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The suppressive effects of *Vochysia divergens* aqueous leaf extract and its 5-methoxyflavone on murine macrophages and lymphocytes

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Abstract

Ethnopharmacological relevance

Leaf infusion from the tree *Vochysia divergens* Pohl (Vochysiaceae), known as "cambará", is used in Brazilian popular medicine against respiratory infections and asthma.

Aim of the study

To evaluate the health-beneficial effects related to the ethnomedicinal uses of *V. divergens* (Vd), a biomonitored fractionation of an aqueous leaf extract was carried out.

Materials and methods

Leaves were extracted by decoction with distilled water, and the extract fractionated by a combination of separation techniques including precipitation, organic partition and chromatography. Chromatographic analyses of active samples were carried out using HPLC-DAD-MS and flavonoid **1** was isolated from *n*-BuOH fraction through classic chromatography techniques. Inhibitory effects of Vd extract, fractions and flavonoid **1** on NO and TNF-α production by macrophages were assessed by Griess method and L929 cell bioassay and cell viability by LDH release assay. Additionally, the suppressive ability on proliferation of BALB/c lymphocytes was estimated by [^{3}H] thymidine incorporation. Antioxidant activity of samples was verified by SNP and DPPH assay and inhibition ability of *i*NOS protein expression through Western blotting.

Results

The LC-MS analysis of Vd extract led to the identification of 5-methoxyluteolin-7-*O*-β-glucopyranoside (**2**), rutin (**4**) and the tannin galloyl-HHDP-glucopyranoside (**3**), besides the main flavonoid 3',5-dimethoxyluteolin-7-*O*-β-glucopyranoside (**1**), which was biologically evaluated in comparison with luteolin aglycone. Vd extract, *n*-BuOH fraction and flavonoid **1** inhibit NO and TNF-α production by LPS-stimulated macrophages. The reduction on NO levels was mediated mainly by suppression of *i*NOS expression. In addition, both Vd extract (IC_{50} 13.6 µg/mL) and flavonoid **1** (IC_{50} 19.8 µg/mL; 41.6 µM) strongly inhibited stimulated lymphocyte proliferation when compared to immunosuppressive agent cyclosporin A (IC_{50} 43.8 µg/mL; 36.4 µM). Vd extract also showed a scavenging activity toward DPPH and NO free radicals. This is the first report describing the immunomodulatory potential of *V. divergens* and its major flavonoid (**1**).

Conclusion

Our findings showed that *V. divergens* leaf extract and its flavonoid can help to suppress indicators of excessive inflammation, which are associated with pathological conditions, explaining, at least partially, the benefits of the ethnomedicinal use of this plant against inflammatory processes. In

addition, this study contributes to the knowledge of the pharmacological properties of 5-methoxy flavones, a poorly investigated subclass of flavonoids.

Abbreviations : ConA, concanavalin A; COSY, correlation spectroscopy; COX-2, Cyclooxygenase -2

CyA, cyclosporin A; DAB, diaminobenzidine; DMEM-F12, Dulbecco's modified eagle medium-nutrient mixture F-12; DMSO, dimethyl sulfoxide; DPPH, 1,1-diphenyl- 2-picrylhydrazyl; FA, CH_2Cl_2/ dichloromethane fraction; FB, EtOAc/ ethyl acetate fraction; FBS, fetal bovine serum; FC, *n*-BuOH/butanol fraction; FD, aqueous fraction; HMBC, heteronuclear multiple-bond correlation; HMQC, heteronuclear multiple-quantum correlation; HPLC-DAD-MS, high-performance liquid chromatography-photodiode array detection-mass spectrometry; IC_{50}, concentration required for 50% inhibition; *i*NOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; L*N*MMA, L-*N*-monomethyl-arginine; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide; NF-kB, factor nuclear kappa B; NMR, nuclear magnetic resonance; NO, nitric oxide; NSAID, non-steroidal anti-inflammatory drugs; PBS, phosphate-buffered saline; PDE-4, phosphodiesterase 4; PGE_2, prostaglandin-2; Private Natural Heritage Reserve, RPPN; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SNP, sodium nitroprusside; TNF-α, tumor necrosis fator-alpha; *V. divergens*/ Vd, *Vochysia divergens*.

Keywords: *Vochysia divergens*; cambará; flavonoids; phenolic profile; inflammation; immunomodulation.

Chemical compounds studied in this article: Acetic acid (Pubchem CID: 176); Acetone (Pubchem CID: 180); Acetonitrile (Pubchem CID: 6342); Actinomycin D (Pubchem CID: 2019); Bromophenol blue (Pubchem CID: 8272); Ceric sulfate solution (Pubchem CID: 10929483); Concanavalin A (Pubchem CID: 16398721); Cyclosporin A (Pubchem CID: 5284373); Diaminobenzidine (Pubchem CID: 7071); Dichloromethane (Pubchem CID: 160586), Dimethyl sulfoxide-d6 (Pubchem CID: 75151); DPPH (Pubchem CID :2735032); Ethanol (Pubchem CID:702); *n*-Butanol (Pubchem CID: 263); Ethyl acetate (Pubchem CID: 8857); Gallic acid (Pubchem CID: 370); Gentamicin (Pubchem CID: 3467); Glycerol (Pubchem CID: 753); Hydrogen peroxide (Pubchem CID: 784); Imidazole (Pubchem CID: 745); Lipopolysaccharide (Pubchem CID: 11970143); L-*N*-monomethyl-arginine (Pubchem CID: 132862); Luteolin (Pubchem CID: 5280445); Methanol (Pubchem CID: 887); MTT (Pubchem CID: 64965); Quercetin (Pubchem CID: 5280343); Sodium Chloride (Pubchem CID: 5234); Sodium dodecyl sulfate (Pubchem CID: 3423265); Sodium nitrite (Pubchem CID: 23668193); Sodium nitroprusside (Pubchem CID: 45469); ß-mercaptoethanol (Pubchem CID: 1567); Tris/Chloride acid (Pubchem

CID: 93573); Triton X-100 (Pubchem CID: 5590); Trypan Blue (Pubchem CID: 124203200); 3',5-Dimethoxy luteolin-7-*O*-β-glucopyranoside (**1**); 5-Methoxyluteolin-7-*O*-β-glucopyranoside (**2**); Galloyl-HHDP-glucopyranoside (**3**); Rutin/quercetin 3-*O*-α-rhamnopyranosyl (1→6) glucopyranoside (**4**) (Pubchem CID: 5280805).

1. Introduction

Vochysia divergens Pohl (Vochysiaceae), known as "cambará", is a tree native to the Amazon Basin and usually found in the Brazilian Pantanal wetland. The flexibility in physiology and ecological characteristics enables their permanence in regions with variable hydrological regime (Dalmagro et al., 2014). The infusion prepared from its leaves is used in popular medicine against microbial infections and respiratory disorders, as well as for healing digestive troubles (Hess et al., 1995a; Neto et al., 2011). Phytochemical studies reported more than 90 different secondary metabolites for Vochysiaceae family (Neto et al., 2011) focusing mainly on triterpenes and steroids (Hess et al., 1995b; Hess and Monache, 1999). Phenolic compounds were isolated from *V. ferruginea*, *V. acuminate*, *V. tyrsoidea*, *V. pacifica*, *V. guianensis*, *V. tucanorum* and *V. cinnamomea* (Corrêa et al., 1975; Lopes et al., 1979; Zucaro et al., 2000; Weniger et al., 2005). So far, there are relatively few studies that have examined the immunomodulatory effect of *Vochysia* species. In spite of the medicinal use of *V. divergens* for treatment of inflammatory processes, this property has not been investigated yet, as well as, the identification of principal compounds that can be responsible for the modulatory effects in the immune system.

