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Artigo

# Screening of Central and South American plant extracts for antimycobacterial activity by the Alamar Blue test

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**RESUMO: "Triagem de extratos de plantas das Américas Central e do Sul para atividade antimicobacteriana pelo teste do Alamar Blue".** Quarenta e oito extratos brutos etanólicos e frações (em hexano, diclorometano, acetato de etila e n-butanol) de dez plantas brasileiras pertencentes às famílias Leguminosae, Monimiaceae e Verbenaceae; uma da Costa Rica (Verbenaceae) e uma da Argentina (Verbenaceae) foram ensaiados para verificação da atividade anti-micobacteriana contra *Mycobacterium tuberculosis* (ATCC-27294 $H_{37}Rv$), pelo teste do Alamar Blue, a uma concentração fixa de 100 µg/mL. Dentre os quarenta e oito extratos e frações estudados, sete mostraram-se ativos na concentração ensaiada - frações em hexano e diclorometano de folhas de *Lantana trifolia*, extrato em metanol:água, 1:1 de cascas de *Vitex cooperi*, frações em hexano e diclorometano de folhas de *Lippia lacunosa* e de *Lippia rotundifolia,* sendo que todas essas plantas pertencem à família Verbenaceae.

**Unitermos:** *Mycobacterium tuberculosis, Lantana trifolia, Vitex cooperi*, *Lippia lacunosa*, *Lippia rotundifolia*, atividade antimicobacteriana, 20-hidroxi-ecdisona.

**ABSTRACT:** Forty eight ethanolic crude extracts and fractions (hexane, dichloromethane, ethyl acetate and n-butanol) from ten Brazilian plants (Leguminosae, Monimiaceae and Verbenaceae), 1 from Costa Rica (Verbenaceae) and 1 from Argentina (Verbenaceae) were screened for anti-mycobacterium activity against *Mycobacterium tuberculosis* (ATCC-27294 $H_{37}Rv$), by the Alamar Blue test, at a fixed concentration of 100 µg/mL. Out of the forty eight, seven were active at this concentration, corresponding to *Lantana trifolia* (hexane and dichloromethane extracts from leaves), *Vitex cooperi* (methanol:water, 1:1 extract from barks), *Lippia lacunosa* (hexane and dichloromethane extracts from leaves) and *Lippia rotundifolia* (hexane and dichloromethane extracts from leaves), all from the Verbenaceae family.

**Keywords:** *Mycobacterium tuberculosis, Lantana trifolia, Vitex cooperi*, *Lippia lacunosa*, *Lippia rotundifolia*, antimycobacterial activity, 20-hydroxyecdysone.



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## INTRODUCTION

Tuberculosis (TB) is a systemic disease caused by *Mycobacterium tuberculosis*, the alcohol-acid resistant bacillus, discovered by Robert Koch more than 120 years ago. Despite the discovery of very effective drugs against it more then 40 years ago, it still remains a serious health problem in many regions of the world, specially in developing nations (Lall; Meyer, 1999). Currently, almost one third of the world population is infected with *M. tuberculosis*, and each year 2-3 million people die from it (Newton et al., 2002). It is estimated that between the years 2000 and 2020 nearly one billion people will be newly infected, 20 million will develop TB and 35 million will die from the disease (WHO, 2000). It is estimated that in Latin America approximately 600.000 new cases of tuberculosis occur per year. Brazil, with c.a. 129.000 new cases per year, ranks the 13º position within other 22 countries with high-burden of TB incidence. The estimated incidence of TB in Costa Rica and Brazil is 15 and 62/100.000 respectively (WHO, 2005). The emergence of multidrug resistant strains (MDR) of *M. tuberculosis* as well as human immunodeficiency virus (HIV) infection have greatly amplified the incidence of TB, reaffirming tuberculosis as a primary public health threat. In the past 5 years several reports and review articles appeared in the literature about medicinal plants and natural products with anti-mycobacterium activity (Okunade et al., 2004; Copp, 2003; Newton et al., 2002; Cantrell et al., 2001). Over 350 natural products, mainly from plant species have been assessed for their antimycobacterial activities (Newton et al., 2002). A number have demonstrated significant *in vitro* antimycobacterial activity and active plant-derived compounds belonging to various chemical classes have been isolated (Newton et al., 2002). In recent years, a notable number of natural product-derived agents has been discovered by employing screening approaches involving cellular or biochemical targets in their assay design (Shu, 1998). In this way, we initiated a screening for anti-mycobacterium activity of some plant extracts against *Mycobacterium tuberculosis* (ATCC-27294 $H_{37}Rv$), by the Alamar Blue test.

