

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 flavanone pinostrobin (**17**), 1-hydroxy-benzoisochromanquinone (**23**) and 23-hydroxy-5a-lanosta-7,9(11),24-triene-3-one (**33**). These were followed by the peracetylestrictosidine lactam (**12**) and the quinone aloe-emodin (**24**) which displayed MICs of 6.25 µg/mL. Finally, liriodenine (**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],

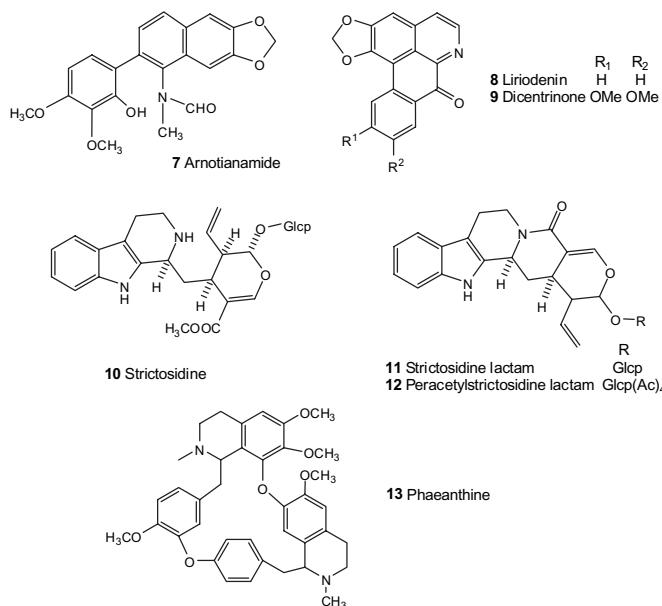
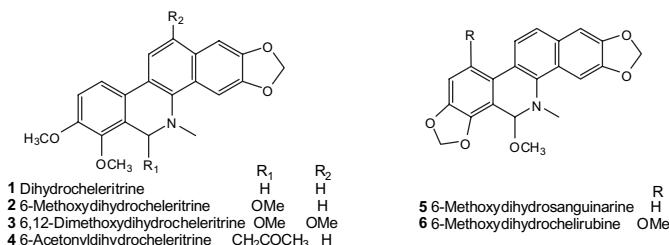
Resumen. Se informan los resultados sobre la bioevaluación de treinta y cinco metabolitos secundarios de plantas frente a una cepa sensible y a tres cepas resistentes a múltiples drogas de *Mycobacterium tuberculosis*. Los resultados frente a la cepa sensible mostraron que cinco metabolitos secundarios poseen valores de CMI de 12.5 µg/mL: los alcaloides 6-metoxidihidroqueliterina (**2**) y 6-metoxi-dihidroquelirubina (**6**), la flavanona pinostrobina (**17**), 1-hidroxi-benzoisochromanquinona (**23**) y el triterpeno 23-hidroxi-5a-lanosta-7,9(11),24-trien-3-ona (**33**). La lactama de peracetilestrictosidina (**12**) y la quinona aloe-emodina (**24**) mostraron CMIs de 6.25 µg/mL. Finalmente, liriodenina (**8**) fue la substancia más activa (CMI: 3.125 µg/mL) de todos los metabolitos secundarios. Los resultados con los aislados clínicos resistentes a multidrogas mostraron que 6-metoxi-dihidroquelirubina (**6**) fue el compuesto más activo (CMI: 12.5 µg/mL).

Palabras clave: *Mycobacterium tuberculosis*, actividad antituberculosis, metabolitos secundarios bioactivos, alcaloides, flavonoides, quinonas, triterpenos, diterpenos.

