Evaluation of Some Plant-derived Secondary Metabolites Against Sensitive and Multidrug-resistant *Mycobacterium tuberculosis*

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**Abstract.** The results on the bioevaluation of thirty five plant-derived secondary metabolites against one sensitive and three multidrug-resistant clinical isolates of *Mycobacterium tuberculosis* are reported. Results toward the sensitive strain showed that five products gave MIC values of 12.5 µg/mL: the alkaloids 6-methoxydihydrochelerytrine (2) and 6-methoxy-dihydrochelirubine (6), the flavane pinostrobin (17), 1-hydroxy-bensoisochromanquinone (23) and 23-hydroxy-5a-lanosta-7,9(11),24-triene-3-one (33). These were followed by the peracetyl-streptosidoline lactam (12) and the quinone aloe-emodin (24) which displayed MICs of 6.25 µg/mL. Finally, lirodenine (8) was the most active (MIC: 3.125 µg/mL) of all secondary metabolites. Results with the multidrug-resistant clinical isolates showed that 6-methoxy-dihydrochelirubine (6) was the most active (MIC: 12.5 µg/mL).

**Keywords:** *Mycobacterium tuberculosis*, antituberculosis activity, bioactive secondary metabolites, alkaloids, flavonoids, quinones, triterpenes, diterpenes.

**Introduction**

Tuberculosis remains one of the major deadliest infectious diseases for humans. Approximately 9.2 million people develop the active disease each year, while 1.7 million cases of active disease result in death in the same period. The situation is worsening primarily because the association between tuberculosis and epidemic HIV/AIDS as well as the growing prevalence of multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains [1]. These acute problems have led to search for structurally effective new drugs against this bacterium.

Part of our area of interest involves the search for structurally novel anti-tuberculosis natural products from higher plants. Plants have been used worldwide in traditional medicines for the treatment of various diseases and it is estimated that even today approximately 65-75% of the world’s population rely only on medicinal plants as their primary source of medicines [2]. The phytochemical study of some of these plants has yielded a number of active natural products [3,4], thus the screening of natural products from higher plants constitutes one avenue in the search for new lead antitubercular agents. In this study we screened thirty five phytochemicals against one drug sensitive and three multidrug resistant clinical isolates of *M. tuberculosis* using the Alamar Blue assay. The tested secondary metabolites included alkaloids (1-13, scheme 1), flavonoids (14-22, scheme 2), quinones (23, 24, scheme 3), triterpenes (25-33, scheme 4) and diterpenes (34-35, scheme 4). The isolation and characterization of the different phytochemicals were described previously [5-14].

**Results and Discussion**

Plant derived secondary metabolites 1-35 were evaluated against *M. tuberculosis* H37Rv and three MDR isolates (345, M-12 and M-20) and the compounds which displayed activity are listed in Table 1. Compounds 4, 7, 11, 16, 18-22, 27, 30 and 31 were considered inactive (MIC > 50 µg/mL for
H37Rv and > 200 µg/mL for 345, M-12 and M-20). Results show that seven secondary metabolites exhibited MIC values of 50 µg/mL, six had MIC values at 25 µg/mL, three presented values of 12.5 µg/mL and two of 6.25 µg/mL and only one had an MIC value of 3.125 µg/mL towards the sensitive strain H37Rv. Among those metabolites with moderate activity (MIC: 12.5 µg/mL) were the flavonoid pinostrobin (17) from Teloxys graveolens, 1-hydroxy-benzoisochromanquinone from Psychotria camponutans (23) and the triterpene 23-hydroxy-5-lanosta-7,9(11),24-triene-3-one (33) isolated from Guarea rhopalocarpa. Those with MIC values of 6.25 µg/mL were the alkaloid peracetylstrictosidine lactam (12) from Cephaelis dichroa and the quinone aloe-emodin (24) from Stephania dinklagei. Liriodenine (8) isolated from S. dinklagei showed the best activity (MIC: value of 3.125 µg/mL).

However, the only secondary metabolites that showed good activity against the three MDR M. tuberculosis clinical isolates were the alkaloids 6-methoxydihydrocleeritetirine (4, MIC 25-50 µg/mL), 6-methoxydihydrosanguinarine (5, MIC 12.5-50 µg/mL) and 6-methoxy-dihydrochelirubine (6, MIC:
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12.5 µg/ml), isolated from *Bocconia arborea*. Interestingly, while 6-methoxy-dihydrochelirubine showed the same activity (MIC 12.5 µg/ml) against the sensitive strain and MDR clinical isolates, the other two alkaloids were more active against the sensitive bacteria. Phaeanthine (13) and 1-hydroxy-benzosichromanquinone (23) showed moderate activity against the sensitive bacteria and displayed only weak activity (MIC: 100–200 µg/ml) against three and two of the resistant bacteria, respectively.

