

## Article

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# *Paepalanthus* spp: Antimycobacterial activity of extracts, methoxylated flavonoids and naphthopyranone fractions

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**Abstract:** *Paepalanthus* spp., Eriocaulaceae, are native plants from Brazil known as "sempre-vivas" (everlasting flowers). In this work, we evaluated the potential anti-mycobacterial activity of two methoxylated flavonoids (flavonoid 7-methylquercetagenin and 7-methylquercetagenin-4'-*O*- $\beta$ -D-glucopyranoside) isolated and identified from *P. latipes* and the naphthopyranone fractions from *P. bromeliodes* ethanolic extracts. The MIC value of 500  $\mu$ g/mL was verified for all compounds tested against *M. tuberculosis* H37Rv. For *M. avium*, the MIC value ranged from 1000-2000  $\mu$ g/mL excepting to naphthopyranone fractions with MIC of 500  $\mu$ g/mL. This is the first report of activity determination of *Paepalanthus* spp. flavonoids activity against *Mycobacterium tuberculosis* and *M. avium*.

## Introduction

Tuberculosis still remains an important public health problem worldwide, accounting for eight million new cases per year (WHO, 2007). The agent of tuberculosis, *Mycobacterium tuberculosis* kills nearly three million people a year throughout the world. Despite improvements in chemotherapy, tuberculosis is severely affected by the development of multi-resistant strains to drugs that are commonly used to treat the disease (WHO, 2007). The treatment of infections caused by other opportunistic environmental *Mycobacterium* species as *M. avium* is difficult, due their intrinsic resistance to a wide range of medications (Nuermberger & Grosset, 2004). Most *Mycobacterium* species are resistant to isonicotinic acid hydrazide (isoniazid), one of the most used therapeutic agents for the treatment of tuberculosis (Chung et al., 2006; Zhang et al., 1992). These challenges have motivated the research of novel antimycobacterial agents to reduce the global burden of tuberculosis and have been discussed in the current biomedical literature (Wilkinson et al., 2007; Pavan et al., 2009; Higuchi et al., 2011, Ma et al., 2010).

Natural products and/or their semi-synthetic derivatives can lead to novel antimycobacterial drugs and may have important role in the chemotherapy of tuberculosis. The literature reports the antimycobacterial activity of many classes of natural products such as alkanes, phenolics, acetogenic quinones, flavonoids, and

triterpenes (Pauli et al., 2005; Copp & Pearce, 2007).

Eriocaulaceae is a pantropical, predominantly herbaceous monocotyledonous family, comprising around 1400 species in ten genera (Giulietti et al., 2012). Plants of this family are known as "everlasting flower" ("sempre-vivas" in Portuguese, since they maintain their appearance and color after drying) and they can be found in "Campos Rupestres" vegetation in Brazil. These plants are exported to Europe, Japan and North America as an ornamental flower, representing an important source of income to the population of Minas Gerais State, Brazil (Giulietti et al., 2012).

Taxonomic studies to delimit the genus, about which there is still some confusion, and the biological investigation into the isolated molecules of Eriocaulaceae, are of great importance, because several molecules possess antioxidant actions (Santos et al., 2003), citotoxic and mutagenic activities (Moreira et al., 2002), macrophage activation (Moreira et al., 2001).

This study aimed to evaluate the antimycobacterial activity against *M. tuberculosis* and *M. avium* of the two flavonoids (6-hydroxy-7-methoxyquercetin (1) and 6-hydroxy-7-methoxyquercetin-4'-*O*- $\beta$ -D-glucopyranoside (2) from 96% crude ethanolic extract from *P. latipes* and the naphthopyranone fractions obtained from *P. bromeliodes*.