The antibacterial activity of *V. divergens* against *Staphylococcus aureus* and *Escherichia coli* were related to the presence of triterpenes to *V. divergens* (Hess et al., 1995a). More recently, Pimenta et al. (2015) reported the presence of methoxyflavones from an ethanol leaf extract of this plant and its *in vitro* effect against *Schistosoma mansoni*.

Since *V. divergens* leaves are used in the popular medicine mostly as water infusion, it is imperative that pharmacological effects of water-soluble compounds are studied. Therefore, with the aim of evaluating health-beneficial effects related to the ethnomedicinal uses of *V. divergens* (Vd), a

biomonitored fractionation of an aqueous leaf extract was carried out. For this, we evaluated the *in vitro* NO and TNF-α production by LPS-stimulated macrophages in the presence of Vd extract, fractions and flavonoid **1**, exploring the mechanism underlying this activity. We also report here the inhibitory effect of these samples on human lymphocyte proliferation, besides their antioxidant effects in a DPPH and SNP assays.

2. Materials and Methods

2.1. General

^{1}H and ^{13}C-NMR spectra (DMSO-d_6 signals as internal reference: δ 2.50) were recorded on a Bruker DRX-300 (^{1}H: 300.13 MHz; ^{13}C: 75.48 MHz) spectrometer. Optical rotation was measured on a JASCO P-2000 polarimeter. Chromatographic purifications were performed on an RP-2 silanized silica (70 – 230 mesh, Merck), RP-18 silanized silica (40 – 63 mesh, Merck) and on Sephadex G-15 gel (40 – 120 μm, Sigma) columns. Eluates from column chromatography were monitored by thin layer chromatography on Silica 60 F_{254} (Merck), using *n*-butanol/acetic acid/water 8:1:1 as eluent solvent, and the spots were visualized under UV light or after ceric sulfate acidic revelation. This research has complied with all relevant federal guidelines and institutional policies related with the use of natural material and animals for research purpose. In addition, the Animal Use Committee of the Institute of Biophysics at the Federal University of Rio de Janeiro (Brazil) approved experimental protocols using mice.

2.2. Plant material

Leaves from *Vochysia divergens* Pohl were obtained from several specimens out of blooming season in a Private Natural Heritage Reserve (RPPN – SESC Pantanal) situated in the State of Mato Grosso (Brazil). A voucher specimen (UPCB 46164) is deposited at the herbarium of the Department of Botany (Federal University of Paraná, Brazil) by Dr. Raquel Negrelle. The plant name has been checked with http://www.theplantlist.org (Accessed: October 22, 2017).

2.3. Extraction and fractionation

Dried leaves (356.9 g) from *V. divergens* (Vd) were powdered and submitted to a decoction process (10% w/v) with distilled water. The lyophilized Vd extract (32.3 g) was re-suspended in distilled water (0.7 L). Ethanol was added in a 1:1 proportion (v/v) and the resulting precipitate (organic salts and/or macromolecules) (Schmourlo et al., 2005) was separated by filtration. After the evaporation of ethanol in a water bath, the supernatant was frozen and lyophilized. The dried material (30.6 g) was re-suspended in distilled water, and partitioned with CH_2Cl_2, EtOAc and *n*-BuOH, affording fractions FA (210.6 mg), FB (1.9 g) and FC (7.3 g), respectively. The residual aqueous fraction was named FD (21.1 g). Among them, the n-butanol fraction (FC) was shown to be richer in phenolic substances and was purified on an RP-2 column (30 x 3.0 cm) using water gradually enriched with ethanol. The flavonoid-enriched fraction (2.7 g) eluted with water/ethanol (70:30) was further injected on an RP-2 column (30 x 2.2 cm) using a gradient of water/ethanol. The fraction eluted with H_2O/EtOH (70:30) was dried affording 1.1 g of a solid material, which was partitioned between distilled water and acetone. The soluble phase was dried (490 mg), suspended in water and submitted to preparative thin-layer chromatography (Silica gel Merck; BAW 8:1:1 elution). The B2 band corresponding to the flavonoid material was extracted successively with ethyl acetate, ethanol and methanol. The solution was dried and the resulting material was purified on a Sephadex G-15 column (25 x 1.4 cm; H_2O). The purification was achieved by means of an RP-18 column (20 x 0.5 cm; H_2O/MeOH gradient). The fraction eluted with H_2O/MeOH (80:20), after drying, gave 18.7 mg of flavonoid **1**, which was obtained as a yellow powder [m.p. 181 – 182ºC; $[\alpha]_D^{25}$ = -35.64 (H_2O; c 1.0)]. This flavonoid was identified as 3',5-dimethoxyluteolin-7-*O*-β-glucopyranoside (syn. 5-methoxycrysoeriol-7-*O*-β-glucopyranoside) by comparison of its ^1H and ^{13}C NMR data with the literature (Ozawa et al., 1995).

2.4. HPLC-DAD-MS analyses

HPLC analyses of samples (10 μL) were performed at University of Florence, Italy, on an HP 1100L instrument with a Diode Array Detector and managed by an HP 9000 workstation equipped with RP-18 column (5 μm, 250 mm, 4.60 mm, Luna, Phenomenex) maintained at 26 ºC. The chromatography was performed in a gradient elution mode at a 1.0 mL min^{-1} flow using formic acid aqueous solution at pH 3.2 (eluent A) and acetonitrile (eluent B). The following solvent gradient, expressed as eluent B percentage (% B), was applied: from 0 to 20 % within 10 min; from 20 to 25 % within 5 min; from 25 to 30 % within 5 min; from 30 to 50 % within 10 min; from 50 to 100 % within 15 min; 45 minutes as total time of analysis. UV–Vis spectra were recorded in the range 200–450 nm. The HPLC system was interfaced with an HP 1100MSD API-electrospray configured as described before (Muzitano et al., 2011).

2.5. Quantification of flavonoid content by HPLC

The Vd extract was analyzed by HPLC in triplicate, and quantification of its phenolic constituents was obtained using calibration graphs with ten data points. Calibration graphs were recorded with sample amounts ranging from 0.20 to 10.0 μg (r^2 = 0.9999). A calibration graph using the flavonoid rutin was carried out for quantitative purposes.

2.6. Effect on LPS-stimulated macrophages: NO and TNF-α production

Murine peritoneal macrophage RAW 264.7 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), and grown at 37 °C with 5% CO_2 in DMEM-F12 supplemented with 10% FCS and gentamicin (50 μg/mL). RAW 264.7 cells were seeded in flat

bottom 96-well tissue culture plates in the presence or absence of Vd extract, Vd fractions (FA, FB, FC and FD) (4, 20, 100 and 500 μg/mL), flavonoid **1** or luteolin (4, 20 and 100 μg/mL), and/or LPS (*Escherichia coli* 055: B5; Sigma, St. Louis, MO, USA). After 24 h incubation, culture supernatants were collected for NO and TNF-α assays. Not treated and not LPS stimulated-macrophages were used as negative control. Macrophages stimulated with 1 μg/mL LPS and not treated were used as positive control. Nitrite, a stable NO metabolite, was determined by the Griess method (Griess, 1864). Briefly, samples were incubated with an equal volume of Griess reagent, and the absorbance at 550 nm was measured. TNF-α was measured by means of L929 fibroblast viability indirect method. For that, murine fibroblast cell line L929 cells (ATCC, Rockville, MD, USA) (2×10^5 cells/well) were seeded in flat bottom 96-well tissue culture plates (Corning Inc., Corning, NY, USA) in the presence or absence of macrophage culture supernatant and in presence of actinomycin D (2 μg/mL). After 24 h incubation, L929 viability was assayed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide] method (Mosmann, 1983). The cytokine levels were calculated by using a purified recombinant mouse cytokine (PMC3016, Biosource, Camarillo, CA, USA) to obtain a standard curve that correlates viability and TNF-α concentration.

