

Antimicrobial Activity of *Persea americana* Mill (Lauraceae) (Avocado) and *Gymnosperma glutinosum* (Spreng.) Less (Asteraceae) Leaf Extracts and Active Fractions Against *Mycobacterium tuberculosis*

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Abstract: Tuberculosis is considered a re-emerging disease and one of the most important health problems worldwide. It causes approximately three million deaths every year. In the last decades multi-drug resistant *Mycobacterium tuberculosis* has been detected, exacerbating the situation. The main objective of this research was to test the effect of *Persea americana* and *Gymnosperma glutinosum* (commonly used in Mexico to treat diverse diseases) leaf extracts and active fractions against *M. tuberculosis* H37Ra and H37Rv strains. Colony-forming units method was used to determine the MICs of plant extracts, active fractions and clofazimine against the mycobacterial strains. Methanol extracts from both plants were observed to possess antimycobacterial activity; however, *P. americana* extracts possessed higher antimicrobial activity against such mycobacteria strains than those of *G. glutinosum*, as determined by their respective MICs of 125 µg/ml versus 250 µg/ml against H37Ra strain, respectively, and 62.5 µg/ml versus 250 µg/ml against H37Rv strain respectively. It was also observed that hexane fraction of *P. americana* extract caused MICs of 31.2 µg/ml against H37Ra and H37Rv strains, whereas *G. glutinosum* GGF5 hexane extract fraction induced MICs of 125 µg/ml against H37Ra strain, fraction GGF6 caused MICs of 250 µg/ml against H37Ra and H37Rv strains, and fraction GGF7 induced MICs of 125 µg/ml against H37Ra and H37Rv strains. MICs of control clofazimine were 0.31 µg/ml against H37Ra and H37Rv strains respectively. We have experiments underway to elucidate the putative antimycobacterial active molecule (s) present in both plants.

Key words: Anti-TB activity • *P. americana* • *G. glutinosum* • *Mycobacterium tuberculosis*

INTRODUCTION

Mycobacterium tuberculosis is a facultative intracellular pathogen that causes high morbidity and mortality worldwide. It is associated with three million deaths every year whose increase has been related to AIDS pandemics and multi-drug resistance mycobacteria strains [1,2]. In the last decade, anti-tuberculosis (TB) drugs, including rifamicines, fluoroquinolones and oxazolidinones have been produced, but have not yet been evaluated in clinical trials (except for KRM-1648 and some quinolones) [3-8]. Because of mycobacterial resistance to current antibiotics, new and more efficient drugs and therapies are required [9].

Interest in botanical medicine has increased over the years, not only by physicians, but also by public in general who seem to prefer products containing “natural extracts” than those based on “synthetic” substances [10, 11]. Although there are an enormous variety of plants in Mexico, the percent of species studied for its antibiotic activity is low, and their effectiveness should be scientifically validated to increase the credibility of their use [12]. Some plant families have been demonstrated to produce antimicrobial substances against a wide variety of bacteria [13] and fungus [14].

Persea americana leaves have been reported to possess anti-inflammatory [15] and antifungal [16] activities; similarly, *Gymnosperma glutinosum*, commonly

used for the treatment of diarrhoea in Mexico, has been recently reported to possess antibiotic activity against *Sarcina lutea*, *Aspergillus niger* and *Trichophyton mentagrophytes* [17]. In addition, a flower extract from the plant chrysanthemum (*Chrysanthemum morifolium*; Asteraceae family) was shown to possess antimycobacterial activity [13]. The present study was undertaken to investigate the potential of *Persea americana* and *Gymnosperma glutinosum* as sources of antimycobacterial agents which has not previously been explored.

