The activity of flavones and oleanolic acid from *Lippia lacunosa* against susceptible and resistant *Mycobacterium tuberculosis* strains

Aline Castellar,[1] Tatiane S. Coelho,[2] Pedro E. A. Silva,[2] Daniela F. Ramos,[2] Maria Cristina S. Lourenço,[3] Celso L. S. Lage,[1] Lisieux S. Julião,[1] Ymira G. Barbosa,[4] Suzana G. Leitão,[4]*

[1]*Programa de Biotecnologia Vegetal, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Brazil,*
[2]*Laboratório de Micobactérias, Fundação Universidade Federal do Rio Grande, Brazil,*
[3]*Laboratório de Bacteriologia e Bioensaios em Micobactérias, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Brazil,*
[4]*Departamento de Produtos Naturais e Alimentos, Faculdade de Farmácia, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Brazil.*

Article

Abstract: Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is the world's number one killer among infectious diseases. The search for new natural products that can act as drugs against TB has received increased attention during the last years. In this work we describe the isolation and identification of the active antimycobacterial principles of the dichloromethane extract from *Lippia lacunosa* Mart. & Schauer, Verbenaceae. Compounds were evaluated for their *in vitro* activity against *Mycobacterium tuberculosis* (susceptible and rifampicin resistant strain) using a redox bioassay. From the dichloromethane extract of *L. lacunosa* leaves, seven methoxy-flavones named cirsimaritin (**1**), eupatilin (**2**), eupatorin (**3**), salvigenin (**4**), 3′-*O*-methyl-eupatorin (**5**), 3′,7-dimethoxy-5,6,4′- trihydroxyflavone (**6**), and 7′-*O*-methylapigenin (**7**), and one triterpene, named oleanolic acid (**8**), were isolated. All compounds were found to display antimycobacterial activity against susceptible strain, with MIC ranging from 25 to 200 μg/mL. None of them was active against rifampicin resistant strain. This is the first report in the antimycobacterial activity of 6-substituted flavones, as well as the first report of the occurrence of these substances in *L. lacunosa*.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is the world's number one killer among infectious diseases (Lin et al., 2002). Each year, about 400,000 people develop TB with isoniazid- and rifampicin-resistant strains (MDR) (Silva et al., 2008). The emergence of MDR strains with human immunodeficiency virus (HIV) co-infection, as well as the decline of socioeconomic conditions in several places around the world, has amplified the difficulty in controlling this disease (Lin et al., 2002; Lechner et al., 2008).

The search for new drugs against TB has received increased attention during the last couple of years. Several candidates are being investigated and an interesting array of compounds constitutes the current portfolio of promising drugs. These drugs should be active against both susceptible and resistant strains (Silva & Ainsa, 2007).

In the last couple of years, several reports and review articles have appeared in the literature about medicinal plants and natural products with antimycobacterium 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* activity and active plant-derived compounds belonging to various chemical classes have been isolated (Newton et al., 2002).

The genus *Lippia* (Verbenaceae) includes approximately 200 species and is found in Central and South America, as well as in some areas of Tropical Africa (Salimena-Pires, 1991). One of the main diversity centers of this genus is located in the

central region of Brazil, mainly in "cerrado" biome, at the "Cadeia do Espinhaço" Mountains, in the State of Minas Gerais (Leitão et al., 2008). Some species, such as *Lippia alba* (Mill.) N. E. Brown, *Lippia origanoides* H. B. K., *Lippia sidoides* Cham., and Lippia graveolens Kunth, are widely used for different purposes such as medicinal and culinary use (Pascual et al., 2001).

The interest in this genus is based on the presence of flavonoids which is a class of low molecular weight phenolic compounds that are widely distributed in the plant kingdom. They exhibit different biological functions that allow interactions between plants and their environment: they are involved in the plant pathogen, plant-plant interactions and plant-insect interactions (Treutter, 2005). Recent studies have also indicated that flavonoids can act as cellular modulators by interaction with enzymes, receptors and transcription factors of intracellular signaling. These interactions could explain their potential anticancer and neurodegeneration-inhibiting activities (Williams et al., 2004).

During the course of our search for Brazilian plant extracts that are active against *Mycobacterium tuberculosis*, the dichloromethane extract from the leaves of *Lippia lacunosa* Mart. & Schauer showed moderate anti-mycobacterial activity (minimal inhibitory concentration, MIC of 25 µg/mL) (Leitão et al., 2006). In this study, we report the fractionation of this extract, which resulted in the isolation of the seven flavonoids and one triterpene, besides the evaluation of their antimycobacterial activity against *Mycobacterium tuberculosis* by the same method.