2.7. Cytotoxicity assay

The cytotoxic effects of samples (Vd extract, Vd fractions, flavonoid **1** and luteolin) on macrophages were examined by LDH (cytoplasmic enzyme lactate dehydrogenase) assay and determined colorimetrically using a commercial kit (Doles Reagentes e Equipamentos para Laboratórios LTDA) according to the manufacturer's instructions. The specific release was calculated as percentage of controls (non-treated macrophages as negative control and 1% Triton X-100 detergent treated macrophages as positive control).

2.8. Antioxidant activity assay

The antioxidant activity of samples was evaluated using sodium nitroprusside (SNP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods. Sodium nitroprusside, a NO donor, spontaneously liberates NO in aqueous solution at physiological pH, which rapidly interacts with oxygen to produce nitrite. To determine whether the samples directly interact with NO, SNP (5 mM; Sigma) was incubated at room temperature for 2.5 h in the presence of Vd extract, FA, FB, FC, FD fractions (4, 20, 100 and 500 µg/mL), flavonoid **1** and luteolin (both at 4, 20 and 100 µg/mL). After this period, nitrite accumulation was determined using the Griess method detailed above (Griess, 1864).

In the radical scavenging method, one milliliter of an aqueous solution of Vd extract was added to 1 mL of a DPPH ethanolic solution (1.10^{-4} M) (Sigma), and the reaction mixture was vigorously shaken at room temperature. DPPH absorption was spectrophotometrically measured at 514 nm. Mean values were obtained from triplicates (Duarte et al., 2007). Quercetin (a flavonol), gallic acid (a phenolic acid) and the commercial plant extract *Gingko biloba* EGb 761 were used as antioxidant controls.

2.9. Western blot analysis

RAW 264.7 cells (1×10^6 cells/well) were seeded in 24-well plates in the presence or absence of either LPS (1 µg/ml), samples at concentration of the 20 and 100 µg/mL for flavonoid **1** and at 100 and 500 µg/mL for the other samples. After 24 h of incubation, cells were washed twice with ice-cold PBS then lysed with the lysis buffer containing Tris/HCl 1M, pH 6.8, 25% distilled H_2O, 10% SDS, 20% glycerol and 5% ß-mercaptoethanol. The thawed cell lysates were mixed with 2% bromophenol blue. Cell lysates (80 µg of protein/lane) from RAW264.7 cells were separated by SDS/PAGE on 10% gels and transferred on to a PVDF membrane (Hybond, GE-Healthcare), which was blocked overnight at 4◦ C with blocking buffer [10 mM Tris/HCl, pH 8.0, 0.15 M NaCl, 0.1% Tween 20 and 5% (w/v) non-fat dried milk]. The blots were then incubated for 1 h at room temperature with 0.2 µg/ml rabbit polyclonal anti-*i*NOS Ab and for 1 h at room temperature with

peroxidase-conjugated anti-rabbit IgG Ab (Santa Cruz Biotechnology Inc.). Then, bound Ab was revealed with a solution containing enzyme substrate and DAB chromogen substance (diaminobenzidine) (100 µL tris HCl 2 M pH 7.5, 4.9 mL distilled H_2O, 5 mg DAB, 0.3 mL imidazole 0.1 M and 5 µL H_2O_2). Protein concentrations were determined by the Bradford method (DC Protein Assay, Bio-Rad). In order to estimate the molecular size of proteins, a protein standard ladder (Full Range Rainbow-GE Healthcare) was used in all electrophoresis experiments.

2.10. T cell proliferation assay

The inguinal lymph nodes were isolated from normal BALB/c mice, and single-cell suspensions in DMEM medium + 10% heat inactivated fetal calf serum and gentamicin (50 µg/mL) were plated (4 × 10^6 cells/mL) in 96-well culture plates (100 µL). Cells were stimulated with 5.0 µg/mL of ConA (Concanavalin A, Sigma Chemical Co.) for 48 h at 37 ºC in the presence of different concentrations of plant material. Cyclosporin A (CyA, Sigma Chemical Co.) was used as positive control. The proliferative response was measured by the incorporation of ^{3}H-thymidine (0.5 µCi/well) added in the last 18 h of culture. Cells were then harvested using a Dot-Blot device, and the radioactivity was measured in a beta-counter (Rossi-Bergmann and Noleto, 1994).

2.11. Statistical analyses

The data were reported as mean ± standard error of the mean (SEM) and were analyzed by one-way analysis of variance followed by a Tukey posttest, and were considered significant when $p<0.05$. In order to allow direct comparison, IC_{50} values were calculated by nonlinear regression analysis.

3. Results and discussion

3.1. Phytochemical investigation

The fractionation of the aqueous leaf extract from *V. divergens* combined an ethanol precipitation step followed by a successive partition of the supernatant with organic solvents, and resulted in

fractions FA (dichloromethane), FB (ethyl acetate), FC (butanol) and FD (remaining aqueous phase). The purification of FC fraction allowed the isolation of flavonoid **1** identified as 3',5-dimethoxyluteolin-7-*O*-β-glucopyranoside (syn. 5-methoxycrysoeriol-7-*O*-β-glucopyranoside) with basis on ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HMQC and HMBC NMR spectroscopic experiments (DMSO-d_6) and in accordance with the literature data (Markham and Geiger, 1994; Ozawa et al., 1995) (**Fig. 1**).

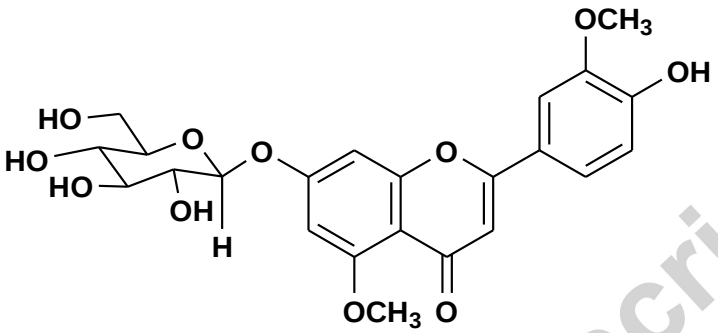

Fig. 1: Flavonoid **1** (3',5-dimethoxy luteolin-7-*O*-β-glucopyranoside) from the aqueous leaf extract of *Vochysia divergens* (Vd extract).

Pimenta et al. (2015) reported recently this flavonoid from an ethanol leaf extract of *V. divergens*, besides two other methoxyflavones. Lopes et al. (1979) found in leaves of *V. cinnamomea* both 7,3',4'-trihydroxyflavone and 3'-methoxy-7,4'-dihydroxyflavone, while for *V. tucanorum* only the first one was reported.

The 5-methoxyflavones are uncommon in nature, and their pharmacological properties are rarely studied (Lopes et al., 1979). Nevertheless, they are reported to exhibit a gastroprotective effect against non-steroidal anti-inflammatory drugs (NSAIDs) (Ares et al., 1995; Blank et al., 1997) and strong chemoprotective activity when compared to their nonmethoxylated counterparts (Walle, 2007). The 5-methoxy flavones also showed cytotoxic effects toward a lung cancer cell line and a cervical carcinoma cell (Rahman and Moon, 2007; Walle, 2007; Ye et al., 2008), and antischistosomal activity (Pimenta et al., 2015).