## Material and Methods

### Plant material

*Paepalanthus* spp., Eriocaulaceae, were collected at Serra do Cipó, Minas Gerais State, Brazil, and authenticated by Prof. Paulo Takeo Sano. Voucher specimens of *Paepalanthus latipes* Silveira (SPF/CFSC 13846) and *P. bromelioides* Silveira (SPF/CFSC 13856), were deposited at the Herbarium of the Institute of Biosciences, University of São Paulo, Brazil.

### Preparation and fractionation of extracts and isolation of compounds

The aerial parts of the *P. latipes* and *P. bromelioides* were dried in an oven with air circulation, at 60 °C for seven days, and then powdered. The dry powders were macerated (528 g of *P. latipes* and 500 g of *P. bromelioides*) for one week, at room temperature, with 96% ethanol. The macerates were filtered and evaporated at 60 °C under reduced pressure, resulting in 11 g of the dry extract of *P. latipes* and 8 g of *P. bromelioides*. The extracts were fractionated by gel permeation column Sephadex LH-20 (Pharmacia) and 96% methanol as eluent, resulting in 110 fractions for *P. latipes* and 150 fractions for *P. bromelioides*. Naphthopyranone fractions were identified by thin layer chromatography (mobile phase: CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O, 43:37:20 v/v/v, developer: NP/PEG and viewing under UV) (Vilegas et al., 1990). The compounds 6-hydroxy-7-methoxyquercetin (**1**) and 6-hydroxy-7-methoxyquercetin-4'-*O*-β-D-glucopyranoside (**2**) were identified by spectroscopic methods (IR, UV, ES-MS, NMR 1D and 2D) (Nehme, 1997).

### Mycobacterial strains

*Mycobacterium tuberculosis* H37Rv ATCC 27294 and *Mycobacterium avium* ATCC 15769 were obtained from the American Type Culture Collection (Manassas, VA, USA). The mycobacteria were cultured in Middlebrook 7H9 supplemented with 10% OADC and additional 0.05% Tween 80 (to avoid clumps), at 37 °C for 7-10 or 3-5 days, respectively for *M. tuberculosis* and *M. avium*.

### Antimycobacterial activity-Determination of in vitro Minimum Inhibitory Concentration (MIC)

The antimycobacterial activity was assessed using the Alamar Blue™ (MABA) method (Collins & Franzblau, 1997). Microplates of 96 wells were used. The wells were filled with different concentrations of extracts and compounds in dimethyl sulfoxide (DMSO),

which varied from 4000 to 250 µg/mL. Isoniazid (Difco, Detroit, MI, USA) was the antimycobacterial drug standard, at concentrations of 0.015-0.50 µg/mL. The tests were performed in triplicate. According to this technique, the MIC of a compound is the concentration that inhibit 90% of the growth of *M. tuberculosis* H<sub>37</sub>Rv, using Alamar Blue™ (resazurin) as a vital fluorescent dye. The bacterial suspensions were prepared at a concentration equivalent to the turbidity standard of McFarland n° 1 and diluted in the ratio 1:25 in Middlebrook 7H9 Broth. The inoculum was supplemented with 10% OADC. Cultures were incubated for seven days at 37 °C and then added Alamar Blue for reading. A negative control was performed without mycobacteria to confirm the absence of reaction of the tested compounds with the resazurin. The dye fluorescence (indicative of bacterial growth) was measured in a microfluorimeter (Tecan SPECTRAfluor Plus microplate reader), with excitation at 530 nm and emission at 590 nm (Collins & Franzblau, 1997).

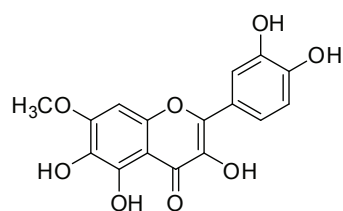
## Results

The fractionation and rechromatographed of crude ethanolic extracts in silica gel CC resulted in isolation of two methoxylated flavonoids previously isolated from *P. latipes* (Vilegas et al., 1999) and naphthopyranone fraction from *P. bromelioides*.