Materials and Methods

Plant material and morphological analysis

Fresh leaves of *Lippia lacunosa* Mart. & Schauer, Verbenaceae, cultivated from clones originally from Diamantina, MG, Brazil (Viccini et al., 2004), were collected in October 2006, at the campus of the Federal University of Juiz de Fora, Juiz de Fora, MG, Brazil, (22º46'48.6"S, 43º22'24.5"W). The plant was authenticated by Dr. Fatima Regina Gonçalves Salimena, and a voucher of specimen is deposited at the Herbarium of the Departamento de Botânica, Universidade Federal de Juiz de Fora (CESJ 41.691).

Plant extracts

Leaves (800 g) of *L. lacunosa* were air-dried, powdered, and extracted by maceration exhaustively with ethanol at room temperature. After filtration and concentration under reduced pressure, the aqueous residue was sequentially extracted with organic solvents
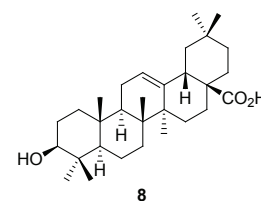
of increasing polarities, to afford the new extracts: *n*-hexane (LLFH, 12.2 g), dichloromethane (LLFD, 6.1 g), ethyl acetate (LLFA, 15.4 g) and *n*-butanol (LLFB, 46.5 g) extracts, in this order, as well as the remaining water-soluble extract (LLFW).

Isolation and identification of substances from the extracts

Part of the dichloromethane extract (LLFD, 3.0 g) was chromatographed on silica gel, and eluted with dichloromethane containing increasing amounts of ethyl acetate. Then it was eluted with ethyl acetate containing increasing amounts of methanol, affording thirty fractions (I-XXX). Fractions V and VI (375 mg), eluted with dichloromethane-ethyl acetate (9:1), were pooled (monitored by TLC) and re-chromatographed on Sephadex LH-20 (methanol), affording oleanolic acid (**8**, 66 mg), (Mahato & Kundu, 1994), and a mixture of flavonoids. The flavonoid mixture was further separated by preparative layer chromatography on silica gel plates using dichloromethane-ethyl acetate (9:1) as the solvent system. Using this procedure, salvigenin (**4**, 1.5 mg) (Skaltsa & Shammas, 1988); 3'-*O*-methyl-eupatorin (**5**, 2 mg) (Valant-Vetshera & Wollemweber, 1988), and cirsimaritin (**1**, 9 mg) (Valant-Vetshera & Wollemweber, 1988) were isolated. Fraction XII (637 mg), eluted with dichloromethane-ethyl acetate (8:2), was re-run on silica gel CC, affording 27 fractions. Fraction XII-8, eluted with dichloromethane-ethyl acetate (9:1), afforded additional 3'-*O*-methyl eupatorin, (**5**, 1.2 mg); fraction XII-15 yielded eupatilin (**2**, 2.3 mg) (Valant-Vetshera & Wollemweber, 1988), and fraction XII-20 afforded eupatorin (**3**, 1.8 mg) (Valant-Vetshera & Wollemweber, 1988). Fractions XIII-XV (466 mg), eluted with dichloromethane-ethyl acetate (**7**:3) on the original column, were re-submitted to purification on silica gel CC and eluted with dichloromethane-ethyl acetate (9:1 to 8:2), to afford 35 fractions. From fraction XIII-XV-23, 3',7-dimethoxy-5,6,4'-trihydroxyflavone (**6**, 10.7 mg) (Christensen & Lam, 1991) was isolated, and from fraction XIII-XV-30, 7'-*O*-methylapigenin (**7**, 12 mg, also known as genkwanin) (Wollemweber, 1986) was isolated.
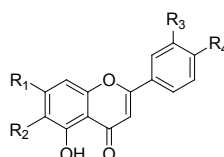
1 R$_1$ = R$_2$ = OCH$_3$; R$_3$ = H; R$_4$ = OH
2 R$_1$ = OH; R$_2$ = R$_3$ = R$_4$ = OCH$_3$
3 R$_1$ = R$_2$ = R$_4$ = OCH$_3$; R$_3$ = OH
4 R$_1$ = R$_2$ = R$_4$ = OCH$_3$; R$_3$ = H
5 R$_1$ = R$_2$ = R$_3$ = R$_4$ = OCH$_3$
6 R$_1$ = R$_3$ = OCH$_3$; R$_2$ = R$_4$ = OH
7 R$_1$ = OCH$_3$; R$_2$ = R$_3$ = H; R$_4$ = OH

The activity of flavones and oleanolic acid from *Lippia lacunosa* against suscep-
tible and resistant *Mycobacterium tuberculosis* strains
Aline Castellar et al.