In addition, Vd extract was submitted to HPLC-DAD-MS analysis (**Fig. 2**). UV/Vis spectra for four phenolic compounds identified in Vd extract are shown in **Fig. 3**. Three major substances were identified (t_R13.201, 13.872 and 15.597 min) in the chromatogram at 254 nm.

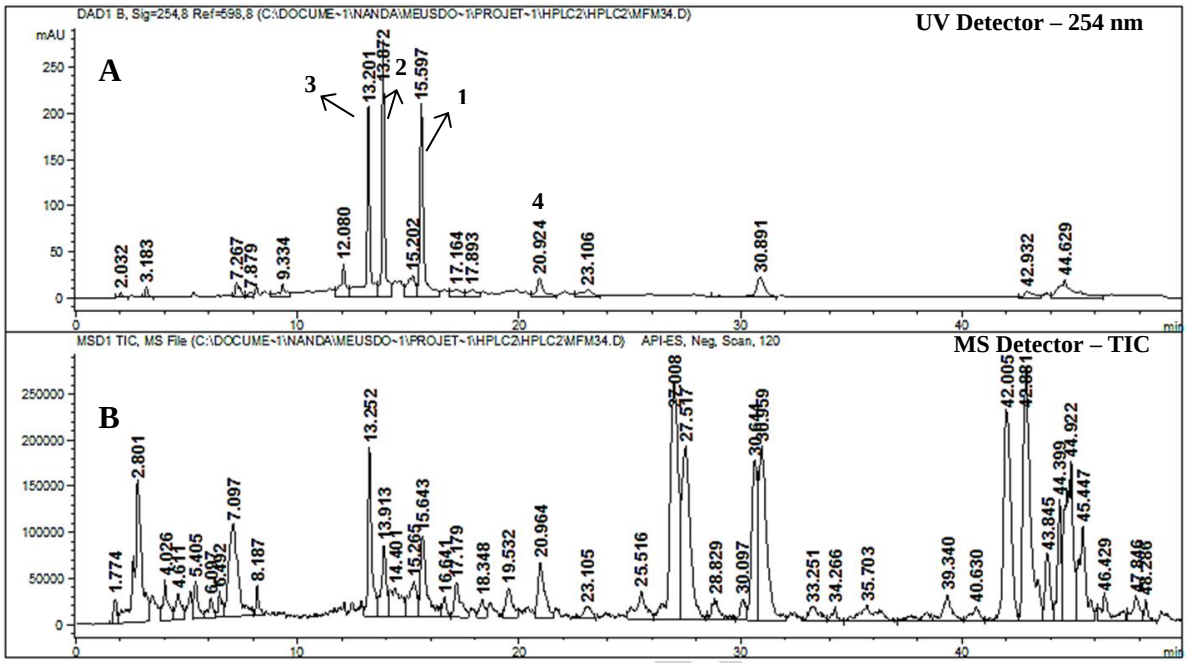

Fig. 2: Chromatograms of the aqueous extract from *Vochysia divergens* leaves (A: UV Detector – 254 nm; B: MS detector – TIC). 3',5-dimethoxy luteolin-7-*O*-β-glucopyranoside (**1**) (t_R 15.597 min), 5-methoxyluteolin-7-*O*-β-glucopyranoside (**2**) (t_R 13.872 min); galloyl-HHDP-glucopyranoside (**3**) (t_R 13.201 min); quercetin 3-*O*-α-rhamnopyranosyl (1→6) glucopyranoside (rutin) (**4**) (t_R 20.924 min) are assigned in A.

Peaks at t_R 13.872 min and 15.597 min showed UV spectra compatible with flavones (typical λ_{max} 251 -271 and 335 - 350 nm) (Greenham et al., 2003; Waridel et al., 2004; Lai et al., 2007). Peak at t_R 15.597 min showed $[M-H]^-$ *m/z* 475 and a characteristic fragment ion at *m/z* 313 due to the loss of a glucose unit. The fragment m/z 313 corresponds to a luteolin skeleton substituted by two methoxy groups. This peak corresponds to isolated 3',5-dimethoxyluteolin-7-*O*-β-glucopyranoside (**1**). The structural hypothesis for peak at t_R 13.872 min was based on UV-Vis and MS spectra, in comparison with **1**. The similarity between their UV spectra indicated that these two flavones have similar structures. A pseudomolecular ion (*m/z* 461) and a fragment ion at m/z 299 due to the loss of

an *O*-glycoside unit were observed for peak at t_R 13.872 min. The fragment *m/z* 299 showed that one hydroxyl group of luteolin skeleton is substituted by a methoxy group (Merken and Beecher, 2000; Sánchez-Rabaneda et al., 2003). A deeper analysis of the UV spectrum allowed the suggestion that this methoxy group is attached to the C-5 position of luteolin, since the UV spectra of compared peaks are practically overlapped. The principal positions susceptible to modification of the UV spectra are 5 (A ring) and 4' (B ring) due to interference in the molecule's electronic availability (Markham et al., 1978; Wolfbeis et al., 1987; Greenham et al., 2003). Thus, we may consider that a hexosyl moiety (probably a glucose molecule) is attached at C-7 and that a methoxy group is linked at C-5. These structural characteristics allow proposing that peak at t_R 13.872 min could correspond to 5-methoxy luteolin-7-*O*-β-glucopyranoside (**2**), a methoxyflavone that has never been reported before for *V. divergens*.

A typical UV absorption of hydrolysable tannin with maxima at about 220 and 280 nm was observed for the peak at t_R 13.201min. Its mass spectrum showed a $[M-H]^-$ at *m/z* 633 with two fragmentation ions at *m/z* 463 and 301, indicating losses of gallic acid and glucosyl units, respectively (Salminen et al., 1999; Soong and Barlow, 2005). This tannin was identified as galloyl-HHDP-glucopyranoside (**3**).

The minor peak at t_R 20.924 min displayed a typical UV absorption of flavonol (λ_{max} 250-280 and 350-380 nm) (Merken and Beecher, 2000; Greenham et al., 2003). Its mass spectrum showed a $[M-H]^-$ at *m/z* 609 with one fragmentation ions at *m/z* 301, indicating loss of rutinosyl unit (glucose + rhamnose). Based on UV and mass spectra, we can propose that this peak corresponds to quercetin 3-*O*-α-rhamnopyranosyl (1→6) glucopyranoside (rutin) (**4**).

The amounts of flavonoids 3',5-dimethoxyluteolin-7-*O*-β-glucopyranoside (**1**), 5-methoxyluteolin-7-*O*-glucopyranoside (**2**), and rutin (**4**) in the Vd extract were calculated as 1.12 %, 0.99 % and 0.27 % w/w, respectively, based on the rutin calibration curve.

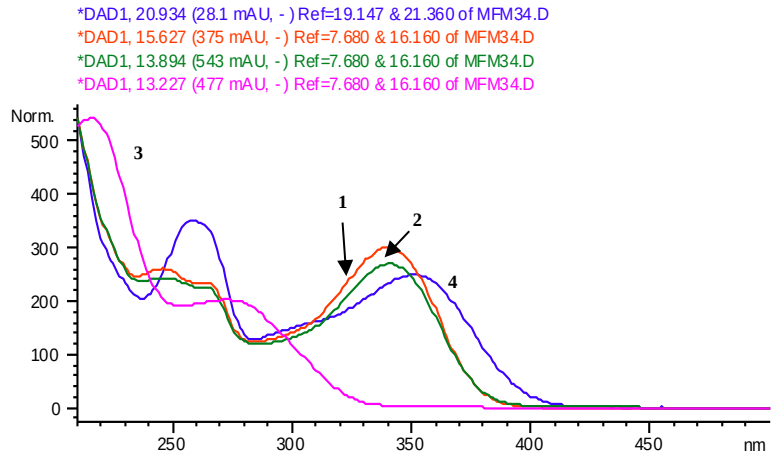

Fig. 3: UV/Vis spectra of the different phenolic compounds identified in the aqueous extract from *Vochysia divergens* leaves (Vd extract) where 3',5-dimethoxy luteolin-7-*O*-β-glucopyranoside (**1**) (t_R 15.597 min), 5-methoxyluteolin-7-*O*-β-glucopyranoside (**2**) (t_R 13.872 min); galloyl-HHDP-glucopyranoside (**3**) (t_R 13.201 min); quercetin 3-*O*-α-rhamnopyranosyl (1→6) glucopyranoside (rutin) (**4**) (t_R 20.924 min) are assigned.