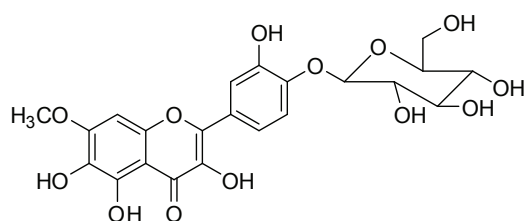
The NMR studies showed that both flavonoids have a methoxy group at position 7, and a hydroxyl group at position 6 which does not occur in quercetin, luteolin and 3-methylquercetin. Besides, this the flavonoid 7-methylquercetagenin (**1**) presents two free hydroxyl at C3' and C4', analogously to the three flavonoids mentioned. The 7-methylquercetagenin (**1**) was identified in fractions 90-102 (9.0 mg) and 7-methylquercetagenin-4'-*O*-β-D-glucopyranoside (**2**) was identified in fractions 69-78 (10.0 mg), as well as in the crude ethanolic extract from *P. latipes*. The results obtained from the ES-MS (70 V, positive mode) confirmed the identification of the flavonoids **1** and **2**, by comparison with the results of Nehme (1997), which also support our NMR data. The naphthopyranones (paepalantine-9-*O*-β-D-glycopyranosyl-(1-6)-β-D-glycopyranoside (**3**), paepalantine-9-*O*-β-D-allopyranosyl-(1-6)-β-D-glycopyranoside (**4**)) were identified in fractions 25-35 (6.5 mg) of the *P. bromelioides* crude ethanolic extract by thin layer chromatography (R<sub>f</sub> 0.40 and 0.45) and the results were confirmed using benchmarks according Vilegas et al (1990).

The antimycobacterial activities of the extracts, fractions and flavonoids from *Paepalanthus* spp. and *P. latipes* against *M. tuberculosis* and *M. avium*, using the Alamar Blue assay (MABA), are showed in Table 1. The

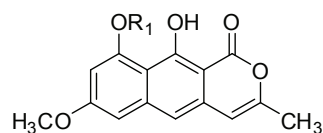
ethanol extracts and isolated compounds tested against *M. tuberculosis* H<sub>37</sub>Rv showed low activity, with MIC value of 500 µg/mL. *M. avium* was even more resistant to the ethanol extracts and compounds (MIC ranging on 1000-2000 µg/mL), excepting to naphthopyranone fraction, whose MIC was 500 µg/mL.



1



2



3 R<sub>1</sub>=Glc-Glc

4 R<sub>1</sub>=Alo-Glc

**Table 1.** Antimycobacterial activity of *Paepalanthus* spp. crude extracts, methoxylated flavonoids and naphthopyranone fraction.

Samples	<i>M. tuberculosis</i> MIC (µg/mL)	<i>M. avium</i> MIC (µg/mL)
<i>P. latipes</i> 96% ethanolic extract	500	1000
<i>P. bromelioides</i> 96% ethanolic extract	500	2000
7- methylquercetagenin	500	1000
7-methylquercetagenin-4'-O-β-D-glucopyranoside	500	1000
naphthopyranone fraction	500	500

## Discussion

Many natural products, including essential oils and phenolic compounds have been extensively tested for antibacterial activity (Friedman et al., 2002; 2003; 2004; 2006).

In this study, the ethanol extract and methoxylated flavonoids (flavonoid 7-methylquercetagenin and 7-methylquercetagenin-4'-O-β-D-glucopyranoside)

from *P. latipes*, and naphthopyranone fractions isolated from *P. bromelioides* ethanolic extract, tested against *M. tuberculosis* and *M. avium*, exhibited no significant antimycobacterial activity, despite their characteristic of polyphenolic compounds. The MIC values ranged from 500 µg/mL against *M. tuberculosis* to 1000-2000 µg/mL to *M. avium*, except for naphthopyranone, that presented the same 500 µg/mL MIC against both bacteria.

