Research Article

Antimycobacterial and HIV-1 Reverse Transcriptase Activity of Julianaceae and Clusiaceae Plant Species from Mexico

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1. Introduction

Tuberculosis (TB) is an illness caused by the slow-growing acid-fast bacillus Mycobacterium tuberculosis. In 1993, TB declared a global emergency by the World Health Organization [1]. In 2013, there were 9 million new cases and 1.5 million deaths; this figure included 0.4 million fatalities associated with HIV patients [2]. Mycobacterium tuberculosis is facultative intracellular bacteria that have developed resistance to first and second line antituberculosis drugs. Antibiotic resistance and multidrug-resistant TB strains are a serious problem due to the lack of results in treatment design directed to disease control and eradication [3, 4]. Due to the recent rise of TB associated with the human immunodeficiency virus VIH and the rapid spread of multidrug resistance TB strains, new classes of antimycobacterial compounds are required [5]. Compounds obtained from plants can be an important source of novel leads in the field of antituberculosis therapeutic agents [6–8], as well as against human immunodeficiency virus (HIV) [9].
Preliminary data indicate that *Amphipterygium adstringens* (Julianaceae) is a promising source of anti-TB compounds, since the stem bark extract inhibited in 95% the growth of *M. tuberculosis* at 50 μg/mL; this tree species is used in Mexican Traditional Medicine for the treatment of tuberculosis and other respiratory diseases [10]. However, other 4 species of this genus found in Mexico have not been investigated yet against *M. tuberculosis* or HIV. Julianaceae species are dioecious; that is, male and female trees are found; some morphological features are useful for sex differentiation; for instance, female specimens show flowers ordinarily in groups of four in a receptacle [11]. So far, the influence of sex in the production of secondary metabolites has been poorly documented; however, in the case of *A. adstringens* bark, an accumulation of masticadienonic and 3α-hydroxymasticadienionic acids has been found to be higher in female plants [12].

The leaf extracts of the 23 species of Clusiaceae distributed in Mexico have been examined against HIV-1 RT [9], but not against *M. tuberculosis*. Among the 5 most active species against HIV-1 RT, the tropical tree *Calophyllum brasiliense* is remarkable [9]; its leaves contain dipipano-tetracyclic coumarins, such as calanolides A, B, and C, as well as inophyllins, mainly soulatroline. Such compounds have been found to be active against HIV-1 RT [13] and *M. tuberculosis* [14]. The calanolide A shows potent and specific inhibition of HIV-RT [15]; this compound has been synthesized and is currently in pharmacological research phases II/III [16]. The hexane leaf extract of *C. brasiliense* has also been proposed for developing a standardized phytodrug; however, to achieve this goal, there is a need to obtain biological material with a high content of active compounds [9, 17]. The active compounds for other Clusiaceae species are still unknown.

The aim of this study was to evaluate Mexican Julianaceae and Clusiaceae crude plant extracts against *Mycobacterium tuberculosis* H37Rv and HIV-RT. Plants were selected according to two criteria: Julianaceae species, based on their use to treat tuberculosis in Mexican Traditional Medicine [18], whereas Clusiaceae species, based on bioprospective and chemotaxonomical data.

## 2. Methods

### 2.1. Plant Material

Clusiaceae and Julianaceae species were collected from different localities in Mexico (Table 1). Voucher specimens were deposited at the Herbarium Facultad de Ciencias (FCME) of the Universidad Nacional Autónoma de México and the Medicinal Herbarium (IMSSM) of Instituto Mexicano del Seguro Social.

### 2.2. Preparation of Extracts

The leaves of Clusiaceae species were used for preparing the tested extracts, whereas, in the case of Julianaceae, the extracts were prepared from the bark.
and leaves of specimens of different genders (male or female). All plant materials (100 g) were dried at room temperature under darkness, ground, and macerated three times for 24 h with a mixture of CH$_2$Cl$_2$–MeOH (1:1, 150 mL). The extracts were concentrated in vacuo to dryness and stored at room temperature until use.

2.3. Stock and Working Plant Extract Solution. Stock solutions of all extracts were prepared in 100% dimethyl sulfoxide (DMSO) at a concentration of 2000 µg/mL and sterilized by filtration throughout a 0.22 µm PTFE membrane. For M. tuberculosis susceptibility tests, extract solutions were prepared by diluting the stock extract in sterile 7H9 broth to obtain a 100 µg/mL concentration, whereas extract solutions for anti-RT tests were diluted in the buffer provided by the kit manufacturer to obtain a working concentration of 200 µg/mL (Lenti RT, Cavidi Tech).

