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Antimycobacterial, anti-inflammatory and genotoxicity evaluation of plants used for the treatment of tuberculosis and related symptoms in South Africa

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ABSTRACT

Ethnopharmacological relevance: Emergence of drug-resistant tuberculosis strains and long duration of treatment has established an urgent need to search for new effective agents. The great floral diversity of South Africa has potential for producing new bioactive compounds, therefore pharmacological screening of plant extracts within this region offers much potential. To assess the *in vitro* antimycobacterial, anti-inflammatory and genotoxicity activity of selected plants that are used for the treatment of TB and related symptoms in South Africa.

Materials and methods: Ground plant materials from 10 plants were extracted sequentially with four solvents (petroleum ether, dichloromethane, 80% ethanol and water) and a total of 68 extracts were produced. A broth microdilution method was used to screen extracts against *Mycobacterium tuberculosis* H37Ra. The cyclooxygenase-2 (COX-2) enzyme was used to evaluate the anti-inflammatory activity of the extracts and the *Salmonella* microsome assay using two *Salmonella typhimurium* strains (TA98 and TA100) to establish genotoxicity.

Results: Six out of 68 extracts showed good antimycobacterial activity. Three extracts showed good inhibition (> 70%) of COX-2 enzyme. All the extracts tested were non-genotoxic against the tested *Salmonella* strains.

Conclusion: The results observed in this study indicate that some of the plants such as *Abrus precatorius* subsp. *africanus*, *Ficus sur*, *Pentanisia prunelloides* and *Terminalia phanerophlebia* could be investigated further against drug-resistant TB strains.

1. Introduction

Tuberculosis (TB) is one of the oldest and most prevalent diseases, having infected about 8.7 million people worldwide in 2011 (Smith, 2003; Okunade et al., 2004; WHO, 2013). In the mid-20th century, the mortality rate of TB began to decrease and the decline is still evident in Europe and America with the incident rate continuing to be low (Daniel, 2006; WHO, 2013). In Africa, approximately half a million people died of TB in 2004. Africa remains the only continent where TB rates are still increasing (Kamatou et al., 2007). TB symptoms are aggravated by the co-infection with Human Immunodeficiency Virus (HIV). Studies done have placed *Mycobacterium tuberculosis* as the most common human cause of TB worldwide (WHO, 2013), responsible for more human fatalities than any single bacterial species and infecting at least one-third of the world's population (Kamatou et al., 2007; Bansal et al., 2009; Knechel, 2009). Streptomyces-based antibiotics and other chemotherapeutic antimycobacterial agents often used in combination in the treatment of TB are no longer effective due to drug resistance exhibited by the organism (Lai et al., 2011). The treatments of TB take a long time resulting in poor compliance of patients which contributes to sustaining multi-drug resistant TB (MDR-TB) (Connolly et al., 2007). Neonates immunization with Bacillus Calmette Guérin (BCG) protects against mainly TB meningitis and miliary TB. However, the efficacy wanes 10–15 years post-vaccination, thus adults are not protected against pulmonary tuberculosis. Therefore, new vaccines and anti-tubercular agents with new modes of activity, shorter treatment duration as well as low toxicity are needed to reduce MDR-TB

Abbreviations: 4-NQO, 4-nitroquinoline 1-oxide; BCG, Bacillus Calmette Guérin; CFU, colony-forming units; CO_2, carbon dioxide; COX, Cyclooxygenase; DCM, dichloromethane; DMSO, dimethylsulfoxide; DPM, disintegrations per minute; EtOH, ethanol; HCL, hydrochloric acid; HIV, human immunodeficiency virus; INH, isoniazid; MDR, multi-drug resistant; MIC, minimum inhibition concentration; No., number; NRF, National Research Foundation; OADC, oleic acid-albumin-dextrose-catalase; PE, petroleum ether; REMA, resazurin microplate assay; TB, tuberculosis

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prevalence and stop the epidemic of TB. One of the TB symptoms is chest pain caused by inflammation of the membranes lining the lungs which leads to development of lung fibrosis. In the case of pulmonary TB, alveolar macrophages control persistant inflammatory responses in the lungs by producing chemical mediators that leads to granuloma formation (Shinohara et al., 2009). In response to infection of the host by *Mycobacteria*, granuloma formation represents protective immunity and inflammatory tissue destruction and repair (Shinohara et al., 2009).

South Africa is endowed with a diverse flora which has potential for the discovery of metabolites that are active against *Mycobacterium tuberculosis* (Lall and Meyer, 1999; McGaw et al., 2008). Screening of plant extracts for antimycobacterial activity within South African medicinal plants with their great diversity offers much potential in the search for active new metabolites that may have activity against *Mycobacterium tuberculosis* and other opportunistic infections. Although medicinal plants have been used in therapy for many years, that does not mean that they are safe, as they may have side effects. Since medicinal plant use and prescription in South Africa is not standardized, the danger of misadministration especially if the plants are toxic is real (Fennell et al., 2004). It is important to determine if the antimicrobial activity exhibited by some plant extracts is not due to toxicity.

