Antimycobacterial Activity of Lichens

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Abstract
Ethanol extracts of nine lichen species, namely Everniastrum cirrhatum (Fr.) Hale ex Sipman (Parmeliaceae), Flavoparmelia caperata (L) Hale (Parmeliaceae), Heteroderma leucomela (L) Poelt (Physciaceae), Lecanora flavidorufa Hue (Lecanoraceae), Leptogium pedicellatum P.M. Jorg (Collemataceae), Lobaria isidiosa (Bory) Trevi-san (Stictaceae), Rimelia reticulata (Taylor) Hale and Fletcher (Parmeliaceae), Phaeophyscia hispidula (Ach.) Essl (Physciaceae), and Stereocaulon foliolosum Nyl. (Stereocaulaceae), were evaluated for antimycobacterial properties against Mycobacterium tuberculosis H37Rv and H37Ra strains using the radiometric BACTEC method. Among the tested lichens, the virulent strain of M. tuberculosis H37Rv was found more susceptible to ethanol extract of F. caperata and H. leucomela (MIC 250 μg/mL). E. cirrhatum, R. reticulata, and S. foliolosum were found active at the concentration of 500 μg/mL. L. isidiosa, L. pedicellatum, P. hispidula, and L. flavidorufa did not exhibit activity at the maximum tested concentration of 1000 μg/mL.

Keywords: Antimycobacterial agents, BACTEC, lichens, Mycobacterium tuberculosis, radiometric assay.

Introduction
Lichens and lichen products have been used in traditional medicines for centuries and still hold considerable interest as alternative treatments in various parts of the world (Richardson, 1991). They produce characteristic secondary metabolites that are unique with respect to those of higher plants (Hale, 1983; Lawrey, 1986). Burkholder et al. (1944) reported for the first time the presence of antibiotic substances in lichens. The lichens Cetraria islandica, Lobaria pulmonaria, and Cladonia species were known for treatment of pulmonary tuberculosis (Vartia, 1973).

Tuberculosis (TB), mainly caused by Mycobacterium tuberculosis, is the leading killer among all infectious diseases worldwide and is responsible for more than 2 million deaths annually. India has 2% of the land area of the world and 15% of total world population but has a disproportionately high rate (30%) of the TB burden. Tuberculosis remains a serious public health problem with an annual incidence of 21 million out of which nearly 1 million are infected smear-positive pulmonary cases (WHO, 2006).

For more than 30 years, no antitubercular agents with new mechanisms of action have been developed. The recent increase in the number of drug-resistant clinical isolates of M. tuberculosis has created an urgent need for the discovery and development of new antituberculosis leads (Cantrell et al., 2001).

India is a rich center of lichen diversity, contributing nearly 15% of the 13,500 species of lichens so far recorded in the world (Negi, 2000). In various systems of traditional medicine worldwide, including the Indian system of medicine, these lichen species are said to effectively cure dyspepsia, bleeding piles, bronchitis, scabies, stomach disorders, and many disorders of blood and heart (Saklani & Upreti, 1992; Lal & Upreti, 1995; Negi & Kareem, 1996; Sochting, 1999).

In the current study, we investigated nine lichen species (Table 1) of traditional importance from the Indian Himalayan flora for antibacterial activity against M. tuberculosis, a highly infectious microorganism, using 460TB assays.

Materials and Methods

Collection of lichens
Lichens were collected from the Narayan Asharam, Pitthoragarh District, Uttaranchal, India, during April 2002. Dr. D.K. Upreti, Lichen Laboratory, National
Antimycobacterial activity of lichens

