Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Chemical Composition, Antimycobacterial and Anti-inflammatory Activities of Iridoids and Triterpene from *Psychotria suterella* (*Rubiaceae*)

Almir Ribeiro De Carvalho Junior, Rafaela Oliveira Ferreira[1], Michel de Souza Passos[2], Milena Gonçalves Curcino Vieira[3], Lorena de Lima Glória das Virgens[4], Sanderson Dias Calixto[4], Thatiana Lopes Biá Ventura[5], Elena Lassounskaia[4], Mario Geraldo de Carvalho[6], Raimundo Braz-Filho[2,6], Ivo José Curcino Vieira[2]

Instituto Federal de Santa Catarina, Câmpus Criciúma, Criciúma, SC, [1]Centro de Ciências Exatas e Tecnológicas, Universidade Federal do Recôncavo da Bahia, Cruz das Almas, BA, [2]Laboratório de Ciências Químicas, Centro de Ciência e Tecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, [3]Instituto Federal Fluminense, Câmpus Centro, Campos dos Goytacazes, [4]Laboratório de Biologia do Reconhecer, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, [5]Laboratório de Produtos Bioativos, Curso de Farmácia, Universidade Federal do Rio de Janeiro, Macaé, [6]Departamento de Química, Instituto de Ciências Exatas, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, Brazil

Submitted: 13-Apr-2020 Revised: 29-May-2020 Accepted: 09-Mar-2021 Published: 12-Jul-2021

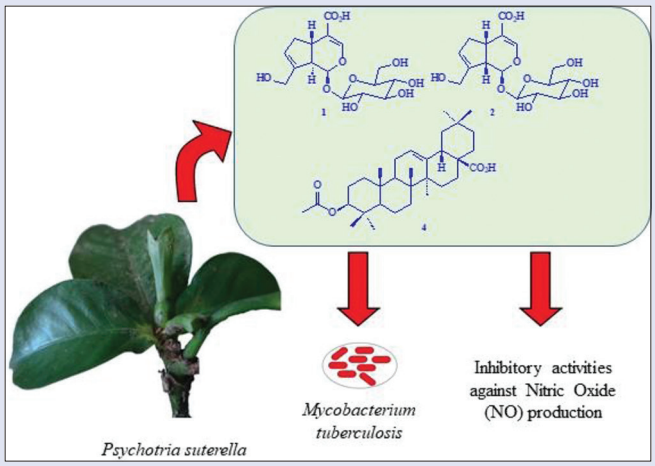
ABSTRACT

Background: *Psychotria* species are known for their medicinal properties and psychoactive activities. Extracts of *Psychotria suterella* showed anti-tuberculosis (TB) activity; however, the substances related to this activity are unknown. **Objectives:** The objective was to study on the chemical constituents of the leaves of plant and evaluate the anti-TB and anti-inflammatory activity. **Materials and Methods:** Solvent extraction, partition, and column chromatography were used to separate the compounds. The structures of these compounds were determined by extensive one dimensional-and two dimensional-Nuclear Magnetic Resonance, infrared and mass spectrometry spectroscopic analyses. Some compounds were evaluated for their *in vitro* activity against *Mycobacterium tuberculosis* and their ability to inhibit nitric oxide (NO) production by lipopolysaccharide-stimulated macrophages. **Results:** This study led to the isolation and characterization of a new iridoid, named *epi*-geniposidic acid (1), together with nine known compounds: geniposidic acid (2), 3-*O*-acethyloleanolic acid (3), pomolic acid (4), spinolic acid (5), maslinic acid (6), tormentic acid (7), methyl oleanolate (8), lyalosidic acid (9), and strictosidinic acid (10). Triterpene 3-*O*-acethyloleanolic acid (3) was found to display antimycobacterial activity against *M. tuberculosis* H37Rv strain and hypervirulent strain (minimum inhibitory concentration 6.7 ± 0.1 and 89.1 ± 1.3 μg/mL, respectively). *Epi*-geniposidic acid (1), geniposidic acid (2), and 3-*O*-acethyloleanolic acid (3) showed promising inhibitory activities against NO production (IC_{50} range 4.12–5.12 μg/mL). The iridoid mixture showed no cytotoxicity against RAW 264.7 macrophages up to a concentration of 100 μg/mL. **Conclusion:** *P. suterella* presents relevant biological properties and should be considered for further *in vivo* studies using a pulmonary TB model.

