Title

_In vitro_ antimycobacterial activity of nine medicinal plants used by ethnic groups in Sonora, Mexico

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Abstract

Background
Sonoran ethnic groups (Yaquis, Mayos, Seris, Guaríjós, Ñimas, Kikapúes, and Pápagos) use mainly herb-based preparations as their first-line medicine. Among the plants used are those with antituberculosis properties; however, no formal research data are available.

Methods
Organic extracts were obtained from nine medicinal plants traditionally used by Sonoran ethnic groups to treat different kind of diseases; three of them are mainly used to treat tuberculosis. All the extracts were tested against Mycobacterium tuberculosis H37Rv using the alamar blue redox method.

Results
Methanolic extracts from Ambrosia confertiflora, Ambrosia ambrosioides, and Guaiacum coulteri showed a minimal inhibitory concentration (MIC) value of 200, 790, and 1000 µg/mL, respectively, whereas no effect was observed with the rest of the methanolic extracts at the concentrations tested. Chloroform, dichloromethane, and ethyl acetate extracts from Ambrosia confertiflora showed a MIC of 90, 120, and 160 µg/mL, respectively.

Conclusions
The present study supports the empiric knowledge and practice of Sonoran ethnic groups only for the use of Guaiacum coulteri as anti-tuberculosis agent, and confirms that plant selection by ethnobotanic criteria improves the possibility for the identification of those species with biological activities. However, the biological activities shown by Ambrosia confertiflora demonstrate that other medicinal plants could also be considered for antimycobacterial research purposes.
Keywords

Medicinal plants, Tuberculosis, Alamar Blue, *Mycobacterium tuberculosis*, *Ambrosia*.
Background

Tuberculosis (TB) is a chronic infectious disease caused mainly by *Mycobacterium tuberculosis* [1,2]. The World Health Organization (WHO) estimated in their last report 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) [3]. Ninety-five percent of TB cases are produced in underdeveloped countries, among which, 80% correspond to the 15-29-year-old group, causing a strong socioeconomic problem [4]. Furthermore, the lack of treatment adherence has given rise to antibiotic-resistant *M. tuberculosis* strains. The resistance is classified in two: multidrug-resistant TB (MDR-TB), which does not respond to the standard treatment with first-line drugs, and extensively drug-resistant TB (XDR-TB), which occurs when resistance to second-line drug develops [5]. According to the new 2012 WHO report on surveillance and response to MDR-TB and XDR-TB, there was an estimate of 310 000 (range, 232 000-400 000) MDR-TB cases among notified TB patients with pulmonary TB in 2011, and there are 84 countries that have reported at least one case of XDR-TB [3]. This emphasizes the need to search for new drugs against tuberculosis [6,7].

According to the National System on Epidemiologic Vigilance (SINAVE), Mexico has shown a reduction in TB incidence and is considered as a medium security risk by WHO. Nevertheless, Mexico still presents 17 000 new cases and approximately 2000 deaths each year [8,9]. Mexico possesses a great geographic diversity, and also has one of the richest floras on the planet, compared with that in Malaysia and some regions in central and South America [10]. Sonora is located on the northwestern region of Mexico and is the second largest state of the country, characterized by more than 3000 plant species widely known and used by the ethnic groups living there: Yaquis, Mayos, Seris, Guarijós, Pimas,
Kikapúes, and Pápagos [11]. These groups use herbal remedies in their traditional medicine for cultural reasons, as well as for the inaccessibility to medical services and/or their expectative of resolving pathologies considered incurable or inoperable by modern medicine [10,11]. Since no report exists yet on studies showing the antituberculosis activity of Sonoran plants, the objective of the present research was to evaluate the potential antimycobacterial activity of medicinal plants used by the Sonoran ethnic groups for the treatment of tuberculosis and other diseases.
Methods

Plants and extract preparation

Plants collection

Nine plants were selected based on their traditional use by Sonoran ethnic groups for tuberculosis or symptom-related diseases, such as cough, fever, lack of appetite, weakness or caquexia, and other diverse illnesses (Table 1). All plants were collected from wild environments located in the surrounding area of the city of Hermosillo, Sonora. 

