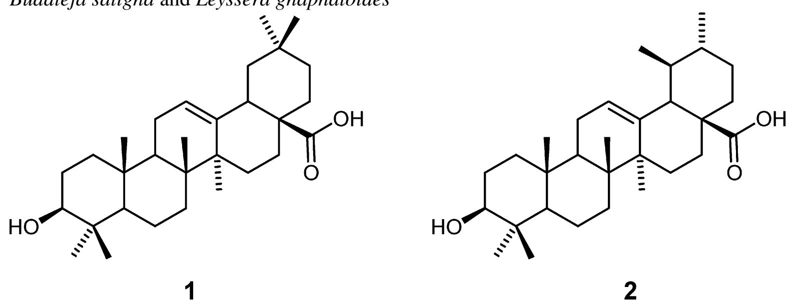
Following identification of the promising anti-mycobacterial activity, the cytotoxicity of the isolated fractions and pure compounds was evaluated using Chinese hamster ovarian cells. Results showed more than 50% CHO cell survival at a concentration as high as 100 μg/ml for the test samples (Table 1) in comparison to the IC_{50} values of 0.06–0.07 μg/mL for the positive control, emetine. These results show that the isolated bioactive compounds/mixtures were not toxic to CHO cells.

It has therefore been established that plant extracts of *Buddleja saligna*, *Leyssera gnaphaloides* exhibit significant anti-mycobacterial activity, and that this activity seems to be primarily associated with the presence of the well-known pentacyclic triterpenoids, oleanolic acid (**1**) and ursolic acid (**2**). This lends credence to the claims for medicinal properties in these plants, and the high level of activity of these two compounds and their low cytotoxicity confirms their potential as lead structures in the search for novel anti-mycobacterial drugs. These two compounds are indeed ubiquitous in the plant world, having been widely identified in a range of plant species, and known to possess a wide spectrum of activity including anti-diabetic, anti-inflammatory, diuretic, anti-spasmodic, anti-atherosclerotic, anti-tumour, antimicrobial, anti-fungal, and anti-HIV (Wachter et al., 1999; Takeoka et al., 2000; Li et al., 2002) The anti-mycobacterial activity reported here correlates well with reported values (Cantrell et al., 2001; Kanokmedhakul et al., 2005). The mechanism of antimicrobial action of **1** and **2** and their derivatives has been investigated (Deng et al., 2000) and found to be based on the inhibition of DNA polymerase.

These results and observations would appear to suggest that all plants containing these compounds will exhibit anti-mycobacterial activity and have potential as natural remedies in the treatment of diseases like TB. This remains to be explored and verified, but one suspects that the activities of plants and their extracts are modulated by the matrix in which the active components exist, with activity amplified or diminished by synergistic association with other plant constituents.

At this time there is no definitive explanation for the fact that the plants under investigation were reportedly used for medicinal purposes in the form of aqueous extracts, while the anti-mycobacterial activity reported here is exclusively associated with non-polar extracts of a lipophilic nature. It is possible that these compounds are also present in the plant as water-

Table 1
Minimum inhibitory concentration (MIC) against selected *Mycobacteria*, and IC_{50} against Chinese hamster ovarian (CHO) cells, of compounds isolated from *Buddleja saligna* and *Leyssera gnaphaloides*



Compound	MICs (μg/disk) (disk diffusion method)	Anti-mycobacterial activity: MIC (μg/spot) (bioautography on TLC plates)				CHO IC_{50} (μg/ml)
	Mtb H37Rv	*Mtb* H37Rv	*Mycobacterium avium* ATCC 25291	*Mycobacterium scrofulaceum* ATCC 19981	*Mycobacterium microti* ATCC 19422	
BS1	5	2.5	1.25	1.25	2.5	>100
BS2	5	2.5	1.25	1.25	2.5	>100
LG1	5	2.5	1.25	1.25	2.5	>100
LG2	5	2.5	1.25	1.25	2.5	>100
Rifampicin	2.5	2.5	2.5	2.5	2.5	
Emetine						0.07

soluble glycosides and/or saponins which act as pro-drugs, and this deserves further examination. One example of such a pro-drug is a derivative of **1**, mimengoside B, consisting of a sugar moiety linked to an aglycon which is a derivative of **1**. It is believed to be the cause of the therapeutic properties of the infusion of leaves of *Buddleja madagascariensis*, a species related to *Buddleja saligna* used in the treatment of asthma, coughs and bronchitis (Emam et al., 1997). Other previous studies have found oleanolic acid-containing saponins to possess broad-spectrum anti-fungal activity (Favel et al., 1994).

4. Conclusion

The results of this investigation have highlighted the importance of assessing the herbal medicines used by traditional medicine practitioners, and the plant kingdom in general.

The fact that the isolated bioactive constituents ursolic acid, oleanolic acid and their derivatives showed no toxicity to CHO cells line adds a considerable pharmaceutical value to the finding of this investigation in the sense that these warrant further investigation as lead anti-TB drugs. Such further studies would include structure anti-TB activity relationships study, the evaluation of the in vitro anti-TB activity, and the in vitro general cytotoxicity of the derivatives.

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