Key words: Inflammation, iridoids, *Mycobacterium tuberculosis,* nitric oxide, *Psychotria suterella,* triterpenes

SUMMARY

- The chemical study of *Psychotria suterella* leaves led to the isolation of 10 compounds, including a new iridoid (1)
- Antimycobacterial and anti-inflammatory activities of compounds 1, 2, and 3 were evaluated. Compound 3 besides present both activities, showed low cytotoxicity.



Abbreviations used: NMR: Nuclear magnetic resonance; $CDCl_3$: Deuterated chloroform; TMS: Tetramethylsilane; HR-ESI-MS: High-resolution electrospray ionisation mass spectrometry; IR: infrared; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; COSY: correlated spectroscopy; MIC: Minimum inhibitory concentration.

Correspondence:

Dr. Almir Ribeiro de Carvalho Junior,
Instituto Federal de Santa Catarina, Câmpus Criciúma, Criciúma, SC, Brazil.
E-mail: almir.ribeiro@ifsc.edu.br

DOI: 10.4103/pm.pm_93_20

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INTRODUCTION

Tuberculosis (TB) has become the communicable disease with the highest mortality rate worldwide, estimated to be responsible for 1.7 million deaths in 2015.[1,2] The emergence of drug-resistant TB strains has reduced the efficacy of first-line anti-TB drugs, such as isoniazid, rifampin, ethambutol, pyrazinamide, and streptomycin.[3] Thus, the development of new antitubercular drugs is essential to cure the disease. Initiation and promotion of the inflammatory process in TB are regulated by proinflammatory

Cite this article as: De Carvalho Junior AR, Ferreira RO, Passos MD, Curcino Vieira MG, Glória das Virgens LD, Calixto SD, *et al*. Chemical composition, antimycobacterial and anti-inflammatory activities of iridoids and triterpene from *Psychotria suterella (Rubiaceae)*. Phcog Mag 2021;17:355-9.

mediators such as nitric oxide (NO) and tumor necrosis factor-α (TNF-α). Lipopolysaccharide (LPS)-activated macrophages stimulate the production of inflammatory mediators, including NO, due to their antimycobacterial action.[4,5] Many diseases involve exacerbated inflammation, which can cause tissue damage in diseases such as rheumatoid arthritis, Alzheimer's disease, multiple sclerosis, malaria, arteriosclerosis, AIDS, and severe forms of TB.[6] Thus, it is important to search for new drugs with dual antimycobacterial and anti-inflammatory activities.

Rubiaceae stands out as a family of complex taxonomy, known to produce a variety of unique metabolites with great biological potential, with indole alkaloids as the main chemical markers.[7] In this family, the genus *Psychotria*, consisting of approximately 2000 species,[8] has received the most attention by chemical and pharmacological studies due to the psychoactive application of these plants. Phytochemical studies with species of this genus have led to the characterisation of metabolites belonging to several classes, including alkaloids, aglycones and heterosides of iridoids, triterpenes and phenolic derivatives. Among the reported activities for substances and/or extracts obtained from this group of plants, their anti-inflammatory, antimicrobial, and cytotoxic properties stand out.[9,10]

Psychotria suterella, more commonly known as the purple coffee tree of the forest, is a species frequently found in the Atlantic Forest whose leaf extracts present antituberculosis, anti-inflammatory, and antitumor activities.[11,12] However, there is no record of its medicinal use and only few studies on the phytochemicals related to this species. In a previous study, Santos *et al.*[13] described the isolation of lyaloside, strictosamide, and naucletin alkaloids from the leaf extracts of *P. suterella*. Continuing the search for substances with pharmacological properties in species of the family *Rubiaceae*,[12,14-17] the current study involved the phytochemical evaluation of leaf extracts of *P. suterella* and the antimycobacterial and anti-inflammatory activities of some of the isolated substances.