The identity of the isolated compounds was confirmed by 1D and 2D-NMR techniques (^{1}H, HMBC and HSQC) on a Bruker Avance DRX400 (Karlsruhe, Germany) at 25 °C, operating at 400.13 MHz for ^{1}H and 100.61 MHz for [13]C. NMR spectra were recorded in CDCl$_3$ (flavonoids) or deuterated pyridine (oleanolic acid), using TMS as an internal standard. The data obtained was in accordance with that found in the literature. Additionally, UV data of the flavonoids in methanol, followed by the use of AcONa as a shift reagent was used for identification purposes (Mabry et al., 1970).

Tested material

Cirsimaritin (**1**), eupatilin (**2**), eupatorin (**3**), salvigenin (**4**), 3'-*O*-methyl-eupatorin (**5**), 3',7-dimethoxy-5,6,4'-trihydroxyflavone (**6**), 7'-*O*-methylapigenin (genkwanin) (**7**) and oleanolic acid (**8**), obtained as described above.

Bacterial strains

M. tuberculosis strain H37Rv (ATCC-27294) was used for all experiments in both of the microbiology laboratories. Additional assays with a rifampicin-resistant strain (ATCC-35838, His-526-Tir) were performed at the FURG laboratories.

The isolates were maintained in Ogawa-Kudoh medium for *ca.* fourteen days. The bacterial suspensions were prepared in sterile water containing 3-mm glass beads. The suspensions were homogenized by vortex agitation and turbidity was adjusted in agreement with tube one of the McFarland scale (3.2 x 10^6 colony-forming units/mL). The inoculums were prepared by diluting the bacterial suspension 1:25 in Middlebrook 7H9 OADC medium (4.7 g Middlebrook 7H9 base; Difco, Becton Dickinson) enriched with 10% (v/v) oleic acid-dextrose-albumin-catalase (BBL).

In vitro *evaluation of anti-tuberculosis activity and Minimum Inhibitory Concentration (MIC)*

Samples were screened against *Mycobaterium tuberculosis* strain H37Rv (ATCC-27294) using microtiter assays containing a redox indicator (Franzblau et al., 1998; Palomino et al., 2002). The final concentration of the substances and fractions was either 100 or 200 µg/mL. Media plus bacteria, with and without rifampicin, were used as controls. MIC determination was carried out as described in literature (Franzblau et al., 1998; Palomino et al., 2002). In brief, redox assays were performed in 96-well microplates using resazurin, Alamar Blue or MTT as the indicator of cellular viability, and the extracts, flavonoids or

oleanolic acid, dissolved in DMSO. The minimal inhibitory concentration (MIC) was carried out by two fold serial dilutions of the drugs, starting from 200 µg/mL.

Results and Discussion

In a previous study from our group (Leitão et al., 2006) the dichloromethane extract from leaves of *L. lacunosa* showed antimycobacterial activity (MIC 25 µg/mL) against *M. tuberculosis* H37Rv. In this work, seven methoxyflavones (**1-7**) and one triterpene (**8**) were isolated from this extract and evaluated against *M. tuberculosis* H37Rv and rifampicin resistant strains (Table 1). These compounds were identified by the combination of 1D and 2D NMR techniques, as well as UV data of flavonoids.

All tested substances showed activity against *M. tuberculosis* H37Rv. The most active compound was 3'-*O*-methyl-eupatorin (**5**, MIC 25 µg/mL), followed by cirsimaritin (**1**), eupatilin (**2**) and eupatorin (**3**, MIC 50 µg/mL). Salvigenin (**4**), and 3',7–dimethoxy-5,6,4'-trihydroxyflavone (**6**) were the least active substances tested, affording MIC of 100 and 200 µg/mL, respectively. Even though there are many reports on the antimycobacterial activity of the triterpene oleanolic acid (MIC of 50-69 µg/mL) in the literature (Jiménez-Arellanes, 2007; Copp, 2003; Cantrell et al., 2001; Caldwell et al., 2000; Koysomboon et al., 2006; Tanachatchairatana et al., 2008) it was re-evaluated against H37Rv as well as against the rifampicin resistant strain. Our results for H37Rv are compatible with those previously reported. However, oleanolic acid was inactive against this rifampicin-resistant strain at the maximum assayed concentration of 200 µg/mL. Due to the isolation of very small amounts of the flavonoids **2-7**, they were not assayed against the rifampicin-resistant strain.