*Phoradendrom californicum* was collected in a zone located at 80 km to the South of Hermosillo; both collecting areas belong to the Sonoran desert ecosystem [12]. The aerial parts, fruits, and/or flowers were obtained and handled separately. The plants were authenticated at the Herbarium of the University of Sonora by Professor Jesús Sánchez-Escalante, where voucher specimens were deposited.

Preparation of methanolic extracts of collected plants

One-hundred grams of dry samples were macerated and kept in 1 L methanol at room temperature for one week, with occasional agitation. Solids were filtered and the extracts were concentrated by evaporation under reduced pressure at 40 °C in a Yamato RE300 rotator evaporator [13]. For their use in the susceptibility testing, working solutions of methanolic extracts were prepared in Middlebrook 7H9 broth (20% DMSO), at four times the maximum desired testing concentration. All solutions were sterilized by filtration through a 0.22-µm pore size nitrocellulose membrane (Millipore). Final concentration of DMSO in the assay was of ≤5%, which does not produce mycobacterial toxicity (internal control).
Preparation of organic extracts of plants with antimycobacterial activity

Those plant whose methanol extracts exhibited antimycobacterial activity at $\leq 200 \mu g/mL$ were further extracted with dichloromethane, chloroform, and ethyl acetate, with a similar protocol to that for methanol extracts preparation.

*Mycobacterium tuberculosis*

*Mycobacterium tuberculosis* strain H37Rv (sensitive to streptomycin, isoniazide, rifampicin, ethambutol, and pirazinamide) was used for the studies, and was provided by the National Institute of Epidemiological Diagnosis and Reference of the Mexican Ministry of Health, and was kept in the State of Sonora Public Health Laboratory.

Preparation of *Mycobacterium tuberculosis* inoculum

An initial inoculum was prepared from a solid *M. tuberculosis* culture, until it reached the log growth phase (approximately 12 days). The bacterium was then transferred to a sterile vial containing five 3-mm glass pearls and 8 mL of sterile saline solution (0.85%). The bacterial suspension was disaggregated by agitation using a Genie II vortex, and left to stand for 15 min at room temperature. The supernatant was then adjusted, using the 1 McFarland standard, to obtain a bacterial concentration of $3.0 \times 10^8$ CFU/mL. The working solution was a 1:25 dilution of this suspension in Middlebrook 7H9 broth supplemented with 0.2% (vol/vol) glycerol and 10% (vol/vol) OADC (oleic acid, albumin, dextrose, catalase enrichment; Becton Dickinson) for the *in vitro* antimycobacterial activity assay.

*In vitro* antimycobacterial assay

Determination of the minimal inhibitory concentration (MIC)
To evaluate the activity of plant organic extracts, the redox Alamar Blue® (AbD Serotec) microplate assay (MABA) was carried out as described by Franzblau and collaborators [14]. The assay was carried out on 96-well polystyrene flat bottom plates with low evaporation cover lids, in which the *M. tuberculosis* inoculum was added to Middlebrook 7H9 supplemented broth medium, and mixed with the extracts at different concentrations. In order to prevent excessive evaporation, sterile distilled water was added to the perimetal wells. As control, rifampicin (Sigma Aldrich) was used. Next, 50 µL of a fresh alamar blue-10% Tween 80 (Sigma Aldrich) mixture (1:1) was added to each well. The microplate was sealed with parafilm and incubated for 48 hours at 37 °C. Finally, the MIC was calculated and defined as the lowest extract or antibiotic concentration at which no color change of the indicator was evident. Each extract was tested in triplicate.

**Qualitative phytochemical analysis**

**Baljet reaction**

A 1% picric acid (w/v) solution in ethanol and a 10% sodium hydroxide (w/v) aqueous solution were combined at 1:1 proportion and added to 2-3 mg of sample. A positive reaction was indicated by an orange to red color [15].
Results and Discussion

Plants used in the present study were selected according to their traditional usage for tuberculosis or symptom-related diseases by the Sonoran ethnic groups. Table 1 shows the identification of the studied plants, by scientific and common name, traditional use, parts analyzed, and the ethnic group that uses them as medicinal remedy. The different parts are used as infusions, this was the reason for their evaluation in methanolic extracts. The extraction method was chosen based on the consideration that methanol extracts may contain a wide range of chemical compounds with biological activity such as terpenoids, phenols, flavonoids, saponines, steroids, and others [16-19]. In addition, some studies have reported that methanol extracts are more active than aqueous extracts in their antibacterial activity [20].