MATERIALS AND METHODS

General experimental procedures

For thin-layer chromatography, silica gel PF_{254} (Merck) plates were used. Plates were observed under ultraviolet light at 254 and 366 nm and developed with sulfuric vanillin and iodine vapors. Chromatographic column separations (CC) were performed using silica gel 60, silanized silica gel 60 (0.063 and 0.200 mm, Merck) and Sephadex LH-20 (Sigma-Aldrich). Fourier-transform infrared spectra were recorded on a Shimadzu IRAffinity-1 spectrometer using a KBr disk. The ^1H and ^{13}C (uni-and bidimensional) nuclear magnetic resonance (NMR) experiments were performed on a Bruker Ascend 500 (500/125 MHz) spectrometer using pyridine-*d5* $CDCl_3$ or methanol-*d4* as solvents and the residual signals from Tetramethylsilane as the reference. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) mass spectra were obtained on a Bruker Daltonics micrOTOF-Q II mass spectrometer using positive and negative ion analysis modes. Low-resolution mass spectra were obtained using the Shimadzu GCMS-QP5050A spectrometer, operating with an ionization energy of 70 eV.

Collection and identification of botanical material

Leaves of *P. suterella* were collected from the Biological Reserve of Porto das Antas in the municipality of Nova Iguaçu-RJ. The botanical material was identified by Professor Dr. Sebastião José da Silva Neto (UERJ). The exsicta of number H9724 is deposited in the UENF herbarium.

Extraction and isolation of chemical constituents

The powdered, air-dried leaves of *P. suterella* (345.4 g) were exhaustively extracted with methanol at the room temperature, yielding 50.1 g of crude extract. After suspension in MeOH-H_2O (1:3) solution, part of this extract (40.0 g) was partitioned with dichloromethane, ethyl acetate and n-butane, yielding 19.7, 5.1, and 8.4 g of each fraction, respectively. The dichloromethane fraction was suspended in a hexane: MeOH (1:1) solution, obtaining a hexane fraction (11.0 g) and methanol fraction (7.8 g).

The methanolic fraction (7.0 g) was subjected to CC on a silica gel and eluted with CH_2Cl_2 and CH_2Cl_2: MeOH solutions, increasing the polarity to 15% of MeOH, resulting in 10 subfractions. Subfraction 4 (700 mg) was similarly chromatographed, producing compound 8 (25.3 mg). Subfraction 5 (840.0 mg) was analogously chromatographed, consisting of compound 3 (27.0 mg) and a mixture of compounds 4 and 5 (246.0 mg). From fraction 8 (660 mg), by analogous chromatographic procedure, a mixture of compounds 6 and 7 (88.0 mg) was obtained. Fraction 9 (457.3 mg) was also similarly chromatographed, yielding a mixture of compounds 1 and 2 (67.2 mg). N-butanol (8.0 g) was subjected to CC on a silanized silica gel, eluted with isocratic mixture of MeOH: H_2O (1:1), yielding six subfractions. Fraction 3 (300 mg) was chromatographed on Sephadex LH-20 and eluted with MeOH, yielding compounds 9 (27.0 mg) and 10 (34.2 mg). All substances were submitted to NMR analysis of ^1H and ^{13}C and substances 1 and 2 were additionally identified with infrared and HR-ESI-MS.

Epi-geniposidic acid (1): Brown oil. IR (KBr) υ_{max} cm^{-1}: 3418, 2928, 2860, 1686, 1639, 1275. HR-ESI-MS (positive mode) *m/z* (rel. int.): 217.0408 (100), 235.0527 (50) and 397.1064 (25). ^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD), Table 1.