METHODS

Reagents and Culture Media: Middlebrook 7H9 broth and 7H10 agar were purchased from (Becton Dickinson, Cockeysville, MD). Alamar blue 100X was purchased from BioSource International, Inc. (Camarillo, CA). Clofazimine was obtained as powder from Sigma–Aldrich (St. Louis, MO). H37Ra and H37Rv *Mycobacterium tuberculosis* strains were kindly provided by the Instituto de Investigaciones Biomédicas del Noreste, in Monterrey, Mexico. The strains were maintained by subculture on Löwenstein-Jensen media and 7H9 broth (Remel, Lenexa, Kansas City, MO).

Plant Extracts Preparation: Plants used in this study were identified by M.Sci. María del Consuelo González de la Rosa, Chief of the Herbarium of the Biological Sciences College at Autonomous University of Nuevo Leon, with voucher specimen numbers 024782 for *P. americana* and 024784 for *G. glutinosum*; plants were collected in San Nicolás de los Garza and Escobedo N. L., México, respectively. Leaves were washed, dried and macerated. To prepare aqueous extracts, 5 grams of leaves powder were allowed to stand in boiling water (80ml) for 10 minutes, freeze dried (Freeze Dry Systems, Labconco Corporation, KC) and stored at -20°C until use. To produce methanol extracts, 5 grams of leaves powder were allowed to stand in 100% methanol (80 ml) for 24 hours at room temperature, solution was then dried in a rotary evaporator Büchi (Brinkmann Instruments Inc., Switzerland) and stored at 6°C until use. Aqueous and methanol extracts were then diluted to 1mg/ml in sterile media. Under aseptic conditions the products were then filtrated through 0.22µ-pore size diameter filters (Whatman filters, Whatman International Ltd., Maidstone, England) and one milliliter aliquots were stored in 1.5ml Eppendorf tubes at -20°C.

Production of Plant Extract Fractions: Sixty grams of the powdered *G. glutinosum* and *P. americana* leaves were sequentially extracted with 500 ml hexane, 500 ml methylene chloride, 500 ml of a mixture of methanol-methylene chloride (1:1 v/v) and 500 ml methanol by Soxhlet system during 40 h each using a 45 x 123 mm cellulose cartridge (Cellulose extraction thimbles, Whatman, Maidstone, England). Next, the extracts were concentrated using a rotavapor (Rotavapor R-205 Büchi, Brinkman Instruments, inc., Westbury, NY), labeled, and stored until use. Only *G. glutinosum* hexane extract was further dissolved in chloroform and then chromatographed on a 32-63 µm particle size, silica gel 60 column (Universal Scientific Incorporated, Atlanta, GA) using first chloroform and then mixtures of chloroform-ethyl acetate as the eluent. Nine fractions were then obtained containing one to three compounds as observed on thin layer chromatography (silica gel; chloroform-ethyl acetate, 1:1). These fractions were named GGF1 ($R_f = 1$ and 0.879), GGF2 ($R_f = 0.882$), GGF3 ($R_{fs} = 0.823, 0.729$, and 0.635), GGF4 ($R_f = 0.541$), GGF5 ($R_{fs} = 0.541, 0.337$, and 0.216), GGF6 ($R_f = 0.541$), GGF7 ($R_f = 0.337$), GGF8 ($R_{fs} = 0.337$ and 0.216) and GGF9 ($R_f = 0.216$).

Anti-Mycobacterial Drug: Stock solution of clofazimine was prepared in advance at a concentration of 1mg/ml in 500µL of dimethyl sulfoxide (DMSO), 500µL of methanol and 1.5µL of 6N HCl; this solution was then filter sterilized and kept at -20°C for not more than 1 month. Working solutions of the drug were prepared at 1µg/ml in Middlebrook 7H9 Broth [18].

