SEARCHING FOR NATURAL ANTITUBERCULARS FROM MOZAMBICAN MEDICINAL PLANTS

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Abstract

Tuberculosis (TB), an infectious disease mainly caused by Mycobacterium tuberculosis, is considered as one of the major global health problems. Due to an increased drug resistance all over the world including to the first line antibiotics, new efficient drugs are urgently needed. A promising approach to achieve this goal is the study of natural products derived from plants, which have been used to treat human diseases through ages. Currently, in Mozambique only 40% of inhabitants can access public health system and most of them still rely on traditional medicine for most of their basic health care needs. Likewise, more than 500 medicinal plant species have been reported for their enrolment by traditional healers to treat various diseases. Here, we show recent findings of our group for new effective antitubercular compounds isolated from medicinal plants traditionally used in Mozambique. Preliminary phytochemical and antimycobacterial evaluation of several crude extracts from fifteen medicinal plants, led us to identify Zanthoxylum capense as a promising species for further investigation. Sixteen compounds with various structural scaffolds were isolated, from the methanol extract of this plant, and evaluated for their antimycobacterial activity against different M. tuberculosis strains. A benzophenanthridine type alkaloid showed a promising activity against M. tuberculosis H37Rv, both in vitro and ex-vivo in human macrophages.

Keywords: Tuberculosis, medicinal plants, antimycobacterial, Zanthoxylum capense

Introduction

Tuberculosis (TB) is a contagious and infectious disease caused by Mycobacterium tuberculosis complex species that typically affects the lungs (pulmonary TB) but can also affect other organs (miliary TB). TB is the second leading cause of death from an infectious disease, remaining a major global health problem (WHO, 2012). World Health Organization (WHO) estimated that there were almost 9 million new cases in 2011 and 1.4 million TB deaths (990 000 among HIV negative people and 430 000 HIV-associated TB deaths). Most of the estimated number of cases occurred in Asia (59%) and Africa (26%); smaller proportions of cases occurred in the Eastern Mediterranean region (7.7%), Europe (4.3%) and America (3%), (Fig. 1). Key factors such as poverty, homelessness and the high prevalence of HIV co-infection mean that tuberculosis will continue to be an important cause of morbidity and mortality worldwide (WHO, 2012).
Nowadays, drugs available for TB treatment are classified into two categories. First line therapy, used for 6 months to treat drug-susceptible TB (DS-TB), includes four drugs: isoniazide, pyrazinamide, ethambutol and rifampicin. Up to 95% of people with DS-TB can be cured with this therapeutic regimen (RAVIGLIONE et al., 2012; KOUL et al., 2011). Nevertheless, inconsistent or partial treatment leads to the development of multidrug-resistant (MDR) and extensively drug resistant TB (XDR), which is considered to be a major threat to the global control of this disease. Infection by MDR strains requires treatment for as long as two years with second-line drugs that includes cycloserine, capreomycin, fluoroquinolones, ethionamide, p-aminosalicylic acid, thioacetazone, rifabutin, clofazimine and some macrolides (LIU et al., 2012). Cure rates for MDR-TB ranges from 50 – 70%. XDR-TB has very high mortality rates and the treatment options are very limited because the bacilli are resistant not only to the first line drugs but also to fluoroquinolones and aminoglycosides. In addition, there are serious toxicity and side effects related with most of the drugs used to treat MDR-TB and XDR-TB (KOUL et al., 2011). Therefore, newer and more effective TB drugs, with novel mechanisms of action, are urgently needed in order to shorten and simplify the therapy and more importantly to target MDR and XDR strains (KOUL et al., 2011).

Trough ages, humans have relied on nature to fulfill their primary needs such as food, shelters, clothing and medicines. In fact, products obtained from natural sources (mostly plants but also minerals, animals and fungi) have been used since antiquity for treating diverse complaints and have provided a rich source of interesting secondary metabolites with biological activity. The vast diversity of natural products represents a powerful tool for drug discovery, since they stand for more than 50% of all the drugs clinically used, playing
also a central role as a source of effective anti-tuberculosis agents (FAKIM, 2006). Currently, there is a re-emerging interest in natural products with the purpose of searching for novel and potential anti-tuberculosis lead compounds, and on the other hand, as a means of identifying those targets that are most vulnerable in the bacterium (COPP and PEARCE 2007; HARTKOORN, et al 2012).

Currently, in Mozambique only 40% of inhabitants can access public health system and most of them still rely on traditional medicine for most of their health care needs. The ratio of doctors (practicing allopathic medicine) to patients is 1:50000. In contrast, the ratio of traditional healers to patients is only 1:200. Thus, inadequate provision of modern allopathic medicine combined with deeply rooted cultural aspects of traditional medicine makes this and in particular the use of medicinal plants an important part of health care in Mozambique (KROG et al 2006). During the last decade, some works have been published gathering the ethnobotanical use of plants in this country and more than 500 plant species were reported to be used by traditional healers to treat several diseases (BRUSCHI et al 2011; RIBEIRO et al 2010; KROG et al 2006; MATAVEL and HABIB 2000;BANDEIRA et al 2001). However, scientific validation and efficacy of many of those plants have not been well documented so far.