Antimycobacterial activity

Samples were evaluated for their antimycobacterial activity using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium

Table 1: ^1H nuclear magnetic resonance (500 MHz) and ^{13}C nuclear magnetic resonance (125 MHz) spectral data of compound 1, including the results of heteronuclear single quantum coherence and heteronuclear multiple bond correlation experiments

	HSQC		HMBC	
	δ_C	δ_H	$^2J_{CH}$	$^3J_{CH}$
C				-
2	112.1	-	H-3	
4	143.3	-	H-7; H-9; 2H-10	H-1
8	170.4	-		H-3; H-5
11				
CH				H-3; H-1'
1	92.5	5.15 (d, 3.4)		H-1; H-5
3	151.5	7.50 (d, 0.9)	-	-
4	-	-		H-1; H-3; H-7
5	35.4	3.22-3.15 (m)		H-5; 2H-10
7	127.2	5.82 (brs)	H-1	H-7
9	45.6	2.73 (t, 7.5)		H-1
1'	96.6	4.51 (d, 7.7)		
2'	73.4	3.22-3.15 (m)		
3'	76.3	3.45 (m)		
4'	70.1	3.55 (m)		
5'	76.9	3.54 (m)		
CH_2				
6	38.4	2.85 (dd, 16.4, 6.2)	H-5; H-7	
		2.14-2.04 (m)		
10	60.1	4.33 (d, 13, 3)		H-7
		4.21 (d, 13.3)		
6'	61.3	3.80 (m), 3.70 (m)		

Chemical shifts δ are given in ppm and coupling constants in Hz.
HSQC: Heteronuclear single quantum coherence; HMBC: Heteronuclear multiple bond correlation

bromide (MTT) assay to quantify bacterial growth in the liquid medium.[18] *Mycobacterium tuberculosis* H37Rv strain (ATCC 27294) and the hypervirulent strain (Beijing M299, isolated from a TB patient in Mozambique)[19] were incubated with Middlebrook 7H9 medium supplemented with 0.5% glycerol, 0.05% Tween 80 and 10% albumin dextrose catalase at 37°C. Cells were plated at a density of 1 × 106 CFU/well in a 96-well plate and treated with the samples at four concentrations (0.8, 4, 20, and 100 µg/mL). The plate was incubated at 37°C and 5% CO_2 for 5 days. Plates were subsequently incubated with the MTT solution (5 mg/mL) for 3 h, then the lysis buffer was added (20% w/v sodium dodecyl sulphate and 50% dimethylformamide in distilled water, pH 4.7). The plate was incubated overnight, and the reading was performed using a spectrophotometer at 570 nm. As a positive control, *M. tuberculosis* treated with the antibiotic rifampicim (95% purity; Sigma-Aldrich) was used (ranging from 0.00032 to 1 µg/mL for *M. tuberculosis* H37Rv and 0.008–10 µg/mL for the *M. tuberculosis* M299 clinical isolate). As a negative control, untreated *M. tuberculosis* was used.

Determination of nitric oxide production from lipopolysaccharide-activated RAW 264.7 cells

The macrophage cell line RAW 264.7 (American Type Culture Collection, Manassas, VA, USA) was grown in Dulbecco's Modified Medium F-12 supplemented with 10% foetal bovine serum and gentamicin (50 µg/mL) at 37°C and 5% CO_2. Macrophages were seeded in 96-well tissue culture plates (1 × 10^5 cells/well) for 24 h at 37°C. The macrophages were stimulated with LPS (1 µg/mL; *Escherichia coli* 055:B5; Sigma-Aldrich) and incubated for a further 24 h in the presence or absence of four concentrations of the samples (0.8, 4, 20, and 100 µg/mL). After a 24-h incubation, supernatants were collected and the concentration of nitrite was determined according to the Griess test.[20] NG-methyl-L-arginine acetate salt (20 µg/mL; L-NMMA, Sigma-Aldrich, 98% purity) was used as a positive control in this experiment.