Culture and Growth of Mycobacteria: *M. tuberculosis* strains were maintained on slants of Lowenstein-Jensen medium (Remel, Lenexa, Kans.). Culture suspensions were prepared by growing an initial inoculum on Middlebrook and Cohn 7H11 agar plates (BBL, Becton Dickinson, Cockeysville, Md.) and subculturing in Middlebrook 7H9 broth (MBB) (Difco Laboratories, Detroit, Mich.) containing 0.5% glycerol (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 10% OADC (oleic acid-albumin-dextrose-catalase) enrichment (Remel) and 0.05% of Tween 80 (polisorbate 80, sorbitan-oleate, polioxietilen; Materiales y Abastos especializados, Jalisco, México) for 72 h at 37°C. The culture suspensions were shaken and sonicated in a bath type ultrasonicator (output power, 80 W; Laboratory Supplies Co., Hicksville, N.Y.) until visible clumps were disrupted (usually 15 to 30 s). Next, suspensions were diluted in the same broth to

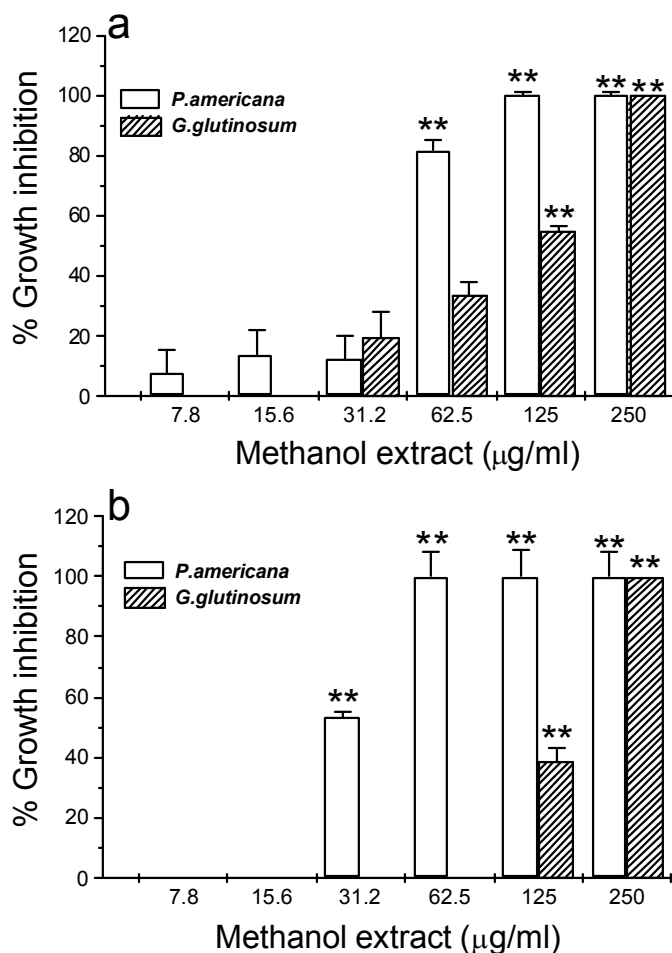


Fig. 1: Antimicrobial activity of *P. americana* and *G. glutinosum* methanol extracts against *M. tuberculosis* strains H37Ra (a) and (b) H37Rv. Percent growth inhibition was measured by the CFU method as explained in the text. Data represent mean \pm SEM of three replicates per treatment from four independent experiments. **, $p < 0.01$. The CFU value of untreated control was $2.8 \times 10^7 \pm 9.1 \times 10^6$ and $4.7 \times 10^7 \pm 3 \times 10^6$ CFU/ml for H37Ra and H37Rv strains respectively.