Liriodenine (8), the most active metabolite against the sensitive strain showed only moderate activity (MIC 100 µg/mL) against only one of the MDR isolates. Similarly, peracetethylstrictosidine lactam (12), 3-oxo-7,24Z-dien-tirucalla-26-oic acid (29) and 5α-lanosta-7,9(11),24-triene-3α,23-diol (32), as well as 5,7-dihydroxy-6-methyl-8-prenyl-flavone (15) and pinostrobin (17) showed moderate to good activity against the sensitive strain and showed only weak activity (MIC: 200 µg/mL) against one MDR isolate.

Table 1. Antimycobacterial activity of some secondary metabolites against *M. tuberculosis* H37Rv and MDR clinical isolates of *M. tuberculosis*. Compounds 4, 7, 11, 16, 18-22, 27, 30 and 31 were considered inactive (MIC > 50 µg/mL for H37Rv and > 200 µg/mL for 345, M-12 and M-20).

| Plant species (Family) | Secondary metabolite (Type) | Reference | *M. tuberculosis* MIC (µg/mL) |
|------------------------|-----------------------------|-----------|-----------------------------|
|                        |                             |           | H37Rv | 345 | M-12 | M-20 |
| *Bocconia arborea* (Papaveraceae) | 1 (A) | 25 | >200 | >200 | >200 |
|                        | 2 (A) | 12.5 | 50 | 50 | 25 |
|                        | 3 (A) | 13 | 50 | >200 | >200 | >200 |
|                        | 5 (A) | 50 | 25 | 50 | 12.5 |
|                        | 6 (A) | 12.5 | 12.5 | 12.5 | 12.5 |
| *Stephania dinklagei* (Menispermaceae) | 8 (A) | 3.125 | >200 | >200 | >200 |
|                        | 9 (A) | 50 | >200 | >200 | >200 |
| *Cephaelis dichroa* (Rubiaceae) | 10 (A) | >50 | 100 | >200 | >200 |
|                        | 12 (A) | 6.25 | 200 | >200 | >200 |
| *Triclisia patens* (Menispermaceae) | 13 (A) | 25 | 200 | 200 | 100 |
| *Eysenhardtia platycarpa* (Leguminosae) | 14 (F) | >50 | 200 | 200 | >200 |
|                        | 15 (F) | 25 | 200 | >200 | >200 |
| *Teloxys graveolens* (Chenopodiaceae) | 17 (F) | 12.50 | 200 | >200 | >200 |
| *Psychotria camponutans* (Rubiaceae) | 23 (Q) | 12.50 | 200 | >200 | 100 |
| *Stephania dinklagei* (Menispermaceae) | 24 (Q) | 7 | 6.25 | >200 | >200 | >200 |
| *E. platycarpa* (Leguminosae) | 25 (T) | 14 | 50 | >200 | >200 | >200 |
| *Celaenodendron mexicanum* (Euphorbiaceae) | 26 (T) | 25 | NTb | NTb | NTb |
|                        | 28 (T) | 50 | >200 | >200 | >200 |
|                        | 29 (T) | 50 | >200 | >200 | >200 |
|                        | 32 (T) | 12.50 | >200 | >200 | >200 |
| *Guarea rhopalocarpa* (Meliaceae) | 33 (T) | 8 | 25 | >200 | >200 | 200 |
|                        | 34 (D) | 25 | >200 | >200 | >200 |
|                        | 35 (D) | 50 | >200 | >200 | >200 |
| **Standard drugs** | | | | | | |
| Isoniazid | 0.06 | <50 | <50 | <50 |
| Ethambutol | 2.0 | 12.50 | 12.50 | 12.50 |
| Rifampicin | 0.06 | <50 | <50 | <50 |
| Streptomycin | 0.50 | 3.125 | 12.50 | 25 |

a: Alkaloid, F: Flavonoid, Q: Quinone, T: Triterpene, D: Diterpene. b: NT: not tested.
In assessing structure-activity relationships, the higher activity of 6-methoxy-dihydrochelerythrine (2), compared to that of dihydrochelerythrine (1) might be explained by a higher lipophilicity of the former, which may facilitate its passage across the cell membrane of the mycobacteria. A similar argument might be made for the following pair of metabolites: per-acetylstrictosidine lactam (12) and strictosidine lactam (11); 5,7-dihydroxy-6-methyl-8-prenyl-flavanone (15) and 5,7-dihydroxy-8-prenyl-flavanone (14); pinostrobin (17) and pinocembrin (16); and 23-hydroxy-5α-lanosta-7,9(11),24-triene-3-one (33) and 5α-lanosta-7,9(11),24-triene-3α,23-diol (32), with the exception of acetyl oleanolic acid (25) and oleanolic acid (26).