Cytotoxicity

Cytotoxicity was assessed by MTT assay.[21] RAW 264.7 cells (5 × 10^5 cells/mL) in 96-well plates, stimulated or not with LPS (1 µg/mL), were incubated with increasing doses of the test compound (0.8, 4.0, 20, and 100 µg/mL of compounds or L-NMMA) at 37°C in 5% CO_2 for 24 h. After treatment, 5 µL MTT solution (5 mg/mL) was added to each well. After incubation for 2 h at 37°C, the plate supernatant was removed, and the crystals that had formed were solubilized in HCl (4 mM) in isopropanol. The absorbance of each well was then read at 570 nm.

Statistical analysis

The experiments were performed in triplicate. All values are expressed as mean ± standard deviation. The results were tabulated using LabChart 7 and statistically analyzed with GraphPad Prism 4 software. Analysis of variance was performed, followed by the comparison of means by the Newman-Keuls and Bonferroni tests, with a reliability index of 95%.

RESULTS AND DISCUSSION

Structural identification

Purification of the extracts of *P. suterella* leaves by chromatographic techniques led to the isolation of 10 compounds including one new iridoid, *epi*-geniposidic acid (1), as well as 9 known compounds (2–10): geniposidic acid (2),[22] 3-*O*-acethyloleanolic acid (3),[23] pomolic acid (4),[24] spinolic acid (5),[25] maslinic acid (6),[26] tormentic acid (7),[27] methyl oleanolate (8),[28] lyalosidic acid (9),[29] and strictosidinic acid (10)[30] [Figure 1]. The identification of the compounds was based on the analysis of the spectra and comparison with data described in the literature.[22-30] All substances are being described for the first time in this species.

Compound 1 [Figure 1] was obtained in a mixture with compound 2 as a brown oil. The molecular formula $C_{16}H_{22}NaO_{10}$ was determined based on HR-ESI-MS (*m/z* 397.1064, [M + Na] +, calculated for 397.1111). The IR spectrum showed absorption bands at 3418 cm^{-1} for the hydroxyl group, at 1639 and 1686 cm^{-1} for conjugated carbonyl groups and at 1415 cm^{-1} for C-O-H, respectively. The ^{1}H NMR spectrum of compound 1 presented similar features to those described for geniposidic acid (2),[22] with signals of two olefinic hydrogens at δ 7.23 (d, J = 0.9 Hz, H-3) and 5.86 (s, H-7), two diastereotopic methylene groups at δ 4.99 (d, J = 14.0 Hz, H-10eq) and 4.87 (d, J = 14.0 Hz, H-10ax) and 2.89 (m, H-6) and 2.12 (m, H-6) and the β-D-glucopyranosyl moiety at δ 4.71 (d, J = 7.7 Hz, H-1′), 3.38 (m, H-5′), 3.33 (m, H-4′), 3.29 (m, H-3′), 3.25 (m, H-2′), 3.66 (dd, J = 12.1, 5.3 Hz, H-6′′) and 3.85 (d, J = 12.1 Hz, H-6′′). The HMBC correlations from H-C (3) to C (4), C (5) and C (11), from H-C (7) to C (5), C (6), C (8) and C (10), along with ^{1}H-^{1}H COSY correlations H-C (5)↔H-C (9)↔H-C (1) established the crude structure of 1 [Figure 1]. Its relative stereochemistry was proposed based on the coupling constant value of H-1 (3.4 Hz), which is typical of an axial-equatorial coupling and the shielding effect on C-1' (δ_C 96.6, suggesting H-9 in equatorial position). All correlations observed by two dimensional NMR experiments are summarized in Table 1.

Antimycobacterial activity

In a previous study by our group,[12] the ethanolic extract from leaves of *P. suterella* showed antimycobacterial activity (MIC_{50} 8.32 ± 2.39 µg/mL) against *Mycobacterium bovis* BCG. In the current work, a mixture of iridoids (1 + 2) and one triterpene (3) were isolated from this species, and their antimycobacterial effect was evaluated against the *M. tuberculosis* H37Rv strain and the hypervirulent strain M299 [Table 2]. The mixture of iridoids was inactive against the H37Rv and hypervirulent M299 strains at the maximum assayed concentration of 100 µg/mL.

