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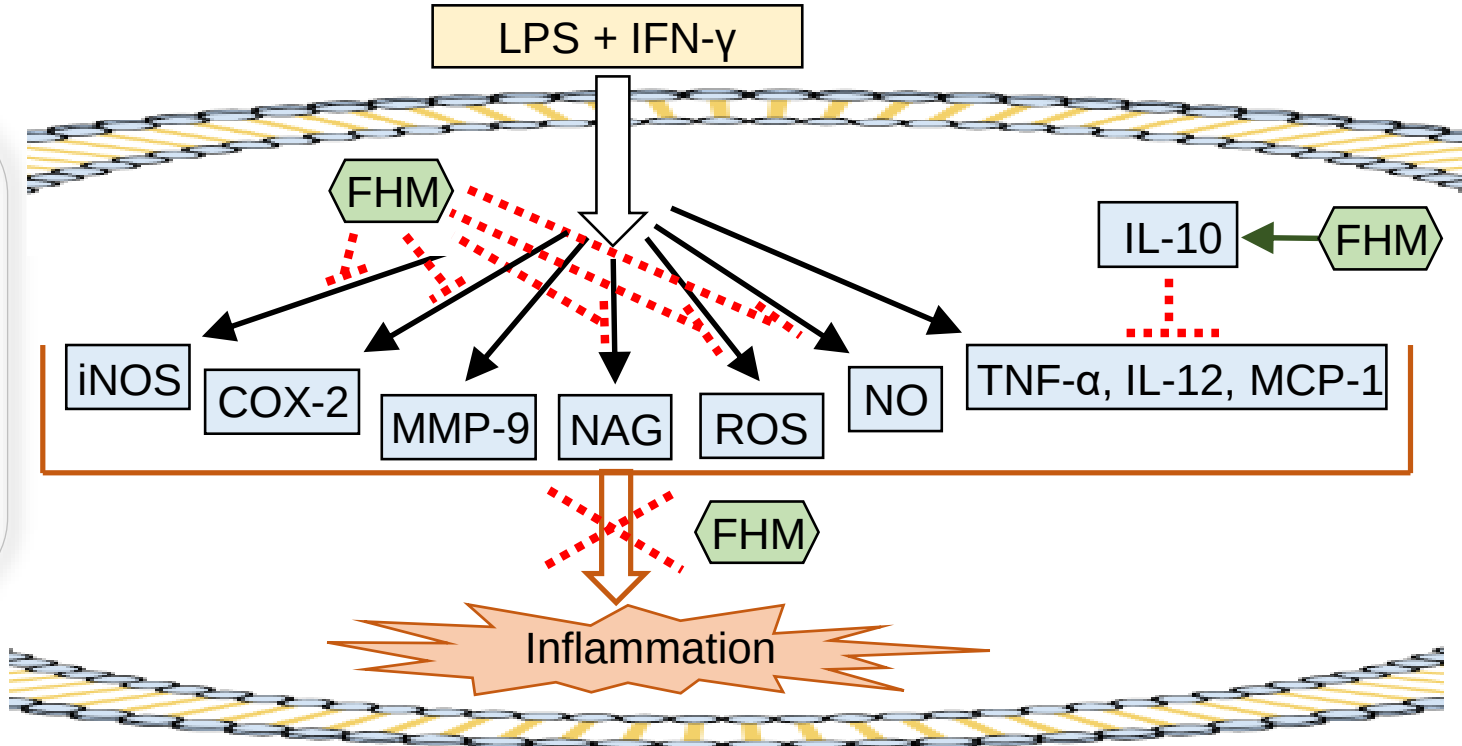
FHM

Hydromethanolic fraction
Protium spruceanum



Anti-inflammatory activity of *Protium spruceanum* (Benth.) Engler is associated to immunomodulation and enzymes inhibition