3.2. Investigation of the immunomodulatory potential

The immunomodulatory potential of Vd extract, fractions (FA, FB, FC and FD) and flavonoid **1** was assessed in the production of NO and TNF-α by LPS-stimulated RAW 264.7 macrophages. Induction by some inflammatory stimuli, e.g., bacterial lipopolysaccharide (LPS), activates the NF-κB pathway and regulates the expression of a wide variety of genes involved in inflammatory responses such as cytokines TNF-α and IL- 1β, and the enzyme inducible nitric oxide synthase (*i*NOS), which catalyzes the conversion of amino acid L-arginine to NO (Duque and Descoteaux, 2014; Mittal et al., 2014). Nitric oxide as an active free radical and inflammatory mediator is part of the host defense. Interestingly, it has been found that increased exhaled NO levels in asthmatic patients compared to that nonasthmatic subjects is probably due to increased expression and activity of the *i*NOS and it could be modulated by nasal glucocorticoids (Pendharkar and Mehta, 2008). *Vochysia divergens* is used in popular medicine against respiratory infections and asthma (Hess et al., 1995a). Thus, it seemed important to investigate the effects of Vd extract, their fractions and main flavonoid on NO production.

As showed in **Fig. 4**, *V. divergens* extract was capable of inhibiting NO production by LPS-stimulated macrophages. This effect was statistically significant when compared to the control groups. Among the four fractions tested, the more pronounced inhibitory activity against NO was exhibited by butanol fraction (FC). The activity of FC was especially noteworthy; reaching more than 90% at 100 µg/mL; even at 20 µg/mL, FC inhibited by 60% the NO production (IC_{50} 15.2 ± 0.1 µg/mL). The ethyl acetate fraction (FB) exhibited moderate ability to inhibit NO production, restricted to the highest concentrations tested. In contrast, the dichloromethane (FA) and aqueous fractions (FD) showed very low activities (IC_{50} greater than the highest concentrations tested). In view of this activity, FC was chosen to be purified by chromatography, affording 3',5-dimethoxyluteolin-7-*O*-β-glucopyranoside (**1**). This 5-methoxyflavone showed to be 20 times more active (IC_{50} 25.2 ± 0.2 µg/mL; 52.8 µM) than Vd extract in inhibiting NO production, although its inhibitory activity has been lower than that observed for FC. We can suppose that other compounds present in the butanol fraction (FC) may contribute to the inhibition of NO production by additive or synergistic effect with flavonoid **1**.

Flavonoid **1** was slightly more active in inhibiting NO production than its aglycone luteolin (IC_{50} 17.2 ± 0.1 µg/mL; 60.1 µM), when IC_{50} values were compared in µM. Park and Song (2013) reported that luteolin and luteolin-7-*O*-glucoside inhibit the NO production through modulation of NF-κB/AP-1/PI3K-Akt signaling cascades. Both are potential candidates for the treatment of inflammation, although luteolin has shown somewhat higher inhibitory activity (Park and Song, 2013). When compared to L*N*MMA (L-*N*-monomethyl-arginine), a known selective *i*NOS inhibitor used as positive control (NO inhibition by 54.7±6.2 % at 20 µg/mL), flavonoid **1** and luteolin presented higher inhibitory activity on NO production, but FC was two times more active.

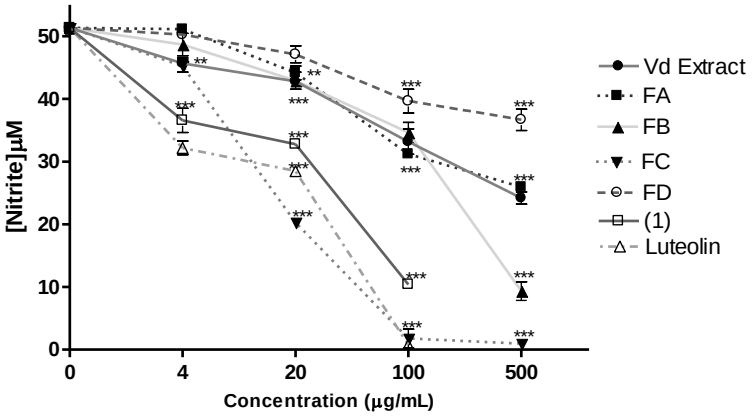

Fig. 4: Effect of the aqueous extract from *Vochysia divergens* leaves (Vd extract), fractions (FA, FB, FC and FD), 3',5-dimethoxy luteolin-7-*O*-β-glucopyranoside (**1**) and luteolin on NO production by LPS-stimulated macrophages RAW 264.7. Cells were plated and stimulated with 1.0 µg/mL de LPS for 24 h at 37 ºC with or without Vd extract, FA (CH_2Cl_2 fraction), FB (EtOAc fraction), FC (*n*-BuOH fraction), FD (aqueous fraction) (4, 20, 100 e 500 µg/mL), **1** (4, 20, 100 µg/mL) or luteolin (4, 20, 100 µg/mL). Nitrite was determined by the method of Griess reaction. Macrophages not treated and not LPS stimulated were used as negative control; and macrophages stimulated with 1 µg/mL LPS (51.3 ± 0.3 µg/mL) and not treated as positive control. L-*N*MMA was also used as positive control at 20 µg/mL inhibiting 54.7 ±6.2 % NO production. Arithmetic means ± standard deviation (n = 3). $p < 0.01$ (**) and $p<0.001$ (***) in relation to non-treated group (0 µg/mL).

Tumor necrosis factor-α (TNF-α) is a major pro-inflammatory cytokine, which regulates inflammation and is related to several disorders. Our findings showed that the plant samples (Vd extract and fractions) and flavonoid **1** were more potent inhibitors of NO than TNF-α. Vd extract shows a poor capacity of inhibiting TNF-α production by LPS-stimulated macrophages RAW 264.7 (**Fig. 5**). The butanol fraction (FC) was notably the most potent inhibitor of TNF-α production, with IC_{50} 206.9 ± 0.4 µg/mL, when compared to other tested fractions and Vd extract. In addition, the flavonoid **1** inhibited significantly TNF-α production, being more potent than FC (IC_{50} 132.5 ± 0.5 µg/mL; 277.6 µM) and showing a similar profile to luteolin (IC_{50} 102.1 ± 0.3 µg/mL; 356.7 µM). It has been observed that some flavonoids are potent inhibitors of the production of TNF-α (Leyva-López et al., 2016; Somerville et al., 2016). Previous studies reported that luteolin and luteolin-7-glucoside inhibited lipopolysaccharide (LPS)-stimulated TNF-α release in RAW 264.7 cells (Paul et al., 2006). Comalada et al. (2006) studied the structure–activity relationship for

several flavonoids using primary bone marrow-derived mouse macrophages. They observed that luteolin was the most potent TNF-α inhibitor, an effect that has been associated with the inhibition of the NF-kB pathway.