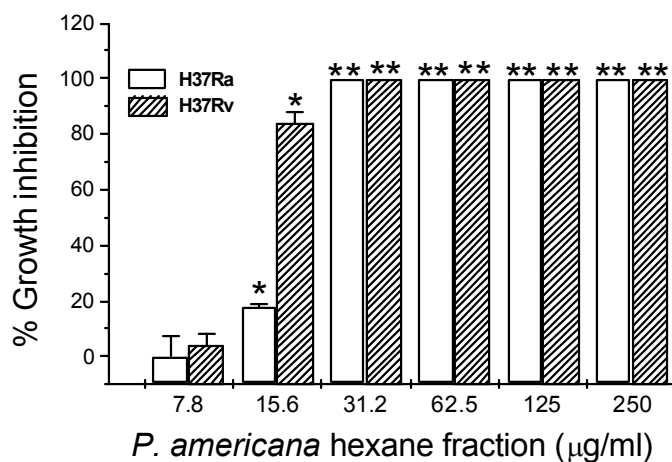


Fig. 2: Effect of *P. americana* soxhlet hexane extracts against *M. tuberculosis* strains H37Ra and H37Rv. Percent growth inhibition was measured by the CFU method as explained in the text. Data represent mean \pm SEM of three replicates per treatment from four independent experiments. *, $p < 0.05$; **, $p < 0.01$. The CFU values of untreated control were $1 \times 10^8 \pm 5 \times 10^6$ and $1 \times 10^8 \pm 3.1 \times 10^6$ CFU/ml for H37Ra and H37Rv strains respectively.

