

ANTIMYCOBACTERIAL ACTIVITY OF THE ETHANOLIC EXTRACT OF THE WOOD OF *BULNESIA SARMIENTOI* LORENTZ EX. GRISEB

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ABSTRACT

In this work the *in vitro* antimycobacterial activity of the ethanolic extract obtained from the wood of *Bulnesia sarmientoi* Lorentz ex. Griseb and its chemical composition were studied. The preliminary phytochemical analysis of the extract showed the presence of steroids and/or triterpenes and tannins, and a GC/MS fingerprint analysis identified guaiol and bulnesol as main volatile components of the extract, based on their retention index and the comparison of their mass spectra with those contained in NIST libraries. The extract showed activity against *Mycobacterium tuberculosis* H37Rv strain with a minimal inhibitory concentration (MIC) of 50 µg/mL, using the microplate Alamar Blue assay. These results suggest that the extract could be a source of antimicrobial substances against *M. tuberculosis*. www.relaquim.com

Keywords: *Bulnesia sarmientoi*, antimycobacterial, Alamar Blue, *Mycobacterium tuberculosis* H37Rv, GC/MS.

RESUMEN

En el presente trabajo se evaluó la actividad antimicobacteriana *in vitro* y se estudió la composición química del extracto etanólico obtenido de la madera de *Bulnesia sarmientoi* Lorentz ex. Griseb. El estudio fitoquímico preliminar mostró la presencia de taninos y esteroides y/o triterpenos. Los componentes volátiles del extracto fueron además analizados por cromatografía gaseosa acoplada a espectrometría de masas (CG/EM) e identificados por comparación con las librerías NIST 21 y 107 del equipo y además con base en sus índices de retención. Este último análisis identificó a los sesquiterpenos guaiol y bulnesol como los principales componentes volátiles del extracto. La evaluación de la actividad antimicobacteriana usando el ensayo de microtitulación con Azul de Alamar mostró que el extracto es activo frente a la cepa H37Rv de *Mycobacterium tuberculosis* con una CMI de 50 µg/mL. Esto demuestra que el extracto puede ser fuente de sustancias con actividad frente a *M. tuberculosis*. www.relaquim.com

Palabras clave: *Bulnesia sarmientoi*, Actividad antimicobacteriana, H37Rv, Azul de Alamar, CG/EM.

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INTRODUCTION

Tuberculosis is a disease that causes a high mortality every year. According to WHO, 1.1 million people died from TB in 2010 among HIV negative cases, and an additional 0.35 million deaths (range, 0.32 million–0.39 million) among people who were HIV-positive were registered. In the same year there were estimated 8.8 million incident cases of TB globally. Additionally, the number of cases of multi-drug resistant tuberculosis (MDR-TB) was 440,000 in 2008 and by July 2010, 58 countries and territories had reported at least one case of extensively drug-resistant tuberculosis (XDR-TB) (WHO, 2011).

According to these data, strong efforts should be directed to find new antimycobacterial drugs, taking into account that MDR-TB and XDR-TB represent serious threats to the world's population health. The need of new leads for such drugs, sustain to consider natural products from plants as sources of antitubercular therapeutically useful substances (Garcia *et al.*, 2012).

Bulnesia sarmientoi Lorentz ex. Griseb (Zygophyllaceae) is an endemic tree of the Gran Chaco area that comprises parts of Paraguay, Argentina, and Bolivia. Its wood is greatly appreciated due to its resistance to insect attack and hardness. The essential oil, which is commercially obtained, is mainly used in perfumery and cosmetic industries. The plant has also an important medicinal use by the local population. The essential oil and the bark are used topically as wound healing agents (Mereles & Degen, 1997; Arenas, 1998) and as insect repellent (Martinez & Barboza, 2010). The leaves decoction is indicated for the treatment of tuberculosis (Filipov, 1997). It is also used for the treatment of rheumatism and nervous system disorders (Amat & Yajía, 1991).

Nevertheless, only few studies have focused on the biological activity of the extracts and components of the plant. The

methanol extract of the bark showed *in vitro* bactericidal activity against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterococcus faecalis* (Salvat *et al.*, 2004). The main components of the essential oil also showed moderate antifungal and insecticidal activities (Rodilla *et al.*, 2011). Anticancer activity and apoptotic effects were also described for the aqueous extract of the plant (Mollah *et al.*, 2009).