## MATERIAL AND METHODS

### Plant material

Plant species, collection sites as well as voucher specimen numbers are described in Table 1.

### Plant extracts

Plants were extracted as follows, or otherwise, as described in Exceptions. Dried and pulverized plant materials were exhaustively extracted with ethanol and the extract was concentrated under reduced pressure to afford a brown syrup. This residue was suspended in water and extracted successively with organic solvents: hexane, dichloromethane, ethyl acetate and *n*-butanol, in this order.

### Exceptions

*Lippia integrifolia* - Organic extracts - Air dried plant material (10 g) was ground and extracted by soaking in dichloromethane (100 ml) at room temperature for 24 h and then filtered. The process was repeated 3 times. The filtrates were combined and taken to dryness under vacuum. The plant material was then extracted with methanol under the same conditions. Aqueous extract - Dried ground plant material (50 g) was extracted with hot water (500 ml) by maceration at room temperature for 20 minutes. The extract was filtered and freeze-dried. *Vitex cooperi* – 90 g of bark and wood were separately extracted twice with ethanol-water (7:3) for 24h. The filtrates were combined and taken to dryness under vacuum.

### Fractionation of *Vitex cooperi* bark methanol-water extract

The methanol-water extract (700 mg) from the bark of *V. cooperi* was chromatographed on silica gel eluted with mixtures of hexane, dichloromethane, ethyl acetate and methanol, to afford 21 fractions. Fractions 7 (24 mg) and 8 (17 mg) eluted with dichloromethane; 10 (13 mg) and 12 (10 mg) eluted with dichloromethane - ethyl acetate (1:1); and 14 (15 mg) and 15 (10 mg), eluted with ethyl acetate, were active by the Alamar Blue test. 20-Hydroxyecdysone was detected on fraction 17 by TCL and co-TLC with an authentic sample isolated from *V. cymosa* bark (Santos et al., 2001). Due to the limited amount of the remaining active fractions (*c.a.* 5 mg were used for the Alamar Blue test), GC-MS analysis was performed in order to identify possible active substances. In fractions 7 and 8 the phytosterols sitosterol and stigmasterol were identified.

### GC-MS analyses

The GC/MS system used was an Agilent 5973 MSD coupled to an Agilent 6890N gas chromatograph equipped with an automatic injector (5683). Conditions: helium as carrier gas (1 $ml.min^{-1}$); fused capillary column: HP-5 (5% phenylmethyl silicone); injector temperature: 270º C, and the column oven program was 70º to 290º C at 4º C $min^{-}1$. Detector was operated at 280º C. Injection volume, 1 µl (split 1:10). Data acquisition was obtained with a HP CHEMISTATION Acquisition Software. Substances were identified by comparison of their mass spectra with those in a spectral database (Wiley 7N) and with literature (Budzikiewicz, 1974).

### Alamar Blue Test

**Table 1.** Susceptibility of *Mycobacterium tuberculosis* $H_{37}Rv$ to the different Verbenaceae, Leguminosae and Monimiaceae plant extracts, as well as their collection sites and voucher specimen numbers.