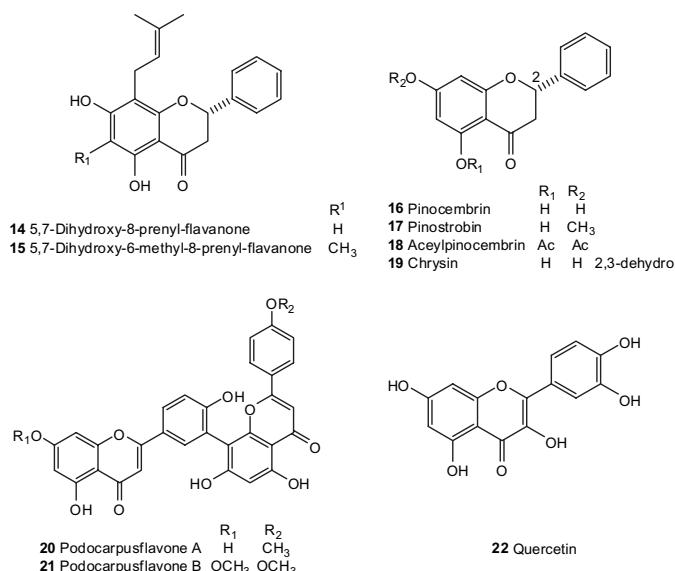
thus the screening of natural products from higher plants constitutes one avenue in the search for new lead antitubercular agents. In this study we screened thirty five phytochemicals against one drug sensitive and three multidrug resistant clinical isolates of *M. tuberculosis* using the Alamar Blue 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

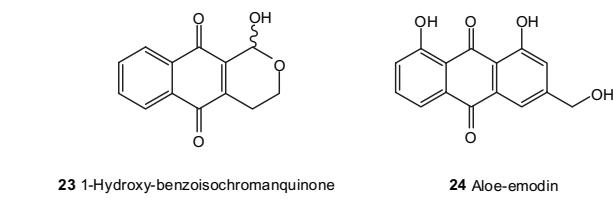


Scheme 1. Structures of tested alkaloids.

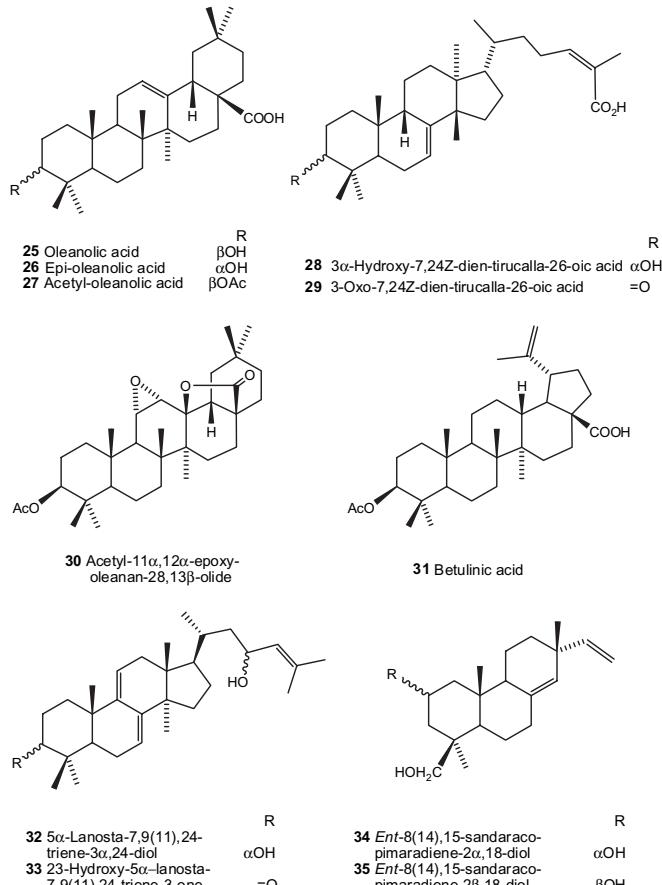


Scheme 2. Structures of tested flavonoids.

H37Rv and $> 200 \mu\text{g/mL}$ for 345, M-12 and M-20). Results show that seven secondary metabolites exhibited MIC values of $50 \mu\text{g/mL}$, six had MIC values at $25 \mu\text{g/mL}$, three presented values of $12.5 \mu\text{g/mL}$ and two of $6.25 \mu\text{g/mL}$ and only one had an MIC value of $3.125 \mu\text{g/mL}$ towards the sensitive strain



Scheme 3. Structures of tested quinones.