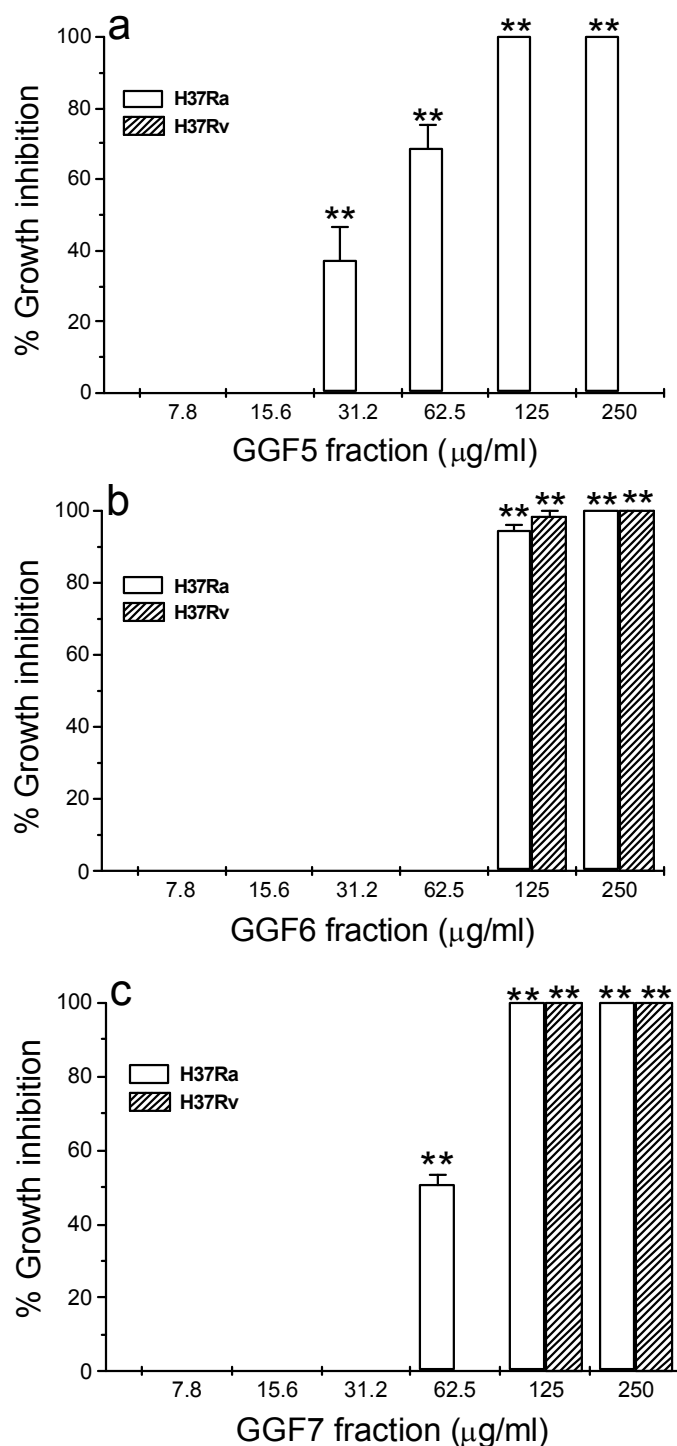


Fig. 3: Effect of fractions GGF5, GGF6 and GGF7 from *G. glutinosum* hexane extract against *M. tuberculosis* strains H37Ra and H37Rv. Percent growth inhibition was measured by the CFU method as explained in the text. Data represent mean±SEM of three replicates per treatment from four independent experiments. **, $p < 0.01$. The CFU values of untreated control were $9.7 \times 10^7 \pm 1.7 \times 10^6$ CFU/ml for H37Ra and $1.2 \times 10^8 \pm 1.7 \times 10^6$ CFU/ml for H37Rv in GGF5 experiments; $1 \times 10^8 \pm 2.6 \times 10^6$ CFU/ml for H37Ra and $1.3 \times 10^8 \pm 8.3 \times 10^6$ CFU/ml for H37Rv in GGF6 experiments, and $1 \times 10^8 \pm 5 \times 10^6$ CFU/ml for H37Ra and $1.4 \times 10^8 \pm 9.4 \times 10^6$ CFU/ml for H37Rv in GGF7 experiments.

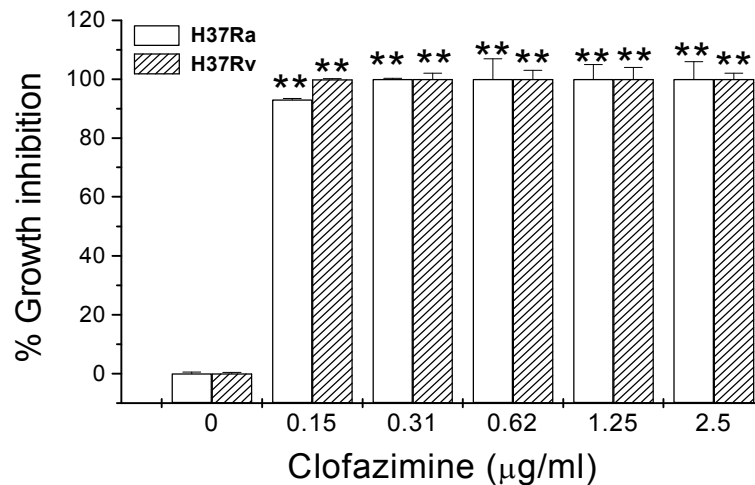


Fig. 4: Effect of clofazimine control on *M. tuberculosis* strains H37Ra and H37Rv. Percent growth inhibition was measured by the CFU method as explained in the text. Data represent mean \pm SEM of three replicates per treatment from four independent experiments. **, $p < 0.01$.

yield a concentration of 10^4 viable bacilli per ml, as confirmed by plate counts on 7H11 agar. Bacterial suspensions were then frozen at -70°C until used (cultures were stored no more than 1 month in a freezer, and fresh cultures were prepared monthly); for the liquid medium cytotoxicity experiments, suspensions were thawed, shaken, and sonicated for 90 s before use [18]. For the solid medium cytotoxicity assays, 7H10 agar (Difco Laboratories, Detroit, MI) was used to determine colony-forming units (CFU).

Drug Susceptibility Testing: Results were confirmed by determining CFU in 7H10 agar plates [19]. Growth inhibition was calculated as follows: Percent growth inhibition = (treatment value/untreated control value) X 100.