The antitubercular activity detected for 25 and 26 is in agreement to that previously reported [17,18]. A comparison of the diterpene epimers of sandaracopimaradiene indicates that the configuration of the hydroxyl group at C-2 is important for activity, since ent-8(14),15-sandaracopimaradiene-2α,18-diol (34) was twice as active as ent-8(14),15-sandaracopimaradiene-2β,18-diol (35) against the sensitive strain. A similar trend was observed between oleanolic acid (25) and epi-oleanolic acid (26).

Finally, it has been reported that the benzo(c)phenanthridine alkaloids chelerythrine and chelirubine inhibited the growth of M. tuberculosis H37Rv by ≥ 94% at 12.5 µg/mL [3]. We found that dihydrochelerythrine (1, MIC: 25 µg/mL) was half as active as chelerythrine against the sensitive strain, whereas 6-methoxy-dihydrochelirubine (6) has the same activity (MIC: 12.5 µg/mL) against the sensitive and multidrug-resistant isolates.

The results obtained in this evaluation indicate that the structural skeletons of the most active alkaloids 6-methoxy-dihydrochelirubine (6) and liriodenine (8) represent useful templates for the development of new anti-tuberculosis drugs.

Experimental

Preparations of samples for testing

Phytochemicals were isolated and identified previously by Camacho et al. [5-10], Solis et al. [11,12], Navarro et al. [13], Narváez-Mastache et al. [14]. The purity of the natural products and derivatives was determined by HPLC. Stock solutions were prepared by dissolving the various metabolites in dimethylsulfoxide at a concentration of 20 mg/mL and stored at -70 °C until use. Before the assay, stock solutions were four-fold diluted in supplemented Middlebrook 7H9 media.

M. tuberculosis strains

For the present study, the following bacteria were used: M. tuberculosis H37Rv American Type Culture Collection (ATCC) 27294, streptomycin, isoniazid, rifampicin, ethambutol, and pyrazinamide-sensitive; MDR M. tuberculosis clinical isolates (M-12, M-20 y 345) obtained from Secretaría de Salud de Tamaulipas, Jurisdicción Sanitaria IV, Reynosa, Tamaulipas, México. The local ethics committee approved all protocols used in this study.

Preparation of test inoculum

Each mycobacteria was cultured at 37 °C in Middlebrook 7H9 broth supplemented with 0.2% glycerol and 10% OADC (oleic acid albumin dextrose catalase, Becton, Dickinson and Company, USA) until logarithmic growth was achieved. Each culture was mixed with a sufficient volume of sterile supplemented Middlebrook 7H9 broth to reach turbidity equivalent to that of McFarland’s nephelometer No. 1 standard. The suspension was then diluted 1:20 with the same culture medium immediately before use in antitubercular test.

Antimycobacterial test

The activity of all phytochemicals against the aforementioned M. tuberculosis strains was tested using the microplate Alamar Blue assay described previously [15, 16]. Sterile distilled water (200 mL) was poured into outer perimeter wells of the microplate. All other wells received 100 mL of supplemented Middlebrook 7H9 broth, then working metabolites solutions (100 mL) were poured into the first well of each row, from which two-fold dilution series were made through the microplate column. The test inoculum (100 mL) was added to all testing wells, as well as to the drug-free control wells. The final concentration of DMSO in wells was < 1% v/v. At the same time, 100:100, 10:100 and 1:100 diluted controls were prepared from the bacterial suspension, representing the growth of 100, 10 and 1% of the bacterial population tested. The final concentrations of metabolites tested ranged from 200 to 0.097 mg/mL. Each concentration was assayed in duplicate. Each microplate was incubated for 5 days at 37°C in a 5% CO₂ atmosphere in a sealed plastic bag. After incubation time, one control growth was developed with a mixture of 20 mL of Alamar blue solution (Trek Diagnostics, Westlake OH) and 12 mL of sterile 10% Tween 80. The plates were re-incubated at 37 °C for 24 h. After incubation time, if the well turned pink, all the wells received the mixture of Alamar blue and Tween solutions in the same way and were incubated for an additional 24 h. Wells with a well-defined pink color were scored as positive for growth. The Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration of sample that prevents a color change to pink. Streptomycin, isoniazid, ethambutol and rifampicin were included as standard drugs. Each experiment was performed at least twice.

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