Little is also known about the chemical constituents of the plant. The essential oil composition was studied (Leung *et al.*, 1996; Retamar, 1988; Rodilla *et al.*, 2011) and saponins were isolated from the seeds (Williams *et al.*, 1984). From an aqueous extract, catechins were identified as the main components (Mollah *et al.*, 2009).

The objectives of the present work were to evaluate the *in vitro* antimycobacterial activity of the ethanolic extract obtained from the wood of *B. sarmientoi*, and to perform preliminary phytochemical analysis and fingerprint characterization of volatile components by GC/MS, in order to improve the knowledge about the chemical composition of the plant and to explore its potential as a source of new drugs against *M. tuberculosis*.

The election of the GC/MS technique was supported by the fact that the extract was obtained from the wood of *B. sarmientoi*, which possesses a high content of volatile compounds, and the antimicrobial activity of the essential oil is well known, and its components could confer that activity to the extract.

MATERIALS AND METHODS

Plant material

Bulnesia sarmientoi wood was collected in Colonia Fernheim, Chaco, Paraguay, at 22° 34' 53' 2" S, 59° 58' 09,4" W. The samples were collected and identified by Mr.

Christian Vogt (Departamento de Botánica, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Paraguay), and a voucher specimen was deposited in the Herbarium FCQ of the Facultad de Ciencias Químicas – Universidad Nacional de Asunción (C. Vogt 1294). The collector is authorized by the Secretaría Nacional del Ambiente (SEAM) of Paraguay for collection of plant material for scientific purposes.

Extract preparation

1.2 kg of triturated wood was macerated in 95% ethanol for 24 hours at room temperature. The process was repeated two more times. The solvent was removed using a vacuum rotary evaporator at 40 °C, resulting 428.6 g of extract, which corresponds to a yield of 35.7%.

Preliminary phytochemical analysis

The chemical analysis was performed using the method of Sanabria-Galindo (1983). In brief, the ethanolic extract was submitted to a series of colour and/or precipitation reactions besides thin layer chromatography (TLC) procedures, in order to identify groups of secondary metabolites. The process allows the characterization of alkaloids, flavonoids, tannins, naphthoquinones and/or antraquinones, saponins, steroids and/or triterpenes. The reagents and solvents used were from Merck KGaA, Darmstadt, Germany and Fisher Scientific, New Jersey, USA. Silica gel 60 TLC plates were from Sigma Aldrich ChemieGmbH, Steinheim, Germany.

GC/MS fingerprint

The chromatographic analysis was performed on a Shimadzu QP-5050A gas chromatograph – mass spectrometer. The extract was dissolved in methanol, to obtain a concentration of 1 mg/mL. To 900 µL of this solution, a 100 µL aliquot of the alkane standard solution C₈-C₂₀ (Sigma-Aldrich, Buchs, Switzerland) was added to determine the retention index of the compounds.

1 µL of the final solution was injected into the gas chromatograph.

The chromatography conditions were as follows: column Zebron ZB-1 30 m × 0.25 mm id × 0.25 µm d_f (Phenomenex, Torrance, CA). Helium gas at 35 cm/sec was employed as carrier. Injection mode split 1:9. Injector temperature 240 °C. Interface temperature 280 °C. Oven program: initial 35 °C, then 180 °C at 4 °C per minute, then 280 °C at 20 °C per minute, holding the final temperature for 3.75 min.

For identification, the mass spectra of the peaks were compared with those of both, NIST 107 and NIST 21 libraries available at the equipment. Besides that, the retention indexes (RI) of the compounds were compared with those existing in the literature (NIST, 2005).

Antimycobacterial activity

Mycobacterium tuberculosis H37Rv (ATCC 27294) was employed in the bioassays of the present study. The strain is sensitive to the five first-line TB drugs (isoniazid, rifampicin, streptomycin, ethambutol and pyrazinamide).

The activity of the extract against the microorganism was tested using the microplate Alamar Blue assay modified by Molina-Salinas (2006). *M. tuberculosis* was cultured in Middlebrook 7H9 broth (Becton Dickinson, Sparks, MD) supplemented with 0.2% glycerol (Sigma Chemical Co., St. Louis, MO) and 10% OADC (oleic acid-albumin-dextrose-catalase; Becton Dickinson) until logarithmic growth phase was reached. Each culture was mixed with a sufficient volume of sterile supplemented Middlebrook 7H9 broth to achieve a turbidity equivalent to McFarland's No.1 standard. To obtain the test inoculum, this suspension was further diluted 1:50 with the same culture medium to approximately 6 × 10⁶ colony-forming units (CFU)/mL, immediately before use.