2.4. Cell Culture. To assess cytotoxicity, human monocytic leukemia THP-1 cells from ATCC were cultured in RPMI 1640 medium supplemented with nonheat-inactivated 20% fetal bovine serum, 1 mM HEPES. For all experiments, THP1 were cultured in 75 cm$^2$ Falcon culture flasks under standard culture conditions of 5% CO$_2$ at 37°C at an initial density of 1.0 × 10$^6$ cells/mL. The cultures were maintained by adding fresh medium with 10% fetal bovine serum every 2-3 days.

2.5. HPLC Analysis of Extracts of Julianaceae and C. brasiliense. The bark extracts of Julianaceae and C. brasiliense leaves were analyzed by HPLC (Agilent 1100 series) according to previous reports [17, 19]. In the case of Julianaceae, the compounds oleanolic acid 1, masticadienonic acid 2, 3α-hydroxymasticadienonic acid 3, and 3β-hydroxymasticadienonic acid 4 were quantified, whereas, for C. brasiliense, the concentrations of apetalic acid 5, calanolide B 6, and soulatrolide 7 were determined (Figure 1). The chromatographic column Kromasil 100 C18, 5 µm, 150 × 4.6 mm was used to analyze Julianaceae species; the mobile phase was a mixture of 0.1% aqueous acetic acid, acetonitrile containing 0.1% acetic acid and grade reagent.
alcohol (90% ethanol + 5% methanol + 5% 2-propanol) in a proportion 18:52:30 v/v for 25 min with an isocratic flowrate of 1.0 mL/min; the injection volume was 10 μL, and the elute was analyzed at 215 nm. Each analysis was followed by a 5 min washing with 100% acetonitrile and an equilibration period with the mobile phase for 15 min.

The components of C. brasilense extract were quantified using the chromatographic column Kromasil 100 C18, 5 μm, 250 x 4.6 mm. The isocratic system acetonitrile water (6:4 v/v) with the flowrate of 1 mL/min was used for 40 min; the injection volume was 10 μL and the detection wavelength 284 nm. Each analysis was followed by a 5 min washing with 100% acetonitrile, 2 min with water, and an equilibration period with the mobile phase for 3 min.

Identification of the compounds in the extracts was carried out by comparison with the retention times (RT) of pure compounds. The calibration graphs of standards were calculated and each compound was injected by triplicate over two different days; Julianaceae compounds were injected in seven different concentrations (20, 40, 60, 100, 140, 200, and 500 μg/mL) whereas standards from C. brasilense, in six different concentrations (20, 50, 80, 120, 150, and 200 μg/mL). The linear regressions and their coefficients of determination (R²) were calculated for each compound as follows: oleicanoic acid 1, y = 3.7085x − 17.043, 0.9987; masticadienonic acid 2, y = 10.766x + 4.3811, 0.9990; mixture of 3α and β-hydroxymasticadienonic acids (3 & 4), y = 11.466x + 22.14, 0.9993; apetalic acid 5, y = 19.547x + 135.64, 0.9933; calanolide B 6, y = 27.786x + 13.369, 0.9995 and soulatrolide 7, y = 35.075x + 209.85, 0.9934. Finally, the percentage of each compound in the extracts was calculated interpolating the linear regression equation. The results are reported as the percentage of extract (Table 3).

2.6. HIV-1 RT Inhibition Test. The extracts were evaluated by a nonradioactive immunocolorimetric assay (Lenty RT Activity Assay, CaviDi Tech) according to the protocol provided by the manufacturer. All extracts were first tested at 50 μg/mL with a final DMSO concentration of 0.5% v/v. Reported values are means of 5 replicates ± SEM. The IC50 values were calculated only for extracts that inhibited ≥50% of the enzymatic activity. These extracts were tested at 7 concentrations 3.125 to 200 μg/mL with increments of 0.3 logarithms. Reported values are means of 3 replicates ± SEM. Nevirapine, a nonnucleoside reverse transcriptase inhibitor (NNRTI), was used as a positive control from 0.01 μM to 1 mM with increments of 1 logarithm.