The antimycobacterial activity of flavonoids has already been reported by many authors. Lin and coworkers (2002) have described the antimycobacterial activity of 142 synthetic chalcones, flavonoids (including some methoxyflavones) and chalcones-like compounds against *M. tuberculosis* H37Rv, and concluded that flavones showed decreased activity with respect to the corresponding chalcones. Another paper on antimycobacterial activity of flavonoids describes the results of the susceptibility of *M. tuberculosis* H37Ra, an avirulent strain, against fourteen flavonoids, including rotenoids and prenylated isoflavones from *Derris indica* (Murillo et al., 2003). In this paper, the authors regret that even with the concomitant isolation of fourteen different compounds from the same class (flavonoids), the careful inspection of the comparison of their molecular structures and their activities did

The activity of flavones and oleanolic acid from *Lippia lacunosa* against suscep-
tible and resistant *Mycobacterium tuberculosis* strains
Aline Castellar et al.

Table 1. Minimal inhibitory concentration (MIC) of flavonoids (**1-7**) and of oleanolic acid (**8**), isolated from the dichloromethane extract from leaves of *Lippia lacunosa*, against *Mycobacterium tuberculosis* strains.

Substances						
					MIC (µg/mL)	
Flavonoids	6	7	3'	4'	H37Rv	35838
cirsimaritin (**1**)	OCH_3	OCH_3	-	OH	50	Inactive at 200 µg/mL
eupatilin (**2**)	OCH_3	OH	OCH_3	OCH_3	50	*
eupatorin (**3**)	OCH_3	OCH_3	OH	OCH_3	50	*
salvigenin (**4**)	OCH_3	OCH_3	-	OCH_3	100	*
3'-O-methyl-eupatorin (**5**)	OCH_3	OCH_3	OCH_3	OCH_3	25	*
3',7– dimethoxy-5,6,4'-trihydroxyflavone (**6**)	OH	OCH_3	OCH_3	OH	200	*
7'-O–methyl-apigenin (**7**)	-	OCH_3	-	OH	50	*
Triterpene						
Oleanolic acid (**8**)					50	Inactive at 200 µg/mL
Rifampicin					1	-

*not tested.

not reveal any useful or consistent trends. Three other publications on antimycobacterial flavonoids report the activity against *M. tuberculosis* H37Rv of methoxyflavones obtained from *Haplopappus sonorensis* (Yenjai et al., 2004), *Kaempferia parviflora* (Gu et al., 2004), and *Valeriana laxiflora* (Haermers et al., 1990). Again, no conclusion on structure-activity could be drawn.

High lipophilicity of substances has been reported as an important feature for antimycobacterial activity (Silva et al., 2007). Due to the fact that the cell wall of mycobacteria contain lipophilic substances such as mycolic acid, more 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). To date, no flavonoid glycosyl derivative has been shown to be active against *M. tuberculosis,* and compounds such as quercetin have demonstrated only weak activity. Thus, if polarity is considered as an important factor of the antimycobacterial activity of flavonoids, it would be expected that in a series of methoxyflavones, that the higher the degree of methyl substitution the higher the activity of the derivatives. Analysis of the MIC of methoxyflavones **1-7** in Table 1, shows, as expected, that the tetramethoxy flavone derivative **5** (3'-*O*-methyl-eupatorin) is the one displaying the best activity (MIC 25 µg/mL). However, this hypothesis does not hold

when we compare the MIC of the dimethoxy flavone **6** (200 µg/mL) with that of monomethoxy flavone **7** (50 µg/mL). Furthermore, a critical analysis of the structures of the trimethyl derivatives **2** and **3**, as well as the dimethyl derivatives **1** and **6** does not reveal any structure-activity relationship.