Statistical Analysis: Data distribution was evaluated by the Kolmogorov-Smirnov one-sample method. The results were expressed as mean \pm SEM of the response of 3 replicate determinations per treatment from three independent experiments. Level of significance was assessed by the Student *t* test and one-way ANOVA. Dunnett's test was used to determine differences between treated and control groups.

RESULTS AND DISCUSSION

As measured by the CFU method, *P. americana* and *G. glutinosum* methanol extracts induced significant ($p < 0.01$) 82% to 100% and 55% to 100% growth inhibition and MICs of 125 $\mu\text{g/ml}$ and 250 $\mu\text{g/ml}$ against H37Ra

strain respectively (Fig. 1a). In addition, *P. americana* and *G. glutinosum* methanol extracts caused significant ($p < 0.01$) 53% to 100% and 39% to 100% growth inhibition and MICs of 62.5 $\mu\text{g/ml}$ and 250 $\mu\text{g/ml}$ against H37Rv strain respectively (Fig. 1b).

It was also observed that only the soxhlet hexane fraction of *P. americana* extract inhibited mycobacterial growth. This fraction induced significant ($p < 0.05$) 18% to 100% and 84% to 100% growth inhibition against H37Ra and H37Rv strains respectively, and MICs of 31.2 $\mu\text{g/ml}$ (Fig. 2). With regard to the effect of *G. glutinosum* hexane extract fractions on *M. tuberculosis* growth, the only fractions that showed antimicrobial activity were GGF5, GGF6 and GGF7. Fraction GGF5 induced significant ($p < 0.01$) 37% to 100% and MIC of 125 $\mu\text{g/ml}$ against H37Ra strain (Fig. 3a); fraction GGF6 caused significant ($p < 0.01$) 94% to 100% and 98% to 100% growth inhibition and MICs of 250 $\mu\text{g/ml}$ against H37Ra and H37Rv strains respectively (Fig. 3b); and fraction GGF7 induced significant ($p < 0.01$) 50% to 100% and 100% growth inhibition and MICs of 125 $\mu\text{g/ml}$ against H37Ra and H37Rv strains respectively (Fig. 3c). As an antimycobacterial drug control, clofazimine caused significant ($p < 0.01$) 93% to 100% and 99.99% to 100% growth inhibition and MICs of 0.31 $\mu\text{g/ml}$ against H37Ra and H37Rv strains respectively (Fig. 4).

Scientific studies with *Asteraceae* and *Lauraceae* products as antibacterial agents are rare, and the selection of the plants for the present study was based on their medicinal traditional use in Mexico. However, renewed interest in herbal pharmacology has generated recent investigation with a wide variety of *Asteraceae* plants

like *Baccharis gaudichaudiana* [20], *Anthemis aciphyla* [21], *Echinops ritro* [22], *Artemisa asiatica* [23], *Pterocaulon spp* [14] and *Lauraceae* plants such as *Machilus odoratissima* [24] and *Cinnamomum verum* [25, 26].

The use of aqueous and methanol plant extracts is common for this type of studies [13, 27, 28]; however, we selected other strong non-polar solvents to isolate antimicrobial activity agents from these plants because of the lipidic nature of mycobacteria cell walls. Other researchers also reported that low polarity fractions, such as hexane- and acetone-based extracts are the most efficient mycobacteria inhibitors [11, 27, 29].

In the present study, it was demonstrated antimycobacterial activity of *P. americana* and *G. glutinosum* extracts, however, *P. americana* hexane extracts possessed higher antimicrobial activity against strains H37Ra and H37Rv than those of *G. glutinosum*, as determined by their MICs (Figs. 1-3). Based on the results, we believe the plants used in this study have potential as sources for antituberculosis drugs, and we have experiments underway leading to the identification of the active molecules present in these plants.

ACKNOWLEDGMENTS

This work was supported by the Programa de Apoyo a la Investigación Científica y Tecnológica (PAICyT) CN1363-06 from Universidad Autónoma de Nuevo León to RGF.

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