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Antimycobacterial activity of selected medicinal plants traditionally used in Sudan to treat infectious diseases

Nadir Abuzeid [a,b,c,1], Sadaf Kalsum [c,1], Richin John Koshy [c], Marie Larsson [d], Mikaela Glader [c], Henrik Andersson [c], Johanna Raffetseder [c], Elsje Pienaar [c], Daniel Eklund [c], Muddathir S. Alhassan [a], Haidar A. AlGadir [a], Waleed S. Koko [a], Thomas Schön [e], M. Ahmed Mesaik [f,g], Omer M. Abdalla [g], Asaad Khalid [a], Maria Lerm [c,*]

[a] Medicinal and Aromatic Plants Research Institute, National Center for Research, P.O. Box: 2404, Khartoum 11111, Sudan
[b] Department of Clinical Microbiology, Faculty of Medical Laboratory Sciences, Omdurman Islamic University, P.O.Box 382, Omdurman,Sudan
[c] Division of Microbiology and Molecular Medicine, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linkoping University, 58185 Linkoping, Sweden
[d] Department of Clinical Microbiology, Linköping University Hospital, 58185 Linkoping, Sweden
[e] Department of Clinical Microbiology and Infectious diseases, Kalmar County Hospital, SE-391 85 Kalmar, Sweden
[f] Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, Kuala Lumpur 50300, Malaysia
[g] Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan

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ABSTRACT

Ethnopharmacological relevance: The emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* underscores the need for continuous development of new and efficient methods to determine the susceptibility of isolates of *Mycobacterium tuberculosis* in the search for novel antimycobacterial agents. Natural products constitute an important source of new drugs, and design and implementation of antimycobacterial susceptibility testing methods are necessary to evaluate the different extracts and compounds. In this study we have explored the antimycobacterial properties of 50 ethanolic extracts from different parts of 46 selected medicinal plants traditionally used in Sudan to treat infectious diseases.

Materials and methods: Plants were harvested and ethanolic extracts were prepared. For selected extracts, fractionation with hydrophilic and hydrophobic solvents was undertaken. A luminometry-based assay was used for determination of mycobacterial growth in broth cultures and inside primary human macrophages in the presence or absence of plant extracts and fractions of extracts. Cytotoxicity was also assessed for active fractions of plant extracts.

Results: Of the tested extracts, three exhibited a significant inhibitory effect on an avirulent strain of *Mycobacterium tubercluosis* (H37Ra) at the initial screening doses (125 and 6.25 μg/ml). These were bark and leaf extracts of *Khaya senegalensis* and the leaf extract of *Rosmarinus officinalis L.* Further fractions of these plant extracts were prepared with n-hexane, chloroform, ethyl acetate, n-butanol, ethanol and water, and the activity of these extracts was retained in hydrophobic fractions. Cytotoxicity assays revealed that the chloroform fraction of *Khaya senegalensis* bark was non-toxic to human monocyte-derived macrophages and other cell types at the concentrations used and hence, further analysis, including assessment of IC50 and intracellular activity was done with this fraction.

Conclusion: These results encourage further investigations to identify the active compound(s) within the chloroform fraction of *Khaya senegalensis* bark.

1. Introduction

Tuberculosis (TB) is a global public health threat and remains one of the major causes of death among infectious diseases. The patient incompliance associated with the long time required to reach sterilizing TB treatment (≥ 6 months on 2–4 antibiotics) contributes to the emergence of multidrug- resistant TB (MDR-TB)

* Corresponding author. Tel.: +46 1010 32088; fax: +46 1010 3478.
E-mail address: maria.lerm@liu.se (M. Lerm).
[1] These authors contributed equally to the study.

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and extensively drug-resistant TB (XDR-TB) (Jain and Dixit, 2008; Pinto and Menzies, 2011). Taking into consideration the low cure rates of MDR-TB and the high relative incidence, 28% of new TB cases in some areas of Eastern Europe, research promoting new antimycobacterial therapies is pivotal (WHO, 2010). In addition, the discovery of new classes of antimycobacterial drugs (Field et al., 2012) and possibly also virulence blockers (Keyser et al., 2008) and drugs stimulating immunity (Lam et al., 2012) may guide the development of a more rational and effective TB therapy that is several months shorter than the present regimen.

A milestone of the development of the standard TB therapy was the implementation of short course chemotherapy in the 1970s (East African-British Medical Research Councils, 1972). The combination of four antibiotics allowed reduction of time of treatment to six months. However, this led to the conclusion that TB was finally defeated, but the emergence of MDR-TB in the United States rekindled the scientific interest in the disease (Frieden et al., 1993).

Natural products can pave the way for new drug leads, since they provide an unlimited source of chemically diverse compounds. Today's first line drug to treat malaria, artemisinin, was found through the systematic search of anti-malarial compounds in herbs used in traditional Chinese medicine (Klayman, 1985). Several broad screening strategies have also been carried out with Sudanese medicinal plants for antibacterial, antifungal, antiviral, antimalarial and antihelminthic properties (Koko et al., 2008).