| Plant species | Family | Plant Part Used | Extract | Susceptibility of $H_{37}Rv$ (MIC)* | Plant collection site | Voucher specimen number |
|---|---|---|---|---|---|---|
| *Bauhinia microstachya var. massambabensis* | Leguminosae | L | E | R | **B**, Rio de Janeiro, Marica restinga | RFA 30813 |
| | | L | H | R | | |
| | | L | D | R | | |
| | | L | A | R | | |
| | | L | B | R | | |
| *Bauhinia microstachya var. microstachya* | Leguminosae | L | E | R | **B**, Rio de Janeiro, Atlantic Forest | RFA 28253 |
| | | L | H | R | | |
| | | L | D | R | | |
| | | L | A | R | | |
| | | L | B | R | | |
| *Lippia origanoides* | Verbenaceae | L | E | R | **B**, Pará State, Oriximiná | CESJ 39532 |
| *Lippia alba forma intermedia* | Verbenaceae | L | E | R | **B**, Pará State, Oriximiná | CESJ 39530 |
| | | L | H | R | | |
| | | L | D | R | | |
| | | L | A | R | | |
| | | L | B | R | | |
| *Lantana trifolia* | Verbenaceae | S | E | R | **B**, Rio de Janeiro | RFA 30801 |
| | | S | H | R | | |
| | | S | D | R | | |
| | | S | A | R | | |
| | | S | B | R | | |
| | | L | E | R | | |
| | | L | H | S (80) | | |
| | | L | D | S (80) | | |
| | | L | A | R | | |
| | | L | B | R | | |
| *Hennecartia omphalandra* | Monimiaceae | L | E | R | **B**, Londrina, Paraná State | RFA 30840 |
| | | F | E | R | | |
| *Vitex cooperi* | Verbenaceae | B | E | S (80) | San Carlos, Alajuela, CO | ¥ |
| | | W | E | R | | |
| *Vitex cymosa* | Verbenaceae | F | E | R | **B**, Corumbá, Mato Grosso do Sul State | CESJ 11.711 |
| | | F | H | R | | |
| *Vitex polygama* | Verbenaceae | L | H | R | **B**, Rio de Janeiro State, Marica restinga | CESJ 45.217 |
| | | L | D | R | | |
| | | L | A | R | | |
| *Lippia integrifolia* | Verbenaceae | A | WI | R | **A**, Tafí del Valle, Tucumán Province, | Slanis 178 # |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | A | M | R | | |
| | | A | D | R | | |
| *Lippia lacunosa* | Verbenaceae | L | E | R | **B**, Juiz de Fora (MG) | CESJ 41.691 |
| | | L | H | S (>100) | | |
| | | L | D | S (25) | | |
| | | L | A | R | | |
| | | L | B | R | | |
| *Lippia rotundifolia* | Verbenaceae | L | E | R | **B**, Juiz de Fora (MG) | CESJ 31.376 |
| | | L | H | S (50) | | |
| | | L | D | S (25) | | |
| | | L | A | R | | |
| | | L | B | R | | |

Used part: L, leaves; S, stems; B, barks; F, fruits; W, woods; R, roots; A, aerial parts.
Extract: **E**, ethanol; **H**, hexane; **D**, dichloromethane; **A**, ethyl acetate; **B**, *n*-butanol; **WI**, water infusion; **M**, methanol.
*** R**, resistant; **S**, sensitive; MIC values in μg/ml.
Plant collection site: **A**, Argentina; **B**, Brazil; **CO**, Costa Rica.
[¥]Identification was accomplished by comparison with an authentic sample deposited in Universidad Nacional Herbarium and confirmed by the botanist Luis J. Poveda.
[#] Voucher specimen deposited at the Herbarium of Fundación Miguel Lillo.

MABA (Microplate Alamar Blue Assay) susceptibility testing was performed according to the method described by Franzblau et al. (1998). Final concentration of plant extract was 100 μg/ml. Media plus bacteria with and without rifampicin were used as controls. The strain $H_{37}Rv$ (ATCC - 27294) was used for all methodologies. The BACTEC ® (Becton & Dickinson) radiometric test was used as reference to confirm antimycobacterium activity of plants extracts. Samples were simultaneously screened by both microbiology laboratories.

## RESULTS

A total of 48 ethanolic crude extracts and fractions (hexane, dichloromethane, ethyl acetate and n-butanol) from 10 Brazilian plants (Leguminosae, Monimiaceae and Verbenaceae), 1 from Costa Rica (Verbenaceae) and 1 from Argentina (Verbenaceae) were assayed at a fixed concentration of 100 μg/mL - Table 1. Out of the assayed extracts, seven, belonging to the Verbenaceae family presented antimycobacterial activity at this concentration. All extracts were simultaneously assayed by the Alamar Blue Test at the two microbiology laboratories (Fiocruz, Rio de Janeiro and FURG, Rio Grande do Sul), in order to assure an interlaboratorial control.

## DISCUSSION

Analysis of the results reported in Table 1 drew our attention to the fact that *Lantana trifolia*, *Lippia lacunosa* and *Lippia rotundifolia* crude ethanolic extracts were inactive, whereas the hexane and dichloromethane fractions obtained from them (except for those from *L. trifolia* stems) were active at the same final concentration of the original extracts. When the search for biological activity is not based on ethno-pharmacological information, a question often arises which is what kind of extract is the best one to be prepared. In the specific case of these three plant extracts we could observe that the pre-purification step of the crude ethanolic extract by liquid-liquid partition played a crucial role in the assessment of the antimycobacterial activity. Literature search (Van Puyvelde et al., 1994; Boily; Van Puyvelde, 1986) showed that a crude ethanolic extract of *Lantana trifolia* collected in Rwanda, and prepared in the same way as ours had been previously assayed for antimycobacterial activity - *M. smegmatis* (Boily; Van Puyvelde, 1986) and clinical strains of *M. tuberculosis* (Van Puyvelde et al., 1994), and similarly, no positive activity was found, corroborating with our results. However, the report on the antimycobacterial activity of another species from the genus *Lantana* - *L. hispida* (Jimenez-Arellanes et al. 2003), prompted us to investigate this activity on the *Lantana trifolia* fractions. The active hexane and dichloromethane fractions from the leaves of *Lantana trifolia*, *Lippia lacunosa* and *Lippia rotundifolia* are now being submitted to bioassay-guided fractionation to locate the active(s) substance(s). Here, we report the bioassay-guided fractionation of *Vitex cooperi*'s methanol-water extract, in order to isolate their antimycobacterial substances. Fractionation by silica gel column chromatography of this extract led to the separation of 21 fractions, one of which containing ecdysteroids, which are common constituents of the barks of *Vitex* species (Santos et al., 2001; Suksamram; Sommechai, 1993). Comparative TLC and also co-TLC of this fraction with an authentic sample of 20-hydroxyecdysone, previously isolated in