<table>
<thead>
<tr>
<th>Lichens</th>
<th>Voucher sample no.</th>
<th>Family</th>
<th>Medicinal use</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Everniastrum cirrhatum</em> (Fr.) Hale ex Sipman</td>
<td>CIMAP-12009</td>
<td>Parmeliaceae</td>
<td>Wound healing, antiseptic, bronchitis</td>
<td>Saklani &amp; Upreti (1992)</td>
</tr>
<tr>
<td><em>Flavoparmelia caperata</em> (L) Hale</td>
<td>CIMAP-12010</td>
<td>Parmeliaceae</td>
<td>Burns, fever, pain, and in the Siddha system of medicine</td>
<td>Saraswathy et al. (1990)</td>
</tr>
<tr>
<td><em>Heterodermia leucomela</em> (L) Poelt</td>
<td>CIMAP-12011</td>
<td>Physciaceae</td>
<td>Wound healing</td>
<td>Saklani &amp; Upreti (1992)</td>
</tr>
<tr>
<td><em>Lecanora flavidorufa</em> Hue</td>
<td>CIMAP-12012</td>
<td>Lecanoraceae</td>
<td>Disorders of blood and heart</td>
<td>Sochting (1999)</td>
</tr>
<tr>
<td><em>Leptogium pedicellatum</em> P.M. Jorg</td>
<td>CIMAP-12013</td>
<td>Collemataceae</td>
<td>No reported use</td>
<td>No report</td>
</tr>
<tr>
<td><em>Lobaria isidiosa</em> (Bory) Trevisan</td>
<td>CIMAP-12014</td>
<td>Stictaceae</td>
<td>Skin diseases, chest complaints</td>
<td>Sochting (1999)</td>
</tr>
<tr>
<td><em>Phaeophyscia hispidula</em> (Arch.) Essl</td>
<td>CIMAP-12015</td>
<td>Physciaceae</td>
<td>No reported use</td>
<td>No report</td>
</tr>
<tr>
<td><em>Stereocaulon foliolosum</em> Nyl.</td>
<td>CIMAP-12018</td>
<td>Stereocaulaceae</td>
<td>Urinary trouble, blister of the tongue</td>
<td>Saklani &amp; Upreti (1992)</td>
</tr>
</tbody>
</table>

Notes:
- "Family" refers to the family to which the lichen belongs.
- "Medicinal use" describes the traditional medicinal uses of the lichen.
- "References" are the sources where the medicinal uses were reported.

**Extract preparation**

Lichens were air-dried at room temperature under shade. After air-drying, they were ground to fine powder in a mixer grinder. The powdered materials (1.0 g) were dipped in absolute ethanol in a percolator for 72 h at room temperature. The extracts were filtered using Whatman filter paper no. 1 and concentrated at 40°C under reduced pressure and then lyophilized to obtain fine crude extract (5–10 mg/mL). Stock solutions of 100 mg/mL were made in DMSO (Merck, India) and filter sterilized for further use in bioassays. The stock solution was stored at 4°C until use.

**Mycobacterium strains**

The strains of *Mycobacterium* used in this study were *M. tuberculosis* H₃₇Rv (ATCC 27294) and *M. tuberculosis* H₃₇Ra (ATCC 25177), which were cultured on Löwenstein–Jansen media slant (Hi Media, India) at 37°C.

**BACTEC radiometric susceptibility assay**

BACTEC 460TB system (Becton-Dickinson Diagnostics Instruments Systems, Sparks, MD) was used to evaluate the antimycobacterial activity of the lichen extracts. This assay is a comparatively rapid, radiometric, drug susceptibility assay system for slow-growing *Mycobacterium* species. The BACTEC 460TB system uses BACTEC 12B medium (Becton Dickinson), which is basically an improved Middlebrook 7H9 base enriched with additional supplements. Mycobacteria utilize a 14C-labeled substrate (fatty acid, e.g., palmitic acid) present in the 12B medium, and 14CO₂ is released as a result of their metabolic activities (Siddiqi, 1996).

**Preparation of inoculum**

Cryopreserved *M. tuberculosis* strains H₃₇Ra and H₃₇Rv were taken out from a −80°C freezer and cultured on Löwenstein–Jensen medium slant. After 18–21 days of incubation, cultures were scraped from slants and transferred in 1.0 mL of BACTEC diluting fluid (Becton-Dickinson) and a complete homogenized suspension made by vortexing with glass beads (2 mm diameter). The suspension was allowed to stand for a few minutes to permit sedimentation of the bacterial clumps, if any. The turbidity of the homogenous suspension was adjusted to McFarland standard 1.0 with diluting fluid. A BACTEC 12B vial (Becton-Dickinson) was injected with 0.1 mL of this suspension. This vial was used as primary inoculum after the growth index (GI) reached a value of about 500 (approximately 1 × 10⁶ CFU/mL).

**Activity evaluation**

The three stock concentrations, that is 100, 50, and 25 mg/mL of lichen extracts, were prepared by dissolving in DMSO. From these stocks, 40 μL was transferred into BACTEC 12B vials containing 4.0 mL of medium so that the final concentration of test compounds became 1000, 500, and 250 μg/mL.
Bacterial suspension (0.1 mL) from the primary inoculum culture vial (GI ∼ 500) was injected into test vials using 1.0 mL insulin syringe. To comply with 1% proportion method (Tarrand & Groschel, 1985), 0.1 mL of primary inoculum was added to 9.9 mL BACTEC diluting fluid to obtain 1:100 dilutions. From this, 0.1 mL was injected into two 12B vials containing 4.0 mL medium along with 40 μL of DMSO that was used as controls.