In our previous study, we reported several plant extracts that exhibited good antibacterial activities against non-pathogenic *Mycobacterium* species and other strains associated with respiratory infections (Madikizela et al., 2013). As a result of research done in our laboratory on antimicrobial activity of plants used for treating TB and that most human fatalities caused by TB are caused by *Mycobacterium tuberculosis*, further investigations were carried out. The investigations included determining the antibacterial activity of selected plants used for treating TB and related symptoms against an *Mycobacterium tuberculosis* strain. The anti-inflammatory (since inflammation is one of TB symptoms) activities of selected medicinal plants used in the previous study, as well as genotoxicity properties of the extracts that showed good antimicrobial activities in that previous study were carried out in the current study.

2. Materials and methods

2.1. Sample collection

Plant materials were collected from the University of KwaZulu-Natal botanical garden and Ukulinga research farm in Pietermaritzburg, South Africa. These plants have already been evaluated for their antimicrobial activity against strains of bacteria related to respiratory ailments in a previous study (Madikizela et al., 2013). The list of the plants used with family, species name, voucher specimen number, traditional uses and previously tested activities are outlined in Madikizela et al. (2013).

2.2. Plant extracts preparation

Plant materials were oven-dried at 50 °C, ground into powder and 10 g was extracted sequentially with 200 ml of petroleum ether (PE), dichloromethane (DCM), 80% ethanol (EtOH) and water. The extracts were then filtered through Whatman No. 1 filter paper and concentrated under vacuum using a rotary evaporator for solvent extracts whereas the aqueous extracts were freeze-dried. The concentrated extracts were then dried under a stream of cold air and kept at 8 °C until required.

2.3. Antimycobacterial activity using resazurin microplate assay

The resazurin microplate assay (REMA) according to the method of Jadaun et al. (2007) was used as a antimycobacterial assay. *Mycobacterium tuberculosis* H37Ra (American type culture collection 25177) stored at −70 °C was thawed at room temperature for 10–15 min and subcultured in Middlebrook 7H9 broth supplemented with 0.2% glycerol and 10% oleic acid–albumin–dextrose–catalase (OADC). The strain was incubated at 37 °C for 4 weeks in 5% carbon dioxide (CO_2) until logarithmic growth was reached. A turbidity equivalent to that of McFarland's no. 1 standard solution was achieved by mixing the culture with a sufficient volume of sterile supplemented Middlebrook 7H9 broth. The test inoculum was obtained by further dilution (1:20) of the suspension with the same culture medium to approximately 6×10^6 colony-forming units (CFU)/ml immediately prior to use. The extracts (50 mg/ml) were dissolved in 10% dimethylsulfoxide (DMSO) and maintained at room temperature for 1 h to assure their sterilization. Rifampicin and streptomycin were used as positive controls. The organic and aqueous extracts from each plant were assayed in duplicates. Each microplate was incubated for 5 days at 37 °C in a 5% CO_2 atmosphere. After 5 days of incubation, 32 μL of freshly prepared resazurin solution was added to one growth control well. The microplates were incubated again at 37 °C in a 5% CO_2 atmosphere for 24 h. When a color shift from blue to pink was observed in the growth control sample, 32 μL of resazurin solution was added to each of the remaining wells, and the microplate was further incubated for 24 h. A well-defined pink color was interpreted as positive bacterial growth, whereas a blue color indicated the absence of growth.

2.4. Anti-inflammatory activity

The cyclooxygenase-2 inhibition assay was performed as described by Zschocke and Van Staden (2000). Cyclooxygenase-2 (COX-2) stock enzyme stored at −70 °C was activated with 1250 μl of co-factor solution and 200 μl of Tris buffer. Organic extracts were tested at a concentration of 250 μg/ml and aqueous extracts at 2 mg/ml. Three controls were used for the assay (background solvent blank and positive control). The negative controls were the background (enzyme inactivated with 4 N HCL before incubation) and the solvent blank (enzyme not deactivated). Indomethacin was used as a positive control at 200 μM. Arachidonic acid (20 μl) was added to the eppendorfs to start the reaction before incubation at 37 °C in a water bath for 10 min. The following formula was used to calculate the percentage of inhibition for the test extracts:

$$COX\ inhibition\ (\%) = \left[1 - \left(\frac{DPM\ sample - DPM\ background}{DPM\ blank - DPM\ background}\right)\right] \times 100$$

2.5. The Ames test

A *Salmonella* microsome assay according to Maron and Ames (1983) modified by Mortelmans and Zeiger (2000) was used to evaluate genotoxicity potential of plant extracts that had good antimicrobial activity. The aliquots were prepared from previously dried plant extracts and dissolved in 10% DMSO to give three concentrations of 5000, 500 and 50 μg/ml. The Ames test was performed with two *Salmonella typhimurium* tester strains, TA98 and TA100 without metabolic activation. Bacterial stocks (100 μl) were incubated in 20 ml of Oxoid No. 2 nutrient broth at 37 °C on a rotary shaker for 16 h. The cultured bacteria (100 μl) were added to 100 μl of plant extract with 500 μl of phosphate buffer and 2 ml of top agar containing biotin–histidine (0.5 mM). The top agar mixture was then poured over the surface of a minimal agar plate and incubated at 37 °C for 48 h. The positive control used was