2.7. Antimycobacterial Screening by Microplate Alamar Blue. The activity of all extracts was tested using the microplate Alamar blue assay as previously described [20, 21]. Outer wells were filled with sterile distilled water (200 μL) to prevent dehydration in experimental wells. Column 2 (B to G wells) was used to evaluate the reference drug rifampin; serial twofold dilutions in 100 μL of Middlebrook 7H9 medium were performed to obtain concentrations from 2.0 to 0.06 μg/mL. Wells 10 E and F were used for DMSO control, and wells 11 B to 11 E for the drug free control. One-hundred μL of supplemented 7H9 broth plus 100 μL the bacterial inoculum (1 x 10^8 UFC/mL) was added to each of these wells. Simultaneously a diluted control 1:100 was prepared from the bacterial suspension, representing 1% growth of the bacterial population tested. All other wells received 100 μL of the extract solution (100 μg/mL) and 100 μL bacterial inoculums. The final concentration of DMSO in well was <1.0% v/v, and all extracts were tested at 50 μg/mL. The IC50 values were calculated only for those extracts that inhibited ≥50% of the mycobacterial growth; these extracts were tested at seven concentrations (3.125 to 200 μg/mL) with increments of 0.3 logarithms. Each microplate was incubated for 7–10 days at 37°C; after incubation, one growth control was developed with a mixture of 20 μL of Alamar blue solution (ABD Serotec) and 5 μL of sterile 20% Tween 80. The plates were reincubated at 37°C for 24 h. After this period, if the control well turned from blue (no growth) to pink (growth), the remaining wells were treated with Alamar–Twee as previously described, and incubated for additional 24 h. Reduction of Alamar blue was calculated according to the manufacturer protocol. Optical density of the plate was measured at 540 and 600 nm with a spectrophotometer. The percentage of inhibition of the crude extracts was defined as 100 – percentage of reduction of Alamar blue.

2.8. Cytotoxicity Assay. Crude extracts were evaluated against human macrophages THP1 cell line. The differentiation of THP1 cells was performed with PMA (phorbol 12-myristate 13 acetate) 50 nM [22]. Twenty thousand cells in the differentiation process were placed in each well, and the plates were incubated for 72 h at 37°C, and 5% CO2 atmosphere. After the incubation, the plates were washed twice with RPMI supplemented medium and 100 μL of the extract solutions (50 μg/mL) was added to each well and reincubated for 24 h. After the reincubation time 10 μL of Alamar blue solution was added to each well, and the plates were reincubated for 24 h. The anthracycline doxorubicin was used as a positive control, and the data were interpreted as indicated by the manufacturer. Cytotoxicity was calculated as the ratio of the average OD (570 and 600 nm) obtained as compared with control wells (untreated macrophages).

3. Results

3.1. Screening of Plant Extracts. The 14 Julianaceae extracts displayed high antimycobacterial activity (>84%). These results are consistent with previously published data for A. adstringens, which inhibited 95% of mycobacterial activity at the same concentration [10]. Regarding the 5 Clusiaceae extracts, only C. brasilense showed similar potency (82%) as compared with Julianaceae; the other Clusiaceae species inhibited the growth of M. tuberculosis H37Rv in the range of 58.3 to 70.3%. Concerning HIV-RT, the Clusiaceae extracts showed inhibition in a range of 27.3 to 67.6%, whereas the Julianaceae extracts inhibited this enzyme in the range of 7.9 to 49.8% (Table 1). Since macrophages are potential targets of M. tuberculosis and HIV, in order to assess the cytotoxicity of the extracts, they were tested against macrophages derived
The HPLC analysis of the three selected Julianaceae bark extracts showed the following metabolites: mastica- 
diolic acid (3 and 4). Under the chromatographic conditions used, 
quantifying individually the isomers 3 and 4 was not possible 
due to their similar retention times (RT = 20.7 and 20.9 min, 
resp.); therefore, these compounds were quantified as the 
mixture of acids. Oleanolic acid 1, which has been previously 
reported as a constituent of A. adstringens bark, was not 
detected in the extracts studied (Table 3). The concentration 
of compounds 2 and mixture of 3 and 4 may not be related to 
*M. tuberculosis* activity since these three compounds from the 
most active extracts (IC<sub>50</sub> < 2.35 μg/mL) were found in high 
(A. *amplifolia*, male), medium (A. *simplicifolium*), and low 
(A. *glaucum*, male) content. In addition, the other Julianaceae 
bark extracts which also showed significant antimycobacterial 
activity (>84.6% at 50 μg/mL) showed no correlation 
with the concentrations of the analyzed compounds, as they 
include the species with the highest concentrations of 2, 3, 
and 4 (A. adstringens from male trees; 14.23% and 10.91%, 
resp.), but also the species devoid of these compounds 
(A. *glaucum* female). The above findings suggest that the 
antimycobacterial active principle in the Julianaceae extracts 
is not compound 2, 3, or 4. The same can be stated for HIV- 
RT, since almost all of these extracts, with the exception of A. 
simplicifolium, showed poor activity (Tables 1 and 2).