The antimycobacterial activity of terpenoids has been reported by many authors. Jyoti *et al.*[31] reported that ursolic acid obtained from *Artemisia cappilares* has a MIC of 12.5 µg/mL and imaging analysis of the H37Rv strain after treatment with triterpene revealed cell wall lysis. Oleanonic acid and *epi*-oleanolic acid isolated from *Junellia tridens* showed similar activities against *M. tuberculosis*, both with MIC values of 16 µg/mL.[32] Stigmasterol and sitosterol obtained from *Morinda citrifolia* (*Rubiaceae*) have MIC values of 32 and 128 µg/mL, respectively.[33] The lipophilicity of the substances is an important factor for the antimycobacterial activity.[34] In this work, we report the antituberculosis action of 3-*O*-acethyloleanolic acid (3) against H37Rv and hypervirulent M299 strains with MIC values of 6.7 ± 0.1 and 89.1 ± 1.3 µg/mL, respectively. Substance 3 was shown to be more active than its structural analogue oleanolic acid, which has a reported MIC of 50 µg/mL against *M. tuberculosis* H37Rv and was found to be inactive

Table 2: Minimal inhibition concentration of iridoids (1+2) and triterpene (3) isolated from the leaves of *Psychotria suterella* against *Mycobacterium tuberculosis* strains

Substances	MIC (µg/mL)	
	H37Rv	M299
1 + 2	Inactive at 100 µg/mL	Inactive at 100 µg/mL
3	6.7±0.1	89.1±1.3
Rifampicin[a]	0.2±0.1	1.1±0.1

[a]Standard anti-tuberculosis drug. MIC: Minimal inhibition concentration

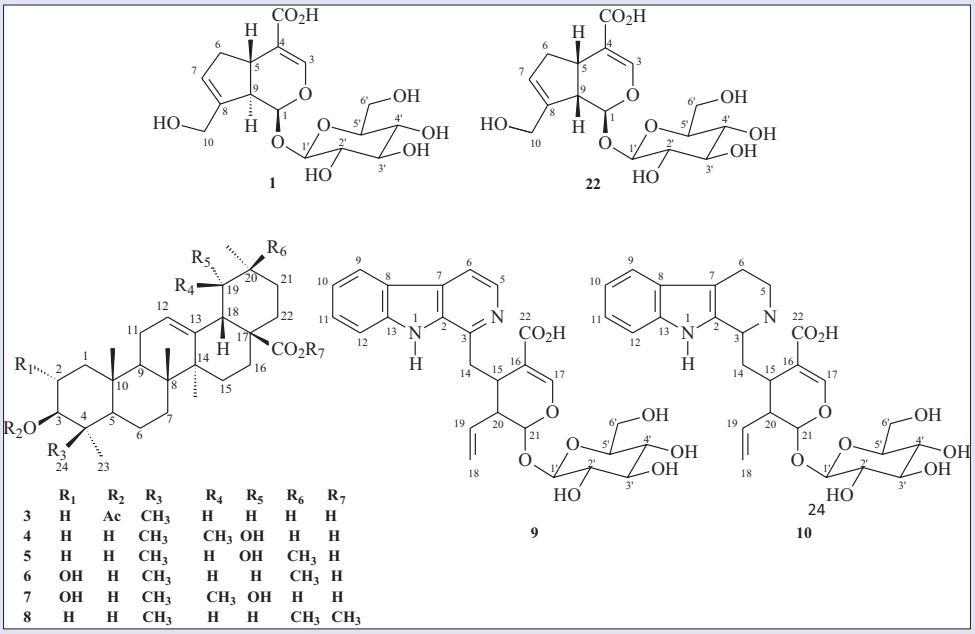

Figure 1: Structures of compounds 1–10

at concentrations up to 200 µg/mL against a rifampicin-resistant strain.[34] These results indicate that *P. suterella* extracts containing 3-*O*-acethyloleanolic acid (3) possess promising chemotherapeutic potency against TB for future use.