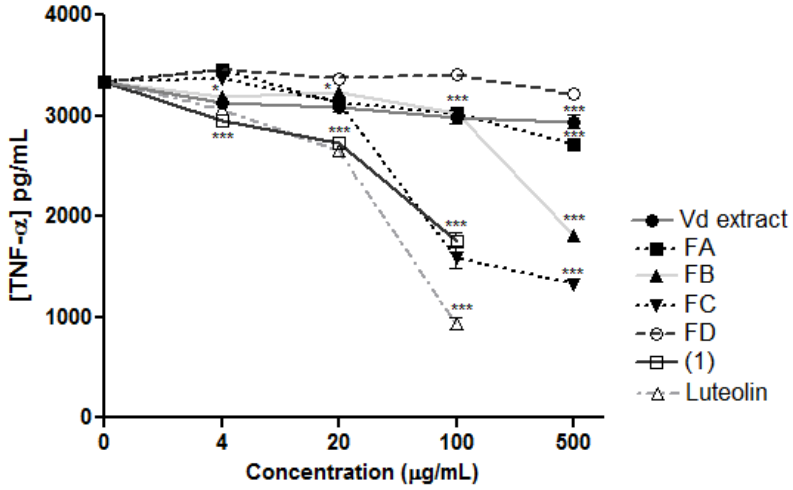

Fig. 5: Effect of the aqueous extract from *Vochysia divergens* leaves (Vd extract), fractions (FA, FB, FC and FD), 3',5-dimethoxy luteolin-7-*O*-β-glucopyranoside (**1**) and luteolin on TNF-α production by LPS-stimulated macrophages RAW 264.7. Cells were plated and stimulated with 1.0 µg/mL de LPS for 24 h at 37 ºC with or without Vd extract, FA (CH_2Cl_2 fraction), FB (EtOAc fraction), FC (*n*-BuOH fraction), FD (aqueous fraction) (4, 20, 100 e 500 µg/mL), **1** (4, 20, 100 µg/mL) or luteolin (4, 20, 100 µg/mL). TNF-α was assayed by L929 fibroblast viability indirect assay. The cytokine levels were calculated by using a purified recombinant mouse cytokine as a standard. As a negative control it was used macrophages not treated and not LPS stimulated and as positive control macrophages stimulated with 1 µg/mL LPS and not treated (3338.6 ± 1.6 µg/mL). Arithmetic means ± standard deviation (n = 3). $p < 0.001$ (*) in relation to non-treated group (0 µg/mL).

To exclude the possibility that the inhibitory effects of Vd samples and flavonoid **1** on macrophages were due to their cytotoxicity, cell viability was assessed by lactate dehydrogenase (LDH) release assay. As observed in **Fig. 6**, all the samples were not toxic at 4 and 20 µg/mL, and exhibited low to moderate toxicity effect (<30% of cell viability at 100 and 500 µg/mL), with $p<0.05$ in relation to non-treated group only at high concentration. However, luteolin showed a statistically significant reduction in cell viability at 100 µg/mL. According to Pimenta et al. (2015), the treatment of V79 lung fibroblasts with luteolin at high concentrations significantly decreases cell viability, while its glycosides derivatives showed the same level of toxicity only when five times more concentrated.

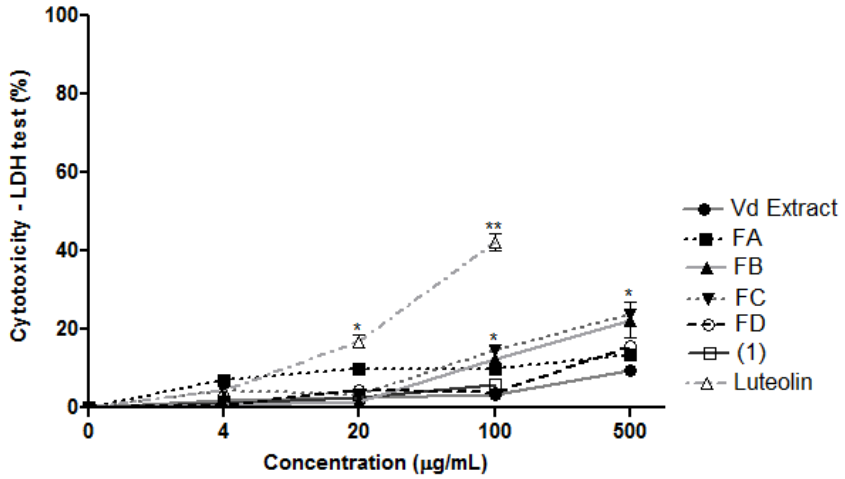

Fig. 6: Cytotoxicity measured by the percentage of specific release of lactate dehydrogenase (LDH) of the aqueous extract from *Vochysia divergens* leaves (Vd extract), fractions (FA, FB, FC and FD), 3',5-dimethoxy luteolin-7-*O*-β-glucopyranoside (**1**) and luteolin. Macrophages RAW 264.7 were plated and stimulated with 1.0 µg/mL de LPS for 24 h at 37 ºC with or without Vd extract, FA (CH_2Cl_2 fraction), FB (EtOAc fraction), FC (*n*-BuOH fraction), FD (aqueous fraction) (4, 20, 100 e 500 µg/mL), **1** (4, 20, 100 µg/mL) or luteolin (4, 20, 100 µg/mL). The LDH content was determined using a commercial kit. The specific release was calculated as percentage of controls (non-treated macrophages as negative control and 1% Triton X-100 detergent treated macrophages as positive control). Arithmetic means ± standard deviation (n = 3). $p < 0.05$ (*) and $p < 0.01$ in relation to non-treated group (0 µg/mL).

V. divergens extract, its fractions and flavonoid **1** were evaluated for their antioxidant activity in order to verify the ability of these samples to scavenge NO radicals derived from a NO donor (SNP) and to suppress the induction of *i*NOS, a key enzyme in NO production by activated macrophages. As seen in **Fig. 7**, the plant samples and flavonoid **1** scavenged only low amounts of SNP-derived NO (about 20–30% of the NO scavenging activity) compared to rutin (IC_{50} 43.3 ± 0.8 µg/mL), a flavonoid known for showing antioxidant activity (Villaño et al., 2007), while Vd extract scavenged about 40 % at 500 µg/mL. The antioxidant activity of the luteolin (IC_{50} of 89.4 ± 1.2 µg/mL; 311.6 µM) was higher than that was observed for flavonoid **1**.