our laboratory from the barks of *Vitex cymosa* (Santos et al., 2001), showed the presence of this substance in *Vitex cooperi*'s bark extract. Owing to the fact that the two extracts from *V. cooperi* (bark and wood) have very similar chromatographic profiles, with the exception of 20-hydroxyecdysone which is present only in the active one (bark), we decided to evaluate the susceptibility of *M. tuberculosis* to this substance. 20-hydroecdysone was assayed at 100 μg/mL but failed to demonstrate any activity against *M. tuberculosis* at this concentration. In this way, we searched for the antimycobacterium activity on the other fractions by the Alamar Blue test. Six fractions turned out to be active. Unfortunately, due to the limited amount of them (10 to 15 mg), further purification steps by traditional open column chromatography were unfeasible. In this way, GC-MS analysis was performed in order to tentatively identify possible active substances. In fractions 7 and 8 the phytosterols sitosterol and stigmasterol were identified by comparison of their mass spectra with those in a spectral database (Wiley 7N) and with those in the literature (Budzikiewicz, 1974). These sterols have already been identified as the antimycobacterial principles of the hexane fraction of *Morinda citrifolia* (Saludes et al., 2002), with MICs of 128 μg/mL (sitosterol) (Okunade et al., 2004) and 32 μg/mL (stigmasterol) (Okunade et al., 2004). Although the MIC reported in literature for sitosterol lies above 100 μg/mL, the fact that fractions 7 and 8 are active at this concentration may be explained by the presence of stigmasterol (MIC 32 μg/mL) in the mixture. Sitosterol has also been found in many active plant extracts against *Mycobacterium tuberculosis* such as *Chamaedora tepejilote* (Jimenez et al., 2005), *Junellia tridens* (Caldwell et al., 2000) and *Euphorbia ebracteolata* (Zhang et al., 1987), together with other antimycobacterium compounds. Also, in a clinical study (randomised placebo-controlled trial) of the efficacy of beta-sitosterol and its glucoside as adjuvants in the treatment of pulmonary tuberculosis significantly improved weight gain and higher lymphocyte and eosinophil counts in positive TB patients receiving sitosterols in addition to an efficacious antituberculosis regimen has been shown (Donald et al., 1998). Authors propose that sitosterols and their possible mode of action should be evaluated in larger numbers of tuberculosis patients and in diseases with a similar immunopathogenesis.

Therefore, we can suggest that the presence of these phytosterols in the bark extract of *Vitex cooperi* may account for the antimycobacterial activity of this plant.

## CONCLUSION

The screening of 48 south and central America plant extracts for antimycobacterium activity led to the discovery of 7 active ones, all from the Verbenaceae family. With the exception of *Vitex cooperi* 's methanol: water extract, all the others were obtained by liquid-liquid partition of their crude ethanolic extracts, showing that pre-purification steps played a crucial role in the assessment of the antimycobacterial activity. The results obtained also pointed out a trend of activity concentration towards the more lipophilic extracts (hexane and dichloromethane). In the case of the active polar methanol-water extract from *V. cooperi*, bioassay-guided fractionation by column chromatography led to the identification of two phytosterols in the less polar (lipophilic) fractions of this extract, which may account for the antimycobacterium activity of this plant. These results are in agreement with recent reports that the cell wall of *Mycobacterium tuberculosis* has high permeability to hydrophobic compounds (Korycka-Machala et al., 2005).

The therapeutic arsenal to the treatment of tuberculosis is reduced; furthermore the treatment is long. This is a serious problem that has worsened in the last times because of the increase of TB cases with resistant *Mycobacterium tuberculosis* strains. Therefore, the search for new drugs from natural origin is urgent and extremely important to developing countries like Brazil and Costa Rica, which are the richest countries in terms of biodiversity.

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