Table 2 shows the MIC of methanol extracts of the plants studied. The methanol extract from *Ambrosia confertiflora* showed a MIC value of 200 µg/mL against *M. tuberculosis* H37Rv. Methanol extracts from *Ambrosia ambrosioides* and *Guaiacum coulteri* showed a MIC of 790 and 1000 µg/mL, respectively. On the other hand, the extracts from *Acalypha californica*, *Schinus molle*, *Vallesia glabra*, *Baccharis glutinosa*, *Phoradendrom californicum*, and *Acacia farnesiana* did not show inhibition even at the highest tested concentration of 1000 µg/mL. The traditionally used plant *G. coulteri* resulted less effective than *A. confertiflora*, which is not traditionally indicated as anti-TB plant; however, *A. confertiflora* is used by Sonoran ethnic groups to treat symptoms closely related to tuberculosis, such as fever and lack of appetite (Table 1). Besides, the family to which it belongs (Asteraceae) contains a high concentration of sesquiterpene lactones (SQL) identified for their wide variety of biological activities, including its antimycobacterial effect [21, 22]. Moreover, although *A. ambrosioides* is not used as
antituberculosis treatment by Sonoran ethnic groups, it is used for placental expulsion (Table 1), given that the placenta is rich in proliferating cells [23] and *A. ambrosioides* acts on them; this fact may explain its antiproliferative effect on mycobacterial cultures. Additional studies are needed to confirm this hypothesis.

*A. farnesiana, G. coulteri, and S. molle* have been referred by the Sonoran traditional medicine as antitubercular agents, but only *G. coulteri* resulted active. We can comment that references of medicinal plants use by ethnic groups are mainly a relevant guide in the research of natural products; however, in many cases it is possible to validate the biological activity referred, but in many others it is not possible to confirm scientifically their medicinal properties.

The difference of the antimycobacterial activity among *A. confertiflora, A. ambrosioides*, and *G. coulteri* may be attributed to the difference in the relative concentrations of the active principle in these plants. However, further investigations are required to demonstrate this statement.

With the exception of *Schinus molle*, none of the plants studied had been evaluated before in Mexico for their antituberculosis properties [24]. Molina-Salinas reported a MIC of 125 µg/mL for the hexane-extract of *S. molle*; however, for the methanol extract evaluated in the present study, no MIC was determined even at the highest concentration tested (1000 µg/ml). The differences between the results obtained by Molina-Salinas and ours are attributed to the fact that a hexane extract contains mainly non-polar compounds, in contrast with the methanolic extract where higher polarity compounds are present.

A diversity of methods and procedures to assess antimycobacterial activity are currently being used resulting in a diversity of cut-off values to define a MIC value as active: MIC ≤100 µg/mL, ≤125 µg/mL, ≤ 200 µg/mL [6, 24-26]. This is the reason why no international
standard has yet being established to adequately define the level of antimycobacterial activity. For the present study we defined as active, those extracts with a MIC value $\leq 200$ $\mu$g/mL, considering the presence in such extracts of the active component(s) at adequate concentration for their further isolation and purification, as in the case of *Ambrosia confertiflora* methanol extracts. Several reports indicate the high content of terpenoid compounds and different biological activities for the *Ambrosia* genus, such as antifungal, antitrypanosomic, and antileishmania, as well as an antimalaria effect [27-30].

From *A. confertiflora*, SQLs have been isolated that could be responsible for the antimycobacterial effect observed in the present study [31, 32]. Cantrell and collaborators, in their review, report plant-derived terpenoids and some synthetic analogs that show variable antitubercular activities, by the BACTEC 460 system. Particularly, over 50 sesquiterpenes are reported [22]; from this evaluation, the most active compounds from *A. confertiflora* were santamarine and reynosin with a MIC value of 64 $\mu$g/mL. The MIC values obtained in our study are higher in comparison with the ones previously reported for reynosin and santamarine because these are pure compounds, whereas ours were crude extracts.