In our search for bioactive compounds from natural sources, we have been studying some Mozambican medicinal plants, namely *Tabernaemontana elegans* and *Momordica balsamina*. Several compounds have been obtained, mainly triterpenoids, isolated from *M. balsamina*, which showed a significant in vitro antimalarial activity and multidrug resistance reversing activity (RAMALHETE et al, 2009; RAMALHETE et al, 2010; RAMALHETE et al, 2011a and 2011b). Moreover, a variety of indole- and β-carboline-type alkaloids were isolated from *T. elegans* and studied for their apoptosis induction activity in human hepatoma HuH-7 cells (MANSOOR et al, 2009).

Here, we provide a summary of our efforts (LUO et al 2011 ; LUO et al, 2012; LUO et al, 2013) to evaluate several medicinal plants traditionally used in Mozambique to treat TB and other respiratory diseases. Our findings validate the use of compounds isolated from these plants as potential antimycobacterial activity required for effective treatment.

**PRELIMINARY ANTIMYCOBACTERIAL SCREENING**

After a literature survey, fifteen traditional medicinal plants were selected according to their traditional use in treatment of TB related symptoms including cough, bronchitis, chest complaints and pneumonia (Table 1) (LUO et al 2011). Seventy-five crude extracts were prepared by sequential extraction with solvents of different polarities or by decoction with boiling water, according to the traditional use (LUO et al, 2011).

The initial screening was performed with two mycobacterial species: *Mycobacterium smegmatis* ATCC 607 and *Mycobacterium tuberculosis* H37Rv, using the broth microdilution method. *Maerua edulis* and
Zanthoxylum capense (n-hexane and dichloromethane extracts, respectively) showed considerable activity against Mycobacterium smegmatis ATCC 607. Among the tested extracts, eight of them exhibited moderate to significant activity against M. tuberculosis H37Rv (Table 2). The most active extracts were Tabernaemontana elegans EtOAc extract and Zanthoxylum capense CH₂Cl₂ extract (LUO et al., 2011).

Table 1. Plant species collected in Mozambique and their traditional uses for treating TB and related diseases (Luo et al. 2011 and references cited therein).

<table>
<thead>
<tr>
<th>Species (plant part)</th>
<th>Traditional use</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adansonia digitata</strong> L. (bark)</td>
<td>Tuberculosis, persistent cough, bronchitis, pneumonia, chest pain.</td>
</tr>
<tr>
<td><strong>Anacardium occidentale</strong> L. (roots)</td>
<td>Cough</td>
</tr>
<tr>
<td><strong>Ansellia africana</strong> Lindl. (whole)</td>
<td>Asthma, respiratory problems</td>
</tr>
<tr>
<td><strong>Artabotrys brachypetalus</strong> Benth. (roots)</td>
<td>Cough, asthma</td>
</tr>
<tr>
<td><strong>Capparis tomentosa</strong> Lam. (roots)</td>
<td>Cough, chest pain, tuberculosis</td>
</tr>
<tr>
<td><strong>Clerodendrum glabrum</strong> E. Mey. Var. Glabrum (leaves)</td>
<td>Cough, fever, tuberculosis, bronchitis, chest pain</td>
</tr>
<tr>
<td><strong>Combretum zeyheri</strong> Sond. (bark)</td>
<td>Cough</td>
</tr>
<tr>
<td><strong>Maerua edulis</strong> (Gilg &amp; Gilg-Ben.) DeWolf (roots)</td>
<td>Cough, tuberculosis</td>
</tr>
<tr>
<td><strong>Maerua juncea</strong> Pax (roots)</td>
<td>Respiratory problems</td>
</tr>
<tr>
<td><strong>Opuntia</strong> spp. (whole)</td>
<td>Cough, bronchitis</td>
</tr>
<tr>
<td><strong>Sarcostemma viminale</strong> (L.) R. Br. (roots)</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td><strong>Securidaca longipedunculata</strong> Fresen (roots)</td>
<td>Cough, chest complaints, tuberculosis</td>
</tr>
<tr>
<td><strong>Tabernaemontana elegans</strong> Stapf (roots)</td>
<td>Chest complaints, tuberculosis</td>
</tr>
<tr>
<td><strong>Zanthoxylum capense</strong> (Thunb.) Harv. (roots)</td>
<td>Violent chronic coughing, tuberculosis, bronchitis</td>
</tr>
<tr>
<td><strong>Vernonia colorata</strong> (Willd.) Drake subsp. colorata (leaves)</td>
<td>Cough, pneumonia</td>
</tr>
</tbody>
</table>
Table 2. Antimycobacterial activity of the active extracts against *M. tuberculosis* H37Rv (LUO *et al* 2011).