Effects of iridoids and triterpene on the production of nitric oxide

Plants belonging to the family *Rubiaceae,* and their extracts have been traditionally used in folk medicine for the treatment of inflammation.[9,35] Iridoids, the chemosystematic markers of the family *Rubiaceae*,[36] also exhibit anti-inflammatory activities *in vitro* and *in vivo*.[37] These data suggest that extracts of these species and iridoids could serve as the models for the development of new anti-inflammatory drugs. In this study, the anti-inflammatory activities of the iridoid mixture (1 + 2) and triterpene (3) were tested in LPS-induced RAW 264.7 cells.

One of the manifestations of inflammation is oxidative stress, which is responsible for inducing the synthetic pathways of inflammatory mediators such as NO and TNF-α.[4] Inflammation is a hallmark of various infectious diseases, including TB.[6] NO is a free radical that plays a role in the host's defence process against different pathological organisms, responsible for increasing the antibacterial activity of activated macrophages.[4] However, in cases of infection with resistant strains, such as multidrug-resistant TB (MDR-TB), exacerbated NO production can lead to tissue damage associated with acute and chronic inflammation.[4,38] In these cases, an alternative drug with dual antimycobacterial and anti-inflammatory properties would be desirable.

Iridoids, including geniposidic acid (2), demonstrated *in vitro* anti-inflammatory activity in trials with cyclooxygenase (COX-1 and COX-2) enzymes, cytokines, and pro-inflammatory mediators such as TNF-α and NO.[37] In the current study, the newly identified *epi*-geniposidic acid (1), obtained in a mixture with substance 2 and triterpene 3-*O*-acethyloleanolic acid (3), both isolated from *P. suterella* leaves, demonstrated potent inhibition of NO in RAW 264.7 cells [Table 3] with IC_{50} values of 5.12 ± 0.1 and 4.12 ± 0.1 µg/mL, respectively.

Table 3: The half maximal inhibitory concentration of compounds for inhibiting nitric oxide production by stimulated macrophages and their cytotoxicity, assessed by succinate dehydrogenase activity 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Compound	IC_{50} (µg/mL)	
	NO	MTT
1 + 2	5.12±0.1	258.9±0.2
3	4.12±0.1	98.07±0.1
L-NMMA[a]	78.3±6.5	>100

[a]NG-methyl-L-arginine acetate salt (standard inhibitor of NO). NO: Nitric oxide; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; IC_{50}: Half maximal inhibitory concentration

To exclude the possibility that the inhibitory effect of the substances on NO production was due to a cytotoxic effect, we carried out viability testing using the MTT assay [Table 3]. Although the inhibitory effect on NO production was associated with low cytotoxicity, especially for triterpene (3), its capacity for inhibiting NO production was greater than its cytotoxicity. For example, for all tested samples at 20 µg/mL, the NO inhibitory activity was >80% while cytotoxicity was between 20% and 40%.

The iridoid mixture showed considerable anti-inflammatory activity and no cytotoxicity; however, it was not active against *M. tuberculosis* strains in concentrations up to 100 µg/mL. Triterpene (3) has been shown to be a dually active compound with antimycobacterial and anti-inflammatory properties, which presents low cytotoxicity. The importance of developing new drugs with dual activities is highlighted by the increasing prevalence of both MDR-TB and extensively drug-resistant TB.[38]

CONCLUSION

The chemical study of *P. suterella* resulted in the identification of 10 substances. One of these, *epi*-geniposidic acid (1) is a newly described iridoid. In addition, triterpene 3-*O*-acethyloleanolic acid (3) exhibits excellent antitubercular activity against the H37Rv and hypervirulent M299 strains of *M. tuberculosis* at high concentrations, as well as inhibition of the production

of proinflammatory mediator NO with high selective toxicity. Therefore, it seems worthwhile to carry out the next phase of experiments necessary for new drug development.

Financial support and sponsorship

The authors are grateful to FAPERJ, CAPES, and to CNPq for scholarships and financial support.

Conflicts of interest

There are no conflicts of interest.

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