With regard to gender and production of secondary 
metabolites, male specimens showed the higher levels of 
masticadienonic acid 2 and α-hydroxymasticadienonic acid 3, 
as compared to female specimens (Figure 2). Our 
results are opposite to those previously published, in which 
the accumulation of compounds 2 and 3 was higher in female 
plants [12].

In the case of Clusiaceae species, only *C. brasiliense* was 
analyzed by HPLC (Figure 3). Apetalic acid (RT = 18.64), 
calanolide B (RT = 23.34), and soulatrolide (RT = 25.30) 
were present in 0.01%, 2.4%, and 6.8%, respectively. Previously, 
a high antimycobacterial and anti-HIV-RT activity of 
soulatrolide and calanolide B has been reported [14]. Hence, 
they can be considered, respectively, as the antimycobacterial 
and anti-HIV active principles.

### 4. Discussion

HIV infection decreases the number of CD4+ lymphocytes, 
so it is quite probable that an HIV+ patient can acquire or 
reactivate tuberculosis disease [25]. During the last 30 years, 
24 anti-HIV drugs have been approved by the FDA [9] but any 
novel anti-TB drug. The rapid spread of multidrug resistance 
to TB strains remarks that new classes of antimycobacterial 
compounds are now required [26, 27]. The treatment of 
patients coinfected with TB/HIV presents also additional 
challenges, such as intolerance and contraindications for the 
use of combined drugs and low attachment to medication 
regime due to the administration of a large number of 
medications. The highly active retroviral therapy (HAART) 
for HIV patients involves the administration of a protease 
inhibitor and two reverse transcriptase inhibitors (1 nonnu-
cleoside + 1 nucleoside type), which represent administration 
of 20 pills/daily; in addition, monotherapy for TB adds 10 to 
drugs [28]. Moreover, HIV-1 protease inhibitors nullify the 
effect of the rifampin used as first-line drug for the treatment of 
TB [26, 27]. In this context, new drugs are needed, if at all 
possible, active to both targets.

A previous report indicated that one *Amphipterygium* 
species had a promising activity against TB [10], and our 
results confirm this finding and extend it to the five species 
of this genus present in Mexico, which are quite potent 
against *M. tuberculosis*; however, these extracts showed 
moderate or poor activity to HIV-RT. No correlation with 
the content of triterpenoids as masticadienonic acid, 3α,
and 3β-hydromasticadienonic acids was detected for anti-TB or anti-RT activities for the extracts of these species and deserves future investigations in order to identify the active compounds. According to our results Amphipterygium species are a source of potent anti-TB extracts with low cytotoxicity to macrophages.

A previous report indicated that the five Clusiaceae species here examined have moderate to high activity against HIV-RT [9], and our results confirm this finding but also show for the first time that they are quite potent to \( \text{M. tuberculosis} \). In particular, \( C. \text{brasiliense} \) organic extract from the leaves could be suitable for developing a phytodrug due to its content of active molecules to both targets and the calanolides A, B, C, and soulatrolide. Our results also evidence that biodiversity is a useful and valuable source for molecular leads aimed to \( M. \text{tuberculosis} \) and HIV. To date it has been described at least 84 natural compounds active against \( M. \text{tuberculosis} \) [7]. On the other hand, 120 substances, mainly extracted from plants, have been identified with activity \textit{in vitro} against HIV [13]. Only a few of them have been examined for both properties.

5. Conclusion

In this study, the high antimycobacterial and moderate anti-HIV-RT activities of Julianaceae bark extracts, especially
Amphipterygium simplicifolium, A. glaucum, and A. molle have been showed. These activities are not related to the triterpenes quantified in this study and suggest that other compounds are the active molecules. Our results provide sustenance to the use of species of Julianaceae plants in Mexican Traditional Medicine in the treatment of tuberculosis. Concerning Clusiaceae, the leaf extracts of the 5 species tested showed good activity against both targets. All the extracts showed low toxicity to human macrophages. *Calophyllum brasiliense* extract may be suitable for developing a phytodrug with dual activity against HIV-1 and *M. tuberculosis* due to its content of the active molecules calanolides and soulatrolide.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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