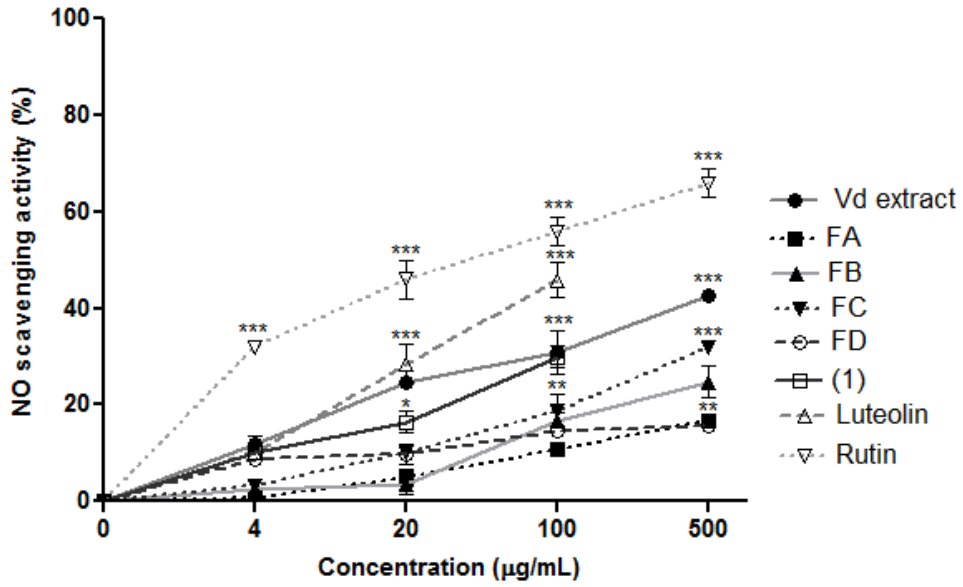

Fig. 7: Effect of the aqueous extract from *Vochysia divergens* leaves (Vd extract), fractions (FA, FB, FC and FD), 3',5-dimethoxy luteolin-7-*O*-β-glucopyranoside (**1**) and luteolin on the scavenging of nitric oxide derived from sodium nitroprusside. Medium with sodium nitroprusside and Vd extract, FA (CH_2Cl_2 fraction), FB (EtOAc fraction), FC (*n*-BuOH fraction), FD (aqueous fraction) (4, 20, 100 e 500 µg/mL), **1** (4, 20, 100 µg/mL) or luteolin (4, 20, 100 µg/mL) was incubated for 2.5 h. Nitrite, a stable NO metabolite, was determined by the method of Griess reaction. As a negative control it was used only medium DMEM-F12 and as positive control of nitrite accumulation: culture medium with 5 mM sodium nitroprusside. Rutin was also used as positive control of NO scavenging (4, 20, 100 and 500 µg/mL). Arithmetic means ± standard deviation (n = 3). $p < 0.01$ (**) and $p<0.001$ (***) in relation to the sodium nitroprusside-treated group. (0 µg/mL).

The antioxidant activity of Vd extract also was evaluated in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Quercetin (flavonoid), gallic acid (phenolic acid) and *Gingko biloba* Egb 761 were used as positive controls (Silva et al., 2005; Kubo et al., 2006; Villaño et al., 2007). Vd extract showed a scavenging activity of 76.3% and 74.5% at 2 µg/mL and 20 µg/mL, respectively (**Table 1**). It is interesting to note that the antioxidant activity observed for Vd extract in the SNP and DPPH assay does not explain what was observed for the most active samples at the same concentration on NO production by macrophages. It is well known that phenolic substances such as flavonoids and tannins have high scavenging ability, contributing to health preservation since they avoid the general inflammatory response caused by free radicals in tissues (Pietta, 2000; Fang and Liu, 2002; Jacobo-Velázquez and Cisneros-Zevallos, 2009). However, some flavonoids have shown

low antioxidant potential with high *in vitro* anti-inflammatory effects (Comalada et al., 2006). These findings suggest that probably the NO inhibitory activity is due to a specific inhibition of *i*NOS activity or expression, rather than a direct scavenging activity of NO species.

Table 1: Antioxidant activity of the aqueous extract from *Vochysia divergens* leaves (Vd extract) and quercetin. Gallic acid and *Gingko biloba* Egb 761 were used as positive controls.

Sample	DPPH assay	
	Concentration (2 μg/mL)	Concentration (20 μg/mL)
	Scavenging activity (%)	Scavenging activity (%)
Quercetin	83.1	89.6
Gallic acid	87.6	94.4
Gingko biloba Egb 761	90.4	71.0
Vd extract	76.3	74.5

Thus, we examined the inhibitory capacity of Vd extract, its fractions and flavonoid **1** on the expression of *i*NOS in LPS-treated macrophages. For these experiments, we used two concentrations of each sample. As can be seen in **Fig. 8**, according to densitometry analysis of the bands, Vd extract and dichloromethane fraction (FA) reduced the *i*NOS expression by about 55–65% at 500 μg/mL. Ethyl acetate (FB) and butanol fractions (FC), when used at a concentration of 500 μg/mL, and flavonoid **1** at 100 μg/mL almost completely inhibited *i*NOS. At the lower concentration, FB and flavonoid **1** retained the *i*NOS expression reduced by about 40%, while FC remained remarkably about 80% inhibition. These results demonstrate that the inhibitory effects observed on NO production were mediated mainly by suppression of *i*NOS expression in activated macrophages. Park and Song (2013) demonstrated that luteolin and luteolin-7-*O*-glucoside inhibited NO and PGE_2, as well as the expression of their corresponding enzymes, iNOS and COX-2. Cruz et al. (2008) have demonstrated that 5-methoxyflavones were able to prevent neurodegeneration through DNA polymerase-β inhibition via antioxidant and anti-inflammatory mechanisms.

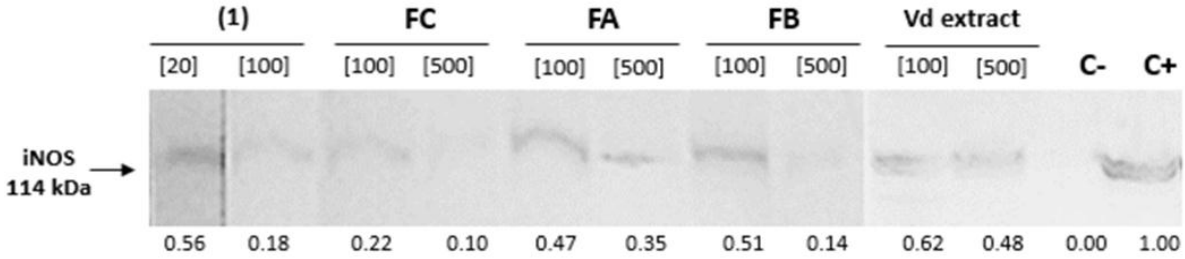

Fig. 8: Effect of the aqueous extract from *Vochysia divergens* leaves (Vd extract), fractions (FA, FB and FC) and 3',5-dimethoxy luteolin-7-*O*-β-glucopyranoside (**1**) on the LPS- induced *i*NOS expression in RAW 264.7 macrophages. Cells were plated and stimulated with 1 μg/mL de LPS for 24 h at 37 ºC with or without samples; 20 and 100 μg/mL (**1**) or 100 and 500 μg/mL (FA (CH_2Cl_2 fraction), FB (EtOAc fraction), FC (*n*-BuOH fraction) and Vd extract. Samples of cell lysates were prepared and analyzed by Western blotting with an *i*NOS- specific antibody. The cells treated with LPS only were used as a positive control of macrophage stimulation (C+). Untreated cells were used as a negative control (C−). Images are representative of two independent experiments that showed similar results. Lower panel, quantification of the protein levels by immunoreactive bands densitometric analysis. Each band was compared to respective positive control band. Relative densities were calculated employing ImageJ software for Windows (NIH, Bethesda, MD, USA). The value for positive control condition (LPS-stimulated cells) was set as 1 and other conditions were recalculated correspondingly to allow ratio comparisons.

We also evaluated the effect of Vd extract and flavonoid **1** on lymphocyte-stimulated proliferation responses (**Fig. 9**). When compared to the classical immunosuppressive agent cyclosporin A, Vd extract inhibited the lymphocyte proliferation (IC_{50} 13.6 ± 0.1 μg/mL) at a higher extent than flavonoid **1** (IC_{50} 19.8 ± 0.1 μg/mL; 41.6 μM) or cyclosporin A, used as a positive control (IC_{50} 43.8 ± 0.2 μg/mL; 36.8 μM; data not shown). Flavonoids such as quercetin glycosides (e.g., rutin) present in Vd are known for their immunomodulatory and antioxidant activities. Although additional substances belonging to several chemical classes compose the crude extract, the higher inhibitory activity observed for Vd extract could be explained, at least partially, by the presence of flavonoids in the leaves of the plant (Corrêa et al., 2008; Merlo et al., 2015).