Recently, the mechanisms of resistance of mycobacteria have garnered a lot of attention. Besides the classic resistance mechanisms, other mechanisms related to intrinsic and acquired resistance, such as efflux pump mechanisms, have been described. Drugs that can decrease resistance by inhibition of the efflux pump have been an important goal in the research of new drugs. For instance, INH resistance has been associated with the activation or induction of an energy-dependent efflux pump (Lechner et al., 2008). In this work, two of the isolated compounds, cirsimaritin (**1**), and oleanolic acid (**8**), were obtained in large enough quantities to be assayed against the rifampicin–resistant *Mycobacterium tuberculosis* strain 35838. One interesting feature about oleanolic acid (**8**), is its inhibitory activity on the multidrug resistance protein ABCC1 (MRP1) (Braga et al., 2007). Furthermore, a number of flavonoids have been described as MRP1 inhibitors (Felipe et al., 2008) to date. However, even at 200 µg/mL, compounds **1** and **8** failed to show any activity against this rifampicin-resistant strain. The antimycobacterial activity of oleanolic acid against a rifampicin-resistant strain has already been reported in literature (Jimenez-Arellanes, 2007) with a MIC of

50 μg/mL. However, as we don't know the molecular basis of rifampicin resistance of the strain assayed by Jimenez-Arellanes (2007) it is very difficult to establish some comparative analysis between these different results, since we can't infer that they are genetically identical.

From the dichloromethane extract of *Lippia lacunosa* seven flavones, including six 6-substituted flavones, were isolated. Our results of the antimycobacterial activity of 6-substituted flavones from *Lippia lacunosa* show the importance of investigating the phytochemistry area to discover new drugs against TB.

This is the first report concerning the non-volatile chemistry of this plant, as well as the first report of the antimycobacterial activity of 6-substituted methoxyflavones.

Acknowledgements

References

Braga F, Ayres-Saraiva D, Gattas CR, Capella MAM 2007. Oleanolic acid inhibits the activity of the multidrug resistance protein ABCC1 (MRP1) but not of the ABDB1 (P-glycoprotein): Possible use in cancer chemotherapy. *Cancer Lett 248*: 147-152.

Caldwell CG, Franzblau SG, Suarez E, Timmermann BN 2000. Oleanane triterpenes from J*unellia tridens. J Nat Prod 63*: 1611-1614.

Cantrell CL, Franzblau SG, Fischer NH 2001. Antimycobacterial plant terpenoids. *Planta Med 65*: 685-694.

Christensen LP, Lam J 1991. Flavones and other constituents from *Centaurea species. Phytochemistry 30*: 2663-2665.

Copp BR 2003. Antimycobacterial natural products. *Nat Prod Rep 20*: 535-557.

Felipe AIA, Duarte MMP 2008. Transportadores de tipo ABC: consecuencias de su interacción con flavonóides. *BLACMA 7*: 296-311.

Franzblau SG, Witzig RS, McLauchlin JC, Torres P, Madico G, Hernandez A, Degnan MT, Cook MB, Quenzer VK, Ferguson RM, Gilman RH 1998. Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. *J Clin Microbiol 36*: 362-366.

Gu JQ, Wang Y, Franzblau SG, Montenegro G, Yang D, Timmermann BN 2004. Antitubercular constituents of *Valeriana laxiflora. Planta Med 70*: 509-514.

Haermers A, Leysen DC, Bollaert W, Zhang M, Pattyn SR 1990. Influence of N substitution on antimycobacterial activity of ciprofloxacin. *Antimicrob Agents Chemother 34*: 496-497.

Jimenez-Arellanes A, Meckes M, Torres J, Luna-Herrera J 2007. Antimycobacterial triterpenoids from *Lantana hispida* (Verbenaceae). *J Ethnopharmacol 111*: 202-205.

Koysomboon S, Altena IV, Kato S, Chantrapromma K 2006. Antimycobacterial flavonoids from *Derris indica. Phytochemistry 67*: 1034-1040.

Lechner D, Gibbsons S, Bucar F 2008. Modulation of isoniazid susceptibility by flavonoids in Mycobacterium. *Phytochem Lett 1*: 71-75.

Leitão SG, Castro O, Fonseca E.N, Julião LS, Tavares ES, Leo RRRT, Vieira RC, Oliveira DR, Leitão GG, Martino V, Sülsen V, Barbosa YAG, Pinheiro DPG, Silva PE A, Teixeira DF, Neves Junior I, Lourenço MCS 2006. Screening of Central and South American plant extracts for antimycobacterial activity by the alamar blue test. *Rev Bras Farnmacogn 16*: 6-11.

Leitão SG, Oliveira DR, Sülsen V, Martino V, Barbosa YAG, Bizzo HR Lopes D, Viccini LF, Salinema FRG, Peixoto PHP, Leitão GG 2008. Analysis of the chemical composition of the essential oil of *Lippia lacunosa* Mart. & Schauer and *Lippia rotundifolia* Cham. (Verbenaceae) by gas chromatography and gas chromatography-mass spectrometry. *J Braz Chem Soc 19*: 1388-1393.