On this basis, and considering that the SQLs isolated by Yoshioka and collaborators are from a chloroformic extract [31], we set out to analyze the activity of less polar extracts such as chloroform, dichloromethane, and ethyl acetate from *A. confertiflora* on the growth of *M. tuberculosis* H37Rv. The results evidenced an increase in the activity of such extracts in comparison to the methanol extract; the most active extract was obtained with chloroform with a MIC value of 90 $\mu$g/mL. On the other hand, the dichloromethane and ethyl acetate extracts showed a MIC value of 120 and 160 $\mu$g/mL, respectively (Table 3). Molecules with high polarity present a reduced transport through the outer lipid
layer of mycobacteria and, in consequence, present lower antimycobacterial activity; whereas, less polar molecules present higher permeability [33, 34]. These reports are in agreement with our results, where the less polar solvent crude extracts showed better activities than those exhibited by the methanolic extracts.

Based on the studies pointing out that both *A. confertiflora* and *A. ambrosioides* are rich in SQLs [21,22], we chose to perform a qualitative Baljet reaction to establish the possible presence of these molecules that resulted positive for all our different extracts of *A. confertiflora* (Table 3) and the methanolic extract from *A. ambrosioides*.

Although this reaction is also positive for cardiac glycosides and others containing α, β-unsaturated lactones, antimycobacterial activity has been attributed to this kind of molecules. Further studies are being carried out on *A. confertiflora* to isolate the active compounds.
Conclusions

The present study supports the empiric knowledge and practice of Sonoran ethnic groups only for the use of *Guaiacum coulteri* as anti-tuberculosis agent. However, *S. molle* and *A. farnesiana*, traditionally used by the Sonoran ethnic groups as antitubercular agents, were inactive. The biological activities shown by *A. confertiflora* and *A. ambrosioides* demonstrate that other medicinal plants could also be considered for antimycobacterial research purposes.

*A. confertiflora* represents a putative species for further phytochemical and pharmacological studies for the identification of the active compound(s) against *M. tuberculosis*. 


Competing interests
The authors declare that they have no competing interests.

Author’s contributions
AGE, RERZ, EWCA, CAVC conceived the study, analyzed data, and drafted the manuscript. EWCA, MNN, ERB were involved in generation of organic extracts. EWCA carried out the biological assay. All authors have read and approved the final manuscript.

Author’s information

Acknowledgments
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References


[http://apps.who.int/iris/bitstream/10665/75938/1/9789241564502_eng.pdf]


Table 1. Plants used against tuberculosis by Sonoran ethnic groups.