All tests were carried out in sterile flat-bottomed 96-well microplates with low-evaporation polystyrene lids (Costar Corning,

Table 1. Preliminary phytochemical analysis of the extract from the wood of *Bulnesia sarmientoi*.

Compound	Test or Reagent	Result
Alkaloids	Dragendorff	-
	Mayer	-
	Valser	-
	Ammonium reineckate	-
Flavonoids	Shinoda	-
	HCl 10%	-
Naphthoquinones and/or antraquinones	Bornträger - Kraus	-
Tannins	FeCl ₃	+
Saponins	Foam test	-
Steroids and/or triterpenes	TLC	+
	Liebermann - Burchard	+

(+): present; (-): absent

New York, NY). The samples were dissolved in DMSO (Sigma) and then diluted with Middlebrook 7H9 broth supplemented with OADC. The working plant extract (100 µL) was poured in the first well of a row, from which two-fold dilution series were made with Middlebrook 7H9 broth. The final concentrations of the extract range from 12.5 to 100 µg/mL. All assays were done in triplicate, and the results were expressed as minimal inhibitory concentration (MIC). In all cases, the solvent concentration was at subtoxic level for the bacterial strain. The TB drug rifampicin was used as positive control at concentrations from 0.06 to 2 µg/mL, and DMSO as negative control.

RESULTS AND DISCUSSION

The chemical preliminary characterization of the wood ethanol extract of *B. sarmientoi* detected the presence of triterpenes and/or steroids and tannins. No alkaloids or flavonoids were detected in the crude extract. The results of preliminary phytochemical analysis are summarized in table 1.

The GC/MS analysis of the extract allowed identifying the sesquiterpenes guaial and bulnesol as principal volatile

components of the extract. The most abundant volatile components identified are summarized in table 2.

Table 2. Main volatile components of the ethanolic extract of *Bulnesia sarmientoi*.

Compound	RI	Yield (%)
α-Bulnesene	1494	0.26
Hedycaryol	1530	1.02
Guaial	1581	27.30
Carotol	1592	2.99
γ-Eudesmol	1613	1.63
α-Eudesmol	1622	0.22
7-epi-α-eudesmol	1631	3.53
Bulnesol	1653	45.00

RI: retention index

The main volatile components have also been reported for the essential oil and it is no surprising to find them in the extract obtained from the wood of *B. sarmientoi*. Other minor components are in agreement with those reported in the literature for the essential oil (Rodilla *et al.*, 2011), except hanamyol that was not detected among the volatile compounds of the wood ethanol extract.

Table 3. *In vitro* antituberculosis activity

Sample	MIC ($\mu\text{g/mL}$) on <i>M. tuberculosis</i> H37Rv	Assayed concentrations ($\mu\text{g/mL}$)
<i>Bulnesia sarmientoi</i>	50	100, 50, 25, 12.5
Rifampicin	0.06	2, 1, 0.50, 0.25, 0.12, 0.06

Concerning the biological assay performed with the extract, it showed activity against *M. tuberculosis* with a MIC of 50 $\mu\text{g/mL}$, using the Alamar Blue microplate assay. The results of the biological assay are summarized in table 3.

This fact demonstrates that some components of the extract have antimycobacterial activity. Although the MIC value is far from the reference drug rifampicin (0.062 $\mu\text{g/mL}$), used as positive control, should be noted that the extract is a mixture of various components and is reasonable to think that the activity is due only to some of them. Taking into account that only tannins and terpenes were detected in the extract, these compounds may be responsible for the observed effect against *M. tuberculosis*. Many triterpenoids have shown antimycobacterial activity (Copp, 2003; Copp & Pearce, 2007; Saleem *et al.*, 2010), and also, the volatile substances present in the extract (sesquiterpenes) could be responsible for it. Tannins also have antimicrobial activity and can as well act improving the effect of other antimicrobial substances (Akiyama *et al.*, 2001). Considering that the microorganism assayed is sensitive to the drugs used clinically, the next step in this work will be the evaluation of the extract against resistant strains of *M. tuberculosis*. In addition, the isolation of individual compounds is necessary to evaluate

if the activity observed is due to some of them or due to the extract as a whole.

CONCLUSIONS

The extract from the wood of *B. sarmientoi* showed antimycobacterial activity against the H37Rv strain of *M. tuberculosis*. This fact confirms that compounds with that activity are present in the extract. Further studies should be performed in order to separate the individual compounds and test them against the same strain and other resistant strains to the drugs used for the treatment of the disease.

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