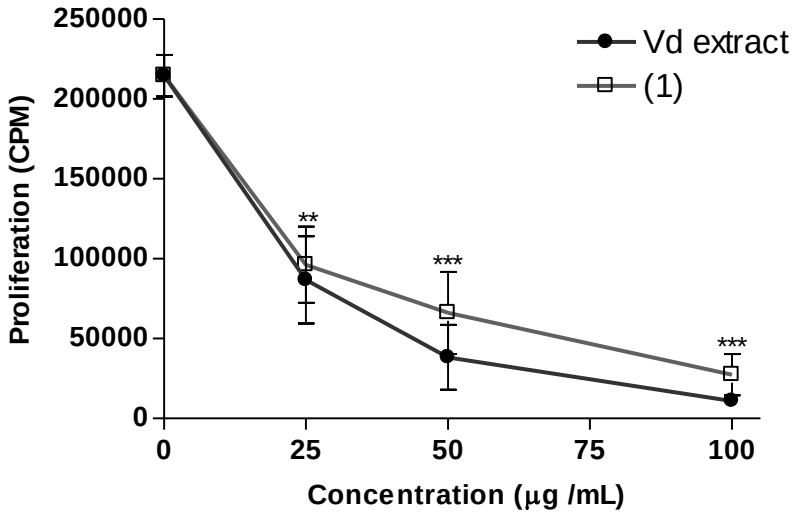

Fig. 9: The inhibitory effect of the aqueous extract from *Vochysia divergens* leaves (Vd extract) and 3',5-dimethoxy luteolin-7-*O*-β-glucopyranoside (**1**) on the stimulated proliferation of BALB/c lymphocytes. Viable Trypan Blue-negative cells (4×10^6 cells/mL) were plated and stimulated with 5.0 µg/mL de ConA for 48 h at 37 ºC. Cyclosporin A (CyA) was used as positive control. The proliferative response was evaluated through the incorporation of ^{3}H-thymidine (0.5 µCi/well) added in the last 18 h of culture. The results were expressed as mean counts per minute (CPM). Arithmetic means ± standard deviation (n = 5). $p < 0.01$ (**) and $p<0.001$ (***) in relation to the non-treated group.

Mookerjee et al. (1986) have reported a reversible lymphoproliferative inhibition effect of flavonoids, such as quercetin, in response to phytomitogens, soluble antigens and phorbol esters by blocking events that follow the exposure to the stimulus. It was also known that the presence of a C-2,3 double bond and of the 3',4'- dihydroxylated system (catechol system) in flavonoid structure are fundamental for the anti-inflammatory properties and suppressive activity against lymphocyte proliferation (Lee et al., 1995; You et al., 1998; Coutinho et al., 2009).

Some biological activities have been described for *Vochysia* species, as anti-allodynic and antiproliferative, attributed to tormentic acid isolated from the stem bark of *V. divergens* (Bortalanza et al., 2002; Fogo et al., 2009); gastroprotective, to *V. tucanorum* methanol extract (Gomes et al., 2009). There are only few studies on immunomodulatory activity of *Vochysia* species. Seven triterpene derivatives isolated from methanol stem bark extracts of *Vochysia pacifica* showed anti-inflammatory activity against phosphodiesterase isozyme (PDE4) (Weniger et al.,

2005). The leaf methanol extract of *Vochysia ferruginea* revealed to be anti-inflammatory by significantly reducing the edema in the rat paw assay (Calderón et al., 2001).

Conclusions

The study of an aqueous leaf extract of this medicinal species allowed the isolation of the flavone 3',5-dimethoxyluteolin-7-*O*-β-glucopyranoside (**1**), and the identification of 5-methoxyluteolin-7-*O*-β-glucopyranoside (**2**), galloyl-HHDP-glucopyranoside (**3**), and rutin (**4**). Our findings showed that Vd aqueous extract, butanol fraction and flavonoid **1** inhibited NO and TNF-α production by LPS-stimulated macrophages. The observed inhibitory activity against NO production was mediated by inhibition of iNOS expression. In addition, Vd aqueous extract and flavonoid **1** also inhibited lymphocyte proliferation *in vitro* suggesting a potential anti-inflammatory activity. This is the first report describing the immunomodulatory potential of *V. divergens* and 3',5-dimethoxy luteolin-7-*O*-β-glucopyranoside (**1**). Our findings emphasize the importance of investigating the immunomodulatory effects of *V. divergens* and encourage the search for therapeutic substances potentially useful against inflammatory processes, especially pulmonary diseases.

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Conflicts of Interest

The authors declare no conflict of interest.

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Author Contribution

Conception and design of experiments: M. F. Muzitano; S. S. Costa; B. Rossi-Bergmann.

Acquisition of data: M. F. P. Corrêa; T. L. B. Ventura; E. A. Cruz; M. F. Muzitano; M. C. Bergonzi.

Analysis and/or interpretation of data: M. F. P. Corrêa; T. L. B. Ventura; M. F. Muzitano; S. S. Costa; M. C. Bergonzi; A. R. Bilia; B. Rossi-Bergmann.

Drafting the manuscript: T. L. B. Ventura; M. F. P. Corrêa; M. F. Muzitano; S. S. Costa.

All the authors have read the final manuscript and approved the submission.

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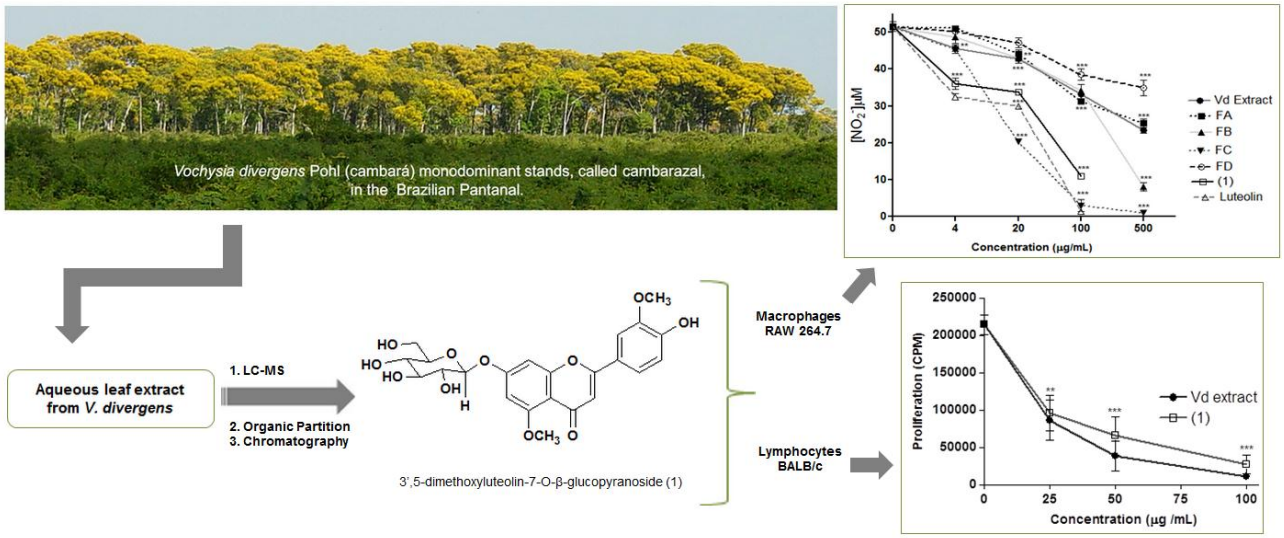

Graphical Abstract