Tosun et al. (2004) considered inactive the plant extracts that could not prevent growth of *M. tuberculosis* up to concentration of 200 µg/mL. According to Gu et al. (2004), a MIC ≤ 128 mg/mL defines a substance as active against *M. tuberculosis* (Gu et al., 2004). For Cantrell et al. (2001), isolated compounds that exhibit a MIC of 64 µg/mL or lower are considered promising (Cantrell et al., 2001).

Thus, the high MIC value of 500 µg/mL, disqualifies the isolated flavonoids compounds or purified naphthopyranone fractions as promising candidates against *M. tuberculosis* and *M. avium*. The antimycobacterial activity of flavonoids has already been reported by many authors. Lin et al. (2002) described the antimycobacterial activity of 142 synthetic chalcones, flavonoids (including some methoxyflavones) and chalconeslike compounds. Murillo et al. (2003) analyzed fourteen flavonoids from *Derris indica*, including rotenoids and prenylated isoflavones (Murillo et al., 2003). The antimycobacterial methoxyflavones from *Kaempferia parviflora* was also report by Gu et al. (2004). However, according to Castellar et al (2011), in all researches, no correlation on structure-activity of flavonoids against mycobacteria could be drawn. In the opinion of Silva et al. (2009), high lipophilicity of a substance is an important feature for its antimycobacterial activity.

Due to the fact that the cell wall of mycobacteria contain high amount of lipids, such as mycolic acid, lipophilic substances are likely to penetrate more easily into the cell (Palomino et al., 2002). It has been demonstrated for a series of terpenes that the activity improves with the lipophilicity of a given substance, when compared to their more polar analogues (Cantrell et al., 2001). Pavan et al. (2009), from 37 plant extracts analyzed, found sixteen with promising activity, twelve of which were chloroform extracts and only four methanol extracts. The chloroform as extractor agent, could extracts more apolar compounds and the lipophilic nature of those compounds allows them to more easily penetrate the mycobacterial lipid rich cell wall. This probably favors their antimycobacterial activity.

In previous reports, we have described the isolation and structure determination of paepalantine, a naphthopyran-1-one isolated from the chloroform extracts of the capitulae of *Paepalanthus bromelioides*, as well as from *Paepalanthus vellozioides*, with potent

antibiotic effect against *S. aureus*, *S. epidermidis*, *Enterococcus faecalis* and *Escherichia coli* (Coelho et al., 2000; Kitagawa et al., 2003; Devienne et al., 2005) and also antiviral activities (Cotterill et al., 2003).

In another study, we verified that ethanol extracts of *P. bromeliodes* and *P. latipes* were able to induce innate immune response through the production of high levels of H<sub>2</sub>O<sub>2</sub> by mouse peritoneal macrophages (Moreira et al., 2001). Since *M. tuberculosis* can survive inside macrophages, their activation is essential for tuberculosis control. Thus, it is possible that the antimicrobial activity of the tested products, poorly in vitro, may be higher in vivo depending on macrophage activation.

### Conclusion

This is the first report of antimycobacterial activity of *Paepalanthus* spp. flavonoids. The results showed that the ethanol extracts, methoxylated flavonoids and naphthopyranone fractions exhibited no significant activity against *M. tuberculosis* and *M. avium*. Further, biological studies are needed to verify whether the antituberculosis activity *in vivo* is more intense, since the alcoholic extracts of the plant species studied were shown to be capable of producing macrophage activation.

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### Authors contributions

RRDM contributed in chromatographic analysis and biological studies, running the laboratory work, analysis of the data and drafted the paper. GZM (PhD student), DNS and FRP contributed to biological studies, running the laboratory work, analysis of the data and drafted the paper. RCLRP contributed to analysis of the data and drafted the paper. SRAL contributed to critical reading of the manuscript. WV contributed in collecting plant sample and identification, confection of herbarium and designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. CQFL contributed to biological studies and analysis of the data and drafted the paper. All the authors have read the final manuscript and approved the submission.

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