Lin YM, Zhou Y, Flavin MT, Zhou LM, Nie W, Chen FC 2002. Chalcones and flavonoids anti-tuberculosis agents. *Bioorg Med Chem 10*: 2795-2802.

Mabry TJ, Markham KR, Thomas MB 1970. *The Systematic Identification of Flavonoids.* New York: Springer-Verlag.

Mahato SB, Kundu A 1994. Review article n.98 13C NMMR spectra of pentacyclic triterpenoids - a compilation and some salient features. *Phytochemistry 6*: 1517-1575.

Murillo JI, Encarnación-Dimayuga R, Malmstrom J, Christophersen C, Franzblau SG 2003. Antimycobacterial flavones from *Haplopappus*

sonorensis. Fitoterapia 74: 226-230.

Newton SM, Lau C, Gurcha SS, Besra GS, Wright CW 2002. The evaluation of forty-three plant species from *in vitro* antimycobacterial activities; Isolation of active constituents from *Psoralea corylifolia* and *Sanguinaria canadensis. J Ethnopharmacol 79*: 57-67.

Okunade AL, Elvin-Lewis MPF, Lewis WH 2004. Natural antimycobacterial metabolites: Current status. *Phytochemistry 65*: 1017-1032.

Palomino JC, Martin A, Guerra H, Swings J, Portaels F 2002. Resazurin microtiler assay plate: Simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis. Antimicrob Agents Chemother 46*: 2720-2722.

Pascual M, Slowing K, Carretero E, Sánchez-Mata D, Villar A 2001. *Lippia*: Traditional uses, chemistry and pharmacology: A review. *J Ethnopharmacol 76*: 201-214.

Salimena-Pires FR 1991. Verbenaceae da Serra do Cipó, Minas Gerais, Brasil. Dissertação de Mestrado, Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, São Paulo.

Skaltsa H, Shammas G 1988. Flavonoids from *Lippia citriodora. Planta Med 54*: 465-465.

Silva PEA, Aínsa JA 2007. Drugs and drug interactions. In: Palomino JC, Leão SC, Ritacco V, editors, Tuberculosis. From Basic Science to Patient Care. http://www.TuberculosisTextbook.com, accessed 2 July 2009.

Silva PEA, Ramos DF, Bonacorso HG, Iglesia AI, Oliveira MR, Coelho TS, Navarini J, Morbidoni HR, Zanatta N, Martins MAP 2008. Synthesis and in vitro antymicobacterial activity of 3-substituted-5-hydroxy-5-trifluoro[chloro]methyl-4,5-dihydro-1-H-1-(isonicotinoyl) pyrazoles. *Int J Antimicrob Agents*

32: 139-144.

Tanachatchairatana T, Remner JB, Chokchaisiri R, Suksamrarn A 2008. Antimycobacterial activity of cinnamate-based esters of the triterpenes betulinic, oleanolic and ursolic acids. *Chem Pharm Bull 56*: 194-198.

Treutter D 2005. Significance of flavonoids in plant resistance and enhancement of their biosynthesis. *Plant Biol 7*: 581-591.

Valant-Vetshera KM, Wollemweber E 1988. Distribuition of externally accumulated flavonoid aglycones within the *Achillea nobilis* group. *Biochem Syst Ecol 16*: 403-409.

Viccini LF, Costa DCS, Machado MA, Campos AL 2004. Genetic diversity among nine species of *Lippia* (Verbenaceae) based on RAPD markers. *Plant Syst Evol 246*: 1-8.

Williams RJ, Spencer JPE, Rice-Evans C 2004. Flavonoids: antioxidant or signaling molecules? *Free Radical Biol Med 36*: 838-849.

Wollemweber E 1986. *Flavones and Flavonols* In: Harborne JB, editor. *The Flavonoids: Advances in research since 1986.* Press: Chapman & Hall/CRC; 259-335.

Yenjai C, Prasanphen K, Daodee S, Wongpanich V, Kittakoop P 2004. Bioactive flavonoids from *Kaempferia parviflora. Fitoterapia 75*: 89-92.

*Correspondence

Suzana G. Leitão
Departamento de Produtos Naturais e Alimentos, Faculdade de Farmácia, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro Bloco A, 2º andar, sala 4, 21.941-590 Rio de Janeiro-RJ, Brazil
sgleitao@pharma.ufrj.br
Tel.: +55 21 2562 6413
Fax: +55 21 2562 6425