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Extract</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Capparis tomentosa</em></td>
<td>70% EtOH</td>
<td>125.0</td>
</tr>
<tr>
<td><em>Securidaca longepedunculata</em></td>
<td>n-hexane</td>
<td>125</td>
</tr>
<tr>
<td><em>Tabernaemontana elegans</em></td>
<td>EtOAc</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>70% EtOH</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>125</td>
</tr>
<tr>
<td><em>Zanthoxylum capense</em></td>
<td>n-hexane</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>CH₂Cl₂</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>EtOAc</td>
<td>125</td>
</tr>
</tbody>
</table>

Based on these results and in order to obtain a wide spectrum antimycobacterium profile, the aforementioned extracts of the four plant species were selected for further studies using *Mycobacterium tuberculosis* H37Ra, *Mycobacterium bovis* BCG ATCC 35734, *Mycobacterium avium* DSM 44156 and DSM 44157, and *Mycobacterium smegmatis* mc² 155 (LUO *et al*, 2011). *Z. capense* appeared to be the most promising species showing MIC values of 31.2 µg/mL against *M. tuberculosis* H37Ra and *M. bovis* BCG, and a MIC value of 62.5 µg/mL against *Mycobacterium smegmatis* mc² 155 (LUO *et al*, 2011).

**PHYTOCHEMICAL STUDY OF ZANTHOXYLUM CAPENSE**

The genus *Zanthoxylum* (Rutaceae) comprises 250 species that are well known for their ethnobotanical uses, and are a rich source of several biologically active compounds, such as alkaloids, amides, coumarins and lignans (SUN and DUAN 1996; LUO *et al*, 2012).

*Zanthoxylum capense*, common name Small Knobwood, is a small to medium-sized (5 – 15 m) multi branched tree indigenous to Zimbabwe, South Africa, and Mozambique (SCHMIDT *et al*, 2002). The bark of young branches is smooth with dark brown thorns and light to dark grey on older branches and stems, with straight spines on cone-shaped knobs ([http://www.plantzafrica.com/plantwxyz/zanthoxylumcapense](http://www.plantzafrica.com/plantwxyz/zanthoxylumcapense)). Traditional healers use the plant to treat several ailments such as flatulent colic, gastric intestinal disorder and intestinal parasites. It has been also employed as a snakebite medicine, administered by rubbing the powdered root into the snakebite wounds. Moreover, the bark has been used in cases of toothache, cold, flu, bronchitis, tuberculosis and blood poisoning in general (STEYN *et al*, 1998).
The methanol extract of the powdered roots of *Z. capense* was successively partitioned into *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol fractions as described by Luo *et al* (2011, 2012). Further antimycobacterial evaluation of these fractions revealed that the non-polar *n*-hexane and dichloromethane soluble parts were the most active, with MIC values of 31.2 and 15.6 µg/mL, respectively. In contrast, the polar ethyl acetate and *n*-butanol fractions showed only weak activity with a MIC value of 250 µg/mL (Luo *et al*, 2013). Therefore, the phytochemical study was carried out with the *n*-hexane and dichloromethane fractions leading to the isolation of sixteen chemically diverse compounds whose structures are represented in Fig.s 2 – 4 (Luo *et al*, 2012; Luo *et al*, 2013).

![Chemical structures](image)

**Fig. 2.** Chemical structures of the alkaloids 1 – 9 isolated from *Zanthoxylum capense*.

The chemical structures of the compounds were deduced from their physical and spectroscopic data, namely low and high resolution mass spectrometry, and extensive Nuclear Magnetic Resonance techniques that included two-dimensional homo and heteronuclear correlation experiments (COSY, HMQC, HMBC and NOESY).

The isolated compounds included the benzophenantridine-type alkaloids decarine (1), norchelerythrine (2), dihydrochelerythrine (3), 6-acetyldihydrochelerythrine (4), tridecanonchelerythrine (5), 6-acetyldihydronitidine (6), zanthocapensine (7) and a quinazoline- and a quinoline-type alkaloid rutaecarpine (8) and skimmianine (9). Moreover, three lignans named (−)-sesamin (10), (−)-episesamin (11), and (−)-savinin (12), two neolignans, zanthocapensol (13) and zanthocapensate (14), an amide N-isobutyl-

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(2E,4E)-2,4-tetradecadienamide (15) and the pentacyclic triterpene lupeol (16) were also isolated (Luo et al, 2012; Luo et al, 2013).

Fig. 3. Chemical structures of the lignans 10 – 12 and neolignans 13 – 14 isolated from Zanthoxylum capense.