Scheme 4. Structures of tested triterpenes and diterpenes.

H37Rv. Among those metabolites with moderate activity (MIC: $12.5 \mu\text{g/mL}$) 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 $6.25 \mu\text{g/mL}$ 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 $3.125 \mu\text{g/mL}$).

However, the only secondary metabolites that showed good activity against the three MDR *M. tuberculosis* clinical isolates were the alkaloids 6-methoxydihydrocheliridine (4, MIC 25-50 $\mu\text{g/ml}$), 6-methoxydihydrosanguinarine (5, MIC 12.5-50 $\mu\text{g/ml}$) and 6-methoxy-dihydrochelirubine (6, MIC:

Table 1. Antimycobacterial activity de 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) ^a	Reference	<i>M. tuberculosis</i> MIC (µg/mL)				
			H37Rv	345	M-12	M-20	
<i>Bocconia arborea</i> (Papaveraceae)	1 (A)	13	25	>200	>200	>200	
	2 (A)		12.5	50	50	25	
	3 (A)		50	>200	>200	>200	
	5 (A)		50	25	50	12.5	
	6 (A)		12.5	12.5	12.5	12.5	
<i>Stephania dinklagei</i> (Menispermaceae)	8 (A)	7	3.125	>200	>200	100	
	9 (A)		50	>200	>200	>200	
<i>Cephaelis dichroa</i> (Rubiaceae)	10 (A)	11	>50	100	>200	>200	
	12 (A)		6.25	200	>200	>200	
<i>Triclisia patens</i> (Menispermaceae)	13 (A)	9	25	200	200	100	
<i>Eysenhardtia platycarpa</i> (Leguminosae)	14 (F)	14	>50	200	200	>200	
	15 (F)		25	200	>200	>200	
<i>Teloxys graveolens</i> (Chenopodiaceae)	17 (F)	5	12.50	200	>200	>200	
<i>Psychotria camponutans</i> (Rubiaceae)	23 (Q)	12	12.50	200	>200	100	
<i>Stephania dinklagei</i> (Menispermaceae)	24 (Q)	7	6.25	>200	>200	>200	
<i>E. platycarpa</i> (Leguminosae)	25 (T)	14	50	>200	>200	>200	
<i>Celaenodendron mexicanum</i> (Euphorbiaceae)	26 (T)	6	25	NT ^b	NT ^b	NT ^b	
	28 (T)		50	>200	>200	>200	
	29 (T)		50	200	>200	>200	
<i>Guarea rhopalocarpa</i> (Meliaceae)	32 (T)	8	12.50	>200	>200	>200	
	33 (T)		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	

^aA: Alkaloid, F: Flavonoid, Q: Quinone, T: Triterpene, D: Diterpene. ^bNT: not tested.

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-benzoisochromanquinone (**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, peracetylstrictosidine lactam (**12**), 3-oxo-7,24Z-dien-tirucalla-26-oic acid (**29**) and 5 α -lanosta-7,9(11),24-triene-3 α ,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.

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-5 α -lanosta-7,9(11),24-triene-3-one (**33**) and 5 α -lanosta-7,9(11),24-triene-3 α ,23-diol (**32**), with the exception of acetyl oleanolic acid (**25**) and oleanolic acid (**26**).

The antimycobacterial 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-2 α ,18-diol (**34**) was twice as active as *ent*-8(14),15-sandaracopimaradiene-2 β ,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)phenanthidine alkaloids chelerythrine and chelirubine inhibited the growth of *M. tuberculosis* H37Rv by $\geq 94\%$ at 12.5 $\mu\text{g}/\text{mL}$ [3]. We found that dihydrochelerythrine (**1**, MIC: 25 $\mu\text{g}/\text{mL}$) was half as active as chelerythrine against the sensitive strain, whereas 6-methoxy-dihydrochelirubine (**6**) has the same activity (MIC: 12.5 $\mu\text{g}/\text{mL}$) 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 antimycobacterial 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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