Vials were incubated at 37°C, and the GI was recorded every 24 h in a BACTEC 460TB instrument. Once the GI of the control vial (1:100) reached 30, then the GI values of the test vials were compared with that of control vials based on difference in growth (ΔGI). The result was interpreted as follows: If the difference (called the ΔGI) of current GI from previous day GI in the case of drug-containing vials was lower than the ΔGI of 1:100 control vial for the same period, then the test compound was termed as active. The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which the ΔGI in the treated vial was less than that of the control.

Results

The ethanol extracts of nine lichens (Table 1) were tested against two strains of *M. tuberculosis*, H37Ra and H37Rv, through the BACTEC radiometric assay. Among these, five lichens, namely, *E. cirrhatum*, *F. caperata*, *H. leucomela*, *R. reticulata*, and *S. foliolosum* were found active against *M. tuberculosis*. The strains H37Ra and H37Rv were found susceptible to these four lichens at a concentration range of 250 to 500 μg/mL after at least 7 days of inhibition with ΔGI unit lesser than that of control (Table 2, Fig. 1). The extract of *F. caperata* was found most active among the lichen species used with the MIC at 250 μg/mL.

The virulent strain H37Rv was found more susceptible to ethanol extract of lichen *H. leucomela* (MIC 250 μg/mL). *E. cirrhatum*, *R. reticulata*, and *S. foliolosum* exhibited MIC at 500 μg/mL against both the strains, whereas *L. isidiosa*, *L. pedicellatum*, *P. hispidula*, and *L. flavidorufa* did not exhibit activity even at maximum tested concentration of 1000 μg/mL. Rifampicin and isoniazid, the front-line antitubercular drugs, were used as positive control.

Discussion

Despite intense efforts to control this disease, tuberculosis remains an expanding global health crisis claiming
2 million to 3 million human lives every year. Therefore, today tuberculosis as a disease to be managed is attracting the attention of researchers globally. With the emergence of drug-resistant strains of \textit{M. tuberculosis}, the need to search for new antituberculosis drugs has become a necessity. Using a BACTEC 460TB assay, we were able to detect the potential of lichens as growth inhibitors of \textit{M. tuberculosis}.

Numerous lichens were screened for antibacterial activity in the beginning of the antibiotic era in the 1950s (Klosa, 1953). Several lichen metabolites were found to be active against Gram-positive organisms (Lauterwein et al., 1995).

The antimycobacterial activity of lichen compounds was reported by Ingolfsdottir et al. (1998) against non-tubercular species of \textit{Mycobacterium}. These compounds exhibited very high minimum inhibitory-concentration MICs against non-pathogenic \textit{Mycobacterium aurum} for example, atranorin from \textit{Stereoaulon alpinum} (250 \(\mu\)g/mL), lobaric acid from \textit{S. alpinum} (125 \(\mu\)g/mL), salazinic acid from \textit{Parmelia saxatilis} (250 \(\mu\)g/mL), and (+)-protolichesterinic acid from \textit{Cetraria islandica} (250 \(\mu\)g/mL), except usnic acid from \textit{Cladonia arbuscula}, which was effective at lower concentration (32 \(\mu\)g/mL).

Although the antibacterial activity of some lichen species has been reported against nonpathogenic and nontubercular mycobacteria, no report was found during a literature search against \textit{M. tuberculosis}. The lichen species used in this study have not been explored earlier for this activity. The radiometric BACTEC assay appears to be more accurate, efficient, and high throughput compared with normal assays where observations are recorded based on visible growth (Chung et al., 1995; Adeniyi et al., 2004).

The results of this study indicated that four out of the nine lichen species commonly used in folk medicine were active against \textit{M. tuberculosis}. Among four lichens, extracts of \textit{Flavoparmelia caperata} and \textit{Heterodermia leucome} exhibited significant activity (MIC 250 \(\mu\)g/mL) at extract level against the virulent strain H\textsubscript{37}Rv using BACTEC assay. Based on our observations and earlier reports, it appears that the molecule(s) responsible for antibacterial activity against tubercular bacilli in active lichen species of our study may be novel with different mode of action. Our laboratory is further investigating bioactive constituents of active lichen species through bioactivity-guided fractionation and also evaluating these against drug-resistant strains of \textit{M. tuberculosis}.

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