In the present study, we screened 50 ethanolic extracts of 46 Sudanese plants with respect to antimycobacterial activity using a luminometry-based method (Eklund et al., 2010). Two hits were identified; *Khaya senegalensis* (bark and leaves) and *Rosmarinus officinalis L.* (leaves), and these extracts were subjected to fractionation with water, n-hexane, chloroform, ethanol, ethyl acetate and n-butane. The n-hexane, chloroform, ethyl acetate and n-butanol fractions were found to effectively inhibit the growth of *Mycobacterium tuberculosis* at a concentration of 6.25 μg/ml. The result was confirmed with other assays for mycobacterial growth.

2. Materials and methods

2.1. Mycobacterial culture

Mycobacterium tuberculosis strains H37Ra and H37Rv (American Type Culture Collection) harboring the pSMT1 plasmid (Eklund et al., 2010), which carry the gene for *Vibrio harveyi* Luciferase (Mtb H37Ra-lux/Mtb H37Rv-lux), were grown at 37 °C for one week before experiment in Middlebrook 7H9 broth (Becton Dickinson) supplemented with albumin dextrose catalase (ADC, Becton Dickinson), 0.05% Tween 80 and 100 μg/ml hygromycin as selection antibiotic.

2.2. Plant extracts and fractionations

Selected plant species were collected between January and April 2010 from different places in Sudan by botanists associated with the Medicinal and Aromatic Plant Institute in Khartoum, Sudan. Ethanolic extraction was carried out at the same institute, according to a method described by Harborne (Harborne, 1984; Rao et al., 2002). For fractionation of selected plant extracts (as shown in Table S1), a specific weight (listed in Table S2) was dissolved in 500 ml of distilled water and extracted three times by gentle shaking with 100 ml of n-hexane, each time using a separating funnel and the three samples were finally pooled. The aqueous fraction was re-extracted three times each with the following solvents: chloroform, ethyl acetate and n-butanol. Finally, the different fractions were evaporated under reduced pressure using a rotary evaporator apparatus and the yield percentage was calculated for each fraction (Table S2). The aqueous layer was finally freezed at −80 °C, stored at −40 °C and freeze-dried using a freeze dryer apparatus until completely dry and the yield was calculated. For experiments, 5 mg of dried plant extracts or fractions were dissolved in 50 μl of 100% DMSO to obtain stock solutions of 100 mg/ml.

2.3. pH measurement

Litmus paper (MERCK) was dipped into the aliquot of plant extracts diluted to 125 and 6.25 μg/ml in broth supplemented with ADC and 0.05% Tween 80. The color obtained determined the pH of the extract as shown in Table S1.

2.4. Bacterial growth assay

One ml of culture containing Mtb H37Ra-lux was centrifuged once at 5000*g* for 5 min at room temperature and the pellet was re-suspended in broth supplemented with ADC and 0.05% Tween 80. In order to remove bacterial aggregates, the suspension was passaged 10 times through a sterile syringe equipped with a 27-gauge needle. The concentration was determined by optical density at 600 nm (OD_{600}) as a function of colony-forming units (CFU) obtained from a standard curve derived from plated bacteria (not shown). For screening, 96-well plates were prepared with dilutions of the extracts at the final concentrations of 2.5 mg/ml, 125 μg/ml or 6.25 μg/ml. 2.5%, 0.125% and 0.00625% DMSO were used as solvent controls, respectively. A high dose of isoniazid (the first-line antibiotic, was selected to insure efficient inhibition) and broth only were included in all experiments as further controls. Before experiment, Mtb H37Ra-lux was added to the wells at a concentration of 10^5 CFU/ml and incubated at 37 °C for 5 days before analysis. As 2.5% DMSO had an inhibitory effect on the bacteria (Fig. S1), the highest concentration of extracts, 2.5 mg/ml was excluded from further investigation.

Hits obtained in the screening procedure were re-confirmed by the same procedure in triplicates for the medium and lowest concentrations, and the isolated fractions were also tested with the same procedure in triplicates.

2.5. Analysis of bacterial growth by luminometry

To measure flash luminescence in the biosafety level (BSL) 2 facility, a Glomax Multiplus Reader (Promega) was used. The instrument is equipped with luminescence, fluorescence and absorbance functions and the presence of an injector module allowed the injection of the luciferase substrate *n*-decanal (Sigma-Aldrich, final concentration 1% decanal) in white, opaque 96-well plates (Eklund et al., 2010).

2.6. Determination of colony forming units (CFU)

For confirmation of the results obtained with the luciferase assay, CFU plating of the 96-well plate cultures was done in parallel. To this end, dilutions of the cultures were streaked on 7H9 agar plates for determining CFU in the presence and absence of plant extracts. The plates were incubated for 3 weeks before evaluation of the number of colonies. All samples were analyzed in triplicates.

2.7. Separation and culture of Human monocyte-derived macrophages (hMDMs)

Heparinized human whole blood was prepared from healthy donors (University Hospital in Linköping, Sweden) as described previously (Welin et al., 2011) and cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 25 mM HEPES. The