Fig. 4. Chemical structures of N-isobutyl-(2E,4E)-2,4-tetradecadienamide (15) and lupeol (16) isolated from Zanthoxylum capense.

ANTIMYCcobacterial Activity Against Mycobacterium Tuberculosis

The isolated compounds 1 – 16 were evaluated for their growth inhibitory activity against Mycobacterium tuberculosis H37Ra and Mycobacterium tuberculosis H37Rv, using the broth microdilution method (LUO et al, 2013). Moreover, a cytotoxicity assay was also performed on human THP-1 macrophages. In most cases, the compounds exhibited low cytotoxic activity (IC50 values between 40.3 and 94.5 µg/mL). The most cytotoxic compounds were found to be 6-acetonyldihydronitidine (6) and (–)-savinin (12) that exhibited IC50 values of 1.7 and 3.7 µg/mL, respectively (LUO et al, 2013).

As reported by Luo et al (2013), decarine (1), 6-acetonyldihydronitidine (6) and N-isobutyl-(2E,4E)-2,4-tetradecadienamide (15) displayed strong antibacterial effect against the two mycobacterial strains (MIC values between 1.6 and 12.5 µg/mL). Decarine (1) showed the most potent inhibitory activity with MIC
values of 1.6 and 3.1 µg/mL against *M. tuberculosis* H37Rv and H37Ra, respectively. The quinoline-type alkaloid, skimmianine (9), and the lignans, (−)-sesamin (10) and (−)-episesamin (11), showed moderate activity (MIC values between 25 and 50 µg/mL) against the tested strains. On the other hand, the remaining compounds did not display any antimycobacterial activity (LUO et al, 2013).

Selectivity index values (SI, calculated by the quotient between the IC<sub>50</sub> values of the cytotoxicity assay and MIC values) were also calculated with the aim of assessing the antimycobacterial specificity of compounds. In this way, when SI is higher than 10, the antimycobacterial activity is considered to be specific. Significant SI values were obtained for decarine (1) and *N*-isobutyl-(2E,4E)-2,4-tetradecadienamide (15) (LUO et al, 2013). Decarine (1) showed the highest selectivity (SI = 21.3 and 41.2 for H37Ra and H37Rv, respectively). As described in Luo *et al* (2013), the remaining compounds 2 – 14 and 16 presented low SI values that could be attributed to their weak antimycobacterial activity or high cytotoxic effect.

Taking into account the favourable selective activity of decarine (1) and *N*-isobutyl-(2E,4E)-2,4-tetradecadienamide (15) against the tested *Mycobacterium tuberculosis* strains, an *ex vivo* assay was performed using THP-1 macrophages infected with *M. tuberculosis* H37Rv. As reported in Luo *et al* (2013), the compounds were tested at various concentrations and at 1, 3 and 5 days post-infection, the surviving mycobacteria were enumerated by colony-counts. In this intracellular model of infection, decarine (1) exhibited a dose-dependent activity, being able to reduce the bacterial survival at a concentration of 6.2 µg/mL, 5 days post drug exposure. On the other hand, *N*-isobutyl-(2E,4E)-2,4-tetradecadienamide (15) showed only a moderate bactericidal activity (LUO *et al*, 2013).

The chemical structures of the benzophenanthridine alkaloids 1 – 7 differ in the substitution pattern of ring A and B (Fig. 2). Decarine (1) and norchelerythrine (2) are very similar; however, the presence of a hydroxyl group at C-8 instead of a metoxyl group seems to improve the antimycobacterial activity of decarine. When analysing the set of compounds 3 – 7, it can be concluded that the oxygenation pattern of ring A and the type of substitution at C-6 also influence their antimycobacterial effect and cytotoxicity. In contrast to the low antitubercular and cytotoxic activities of alkaloids 3 – 5 and 7, 6-acetonyldihydronitidine (6) that has two methoxyl groups at C-8 and C-9 and an acetonyl residue at C-6, displayed a strong inhibitory effect against *M. tuberculosis*. Nevertheless, this compound also showed high cytotoxicity on macrophages, leading to a poor selectivity index.

The benzophenanthridine class of alkaloids is a specific group within the isoquinoline alkaloids, which occur only in higher plants, mainly in the *Papaveraceae* and *Rutaceae* families (ZENK 1994; ISHIKAWA 2001). In previous studies, this type of compounds has shown interesting biological activities, namely antitumor, antibiotic and antituberculosis (ISHIKAWA 2001, PARHI *et al* 2012).
Conclusions

The value of the selected medicinal plants in traditional applications for TB treatment was corroborated by our studies. In particular, some compounds isolated from the roots of *Zanthoxylum capense* presented potential antimycobacterial activity both *in vitro* and intracellularly within human macrophages. This promising activity reinforces the importance of natural products as effective anti-tuberculosis lead compounds.

REFERENCES