<table>
<thead>
<tr>
<th>Scientific name (Family)</th>
<th>Common name</th>
<th>Traditional use</th>
<th>Part of plant used</th>
<th>Ethnic group usage</th>
<th>Voucher</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambrosia confertiflora DC. (Asteraceae)</td>
<td>Estafiate, istafiate, chi’ichibo, chibchibo</td>
<td>Intestinal parasites, stomach ache, fever, lack of appetite, menstrual symptoms</td>
<td>Leaf and branches</td>
<td>Yaquis, Mayos</td>
<td>17401</td>
</tr>
<tr>
<td>Ambrosia ambrosioides (Cav.) W.W. Payne (Asteraceae)</td>
<td>Chicura, nagua, toiwé, jupu, joroguo</td>
<td>Wounds, sores, placental expulsion, menstrual symptoms, hair diseases</td>
<td>Leaf and root</td>
<td>Mayo, Ópata, Seri, Yaqui</td>
<td>17404</td>
</tr>
<tr>
<td>Guaiacum coulteri A. Gray (Zygphyllaceae)</td>
<td>Guayacán, mocni</td>
<td>Dysentery, syphilis, rheumatism, tuberculosis, fever</td>
<td>Fruit, flowers</td>
<td>Seris</td>
<td>17400</td>
</tr>
<tr>
<td>Acalypha californica Benth. (Euphorbiaceae)</td>
<td>Cupper leaf, cancer weed, bajer cupaj buy</td>
<td>Mouth, stomach, intestine and skin cancer</td>
<td>Branches</td>
<td>Pimas</td>
<td>17402</td>
</tr>
<tr>
<td>Schinus molle L. (Anacardiaceae)</td>
<td>Pirul</td>
<td>Tuberculosis</td>
<td>Branches</td>
<td>Mayo</td>
<td>17399</td>
</tr>
<tr>
<td>Vallesia glabra (Cav.) Link (Apocynaceae)</td>
<td>Citabaró, huevito citabaró, otábaré, tonóopa, timóopa, pało verde</td>
<td>Eye inflammation, measles, rheumatism, muscular pain</td>
<td>Fruit and leaves</td>
<td>Mayo, Yaqui, Seri</td>
<td>18150</td>
</tr>
<tr>
<td>Baccharis salicifolia (Ruíz &amp; Pavon) Pers. (Asteraceae)</td>
<td>Batamote, jarilla, pasmo weed, bachomo, guachomo, uubachomo</td>
<td>Obesity, anti-contraceptive, hemorrhoids, hair disease, rabies, wounds, digestive disorders</td>
<td>Branches</td>
<td>Mayo, Opata, Seri</td>
<td>17398</td>
</tr>
<tr>
<td>Phoradendron californicum Nutt. (Loranthaceae)</td>
<td>Toji, chipchia, aaxt</td>
<td>Diarrhea, stomach polyps, venereal disease, “in-body” diseases</td>
<td>Branches, leaves and cortex</td>
<td>Mayo-Guarijíos, Seri</td>
<td>17405</td>
</tr>
<tr>
<td>Acacia farnesiana (L.) Wild (Fabaceae)</td>
<td>Huizache, vinorama, Kuká</td>
<td>Throat and digestive tract inflammation, cold, diarrhea, typhoid, wounds, swollen spleen, weak heart, tuberculosis, and headache</td>
<td>Gum, flower, seed, leaves, cortex and root</td>
<td>Mayo-Guarijíos</td>
<td>17406</td>
</tr>
</tbody>
</table>
Table 2. *In vitro* antimycobacterial activity of medicinal plants’ methanol extracts.

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Part of the plant used</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ambrosia confertiflora</em> DC.</td>
<td>Aerial parts</td>
<td>200</td>
</tr>
<tr>
<td><em>Ambrosia ambrosioides</em> (Cav.) W.W. Payne</td>
<td>Aerial parts</td>
<td>790</td>
</tr>
<tr>
<td><em>Guaiacum coulteri</em> A. Gray</td>
<td>Flower</td>
<td>1000</td>
</tr>
<tr>
<td><em>Acalypha californica</em> Benth.</td>
<td>Aerial parts</td>
<td>NI</td>
</tr>
<tr>
<td><em>Schinus molle</em> L.</td>
<td>Leaf</td>
<td>NI</td>
</tr>
<tr>
<td></td>
<td>Fruit</td>
<td>NI</td>
</tr>
<tr>
<td><em>Vallesia glabra</em></td>
<td>Aerial parts</td>
<td>NI</td>
</tr>
<tr>
<td><em>Baccharis salicifolia</em> (Ruiz &amp; Pavon) Pers.</td>
<td>Aerial parts</td>
<td>NI</td>
</tr>
<tr>
<td><em>Phoradendron californicum</em> Nutt.</td>
<td>Aerial parts</td>
<td>NI</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>NI</td>
</tr>
<tr>
<td><em>Acacia farnesiana</em> (L.) Wild</td>
<td>Fruit</td>
<td>NI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1171</td>
</tr>
</tbody>
</table>

MIC: minimal inhibitory concentration.

NI: no inhibition even at the highest tested concentration of 1000 µg/mL.
Table 3. *In vitro* antimycobacterial activity of organic extracts obtained from the *Ambrosia confertiflora*.

<table>
<thead>
<tr>
<th>Organic extracts</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>90</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>120</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>160</td>
</tr>
<tr>
<td>Methanol</td>
<td>200</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.1171</td>
</tr>
</tbody>
</table>

MIC: minimal inhibitory concentration.