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ABSTRACT

Ethnopharmacological relevance: Protium spruceanum (Burseraceae) is used in Brazilian traditional medicine as anti-inflammatory, but the factors involved in this activity were not yet characterized. *Aims of the study:* analyze the aspects involved in the anti-inflammatory activity of polar fractions obtained from extracts of leaves and branches. *Materials and methods:* Hydromethanolic fraction was obtained by liquid-liquid partition from crude ethanolic extract and its compounds were identified by LC-DAD-MS. Activity tests were performed using LPS+IFN-γ stimulated J774A.1 macrophages. Cytokines were evaluated by CBA kit, NO by Griess method, ROS by DCFH-DA, N-acetylglucosaminidase (NAG) activity by spectrophotometric method, matrix-metalloproteinase (MMP-9) activity by zymography, inducible nitric oxide synthase (iNOS) expression by immunofluorescence and cyclooxygenase (COX-2) expression by Western blot. *Results:* Fractions induced an increase of IL-6 and IL-10 which leads to the

control of pro-inflammatory cytokines levels. The treatment with the fractions also reduced NO production at all concentrations tested in all evaluated periods. ROS production by the macrophages was inhibited by the treatment and the leaves fraction showed the best results with a lower concentration than that observed for the branches. The enzymes assays showed that leaves fraction inhibited NAG and MMP-9 activities, as well as, iNOS and COX-2 expression. These activities can be associated with the presence of procyanidin, catechin, rutin, quercitrin, isoquercitrin and kaempferol-3-*O*-rhamnoside, major compounds that were identified in the fraction. *Conclusions:* Anti-inflammatory activity of *P. spruceanum* is associated to an immunomodulatory effect that leads to inhibition of ROS, NO, NAG, MMP-9, COX-2 and iNOS.

Keywords: *Protium spruceanum*; Traditional medicine; Anti-inflammation; Immunomodulation; Flavonoids

1. Introduction

Inflammation is an immune response that physiologically plays a beneficial role for the body and is essential for the defense against infections and for tissue repair during healing. However, if uncontrolled, this process can be harmful, causing a lot of pathologic processes (Medzitov, 2008). Inflammation has long been recognized as a major cause of diseases, related to cancer, cardiovascular disease, diabetes, pancreatitis, hepatic and pulmonary diseases, colitis, autoimmune and neurodegenerative diseases (Chen et al., 2018a).

The side effects of conventional anti-inflammatory drugs and analgesics stimulate the research with natural products based on their ethnopharmacological information (Maione et al., 2016). The anti-inflammatory activity of medicinal plants can be evaluated by *in vitro* and/or *in vivo* assays, through the quantification of inflammatory mediators as cytokines, nitric oxide (NO), reactive oxygen species (ROS) and enzymes (Oliveira et al., 2014; Torres-Rodriguéz et al., 2016). During the last decades, several plants extracts, containing mainly phenols/flavonoids, terpenoids and glycosides, have been identified as promising anti-inflammatory agents (Maione et al., 2016).

The genus *Protium*, family Buseraceae, comprises about 135 species, many of which have previously described anti-inflammatory activity (Rüdiger, Siani and Veiga Junior, 2007). Several studies about extracts obtained from species of this genus, for example, *P. heptaphyllum*, *P. strumosum*, *P. grandifolium*, *P. lewellyni* and *P. hebetatum* showed their anti-inflammatory effects in a mouse model (Siani et al., 1999).

Protium spruceanum (Benth.) Engler (Burseraceae), known as breu-branco, is used as anti-inflammatory and analgesic in traditional Brazilian medicine and occurs in Atlantic and Amazon rain forests, as well as,in riparian forests of the Cerrado (Vieira et al. 2010; Rodrigues et al. 2013). The use of its resin, leaves and barks as tea, inhalation and topical application has already been reported (Branch and Silva, 1983; Milliken et al. 1986; Hadju and Hohmann; Hajdu and Hohmann, 2012).The anti-inflammatory activity of extracts and fractions from leaves of *P. spruceanum* has already been studied *in vivo* and the potential of the hexanic fraction was attributed to the presence of α and β-amyrins, triterpenes with the mechanisms about inflammation already understood (Vitor et al., 2009; Rodrigues et al. 2013; Krishnan et al., 2014). However, Rodrigues et al. (2013) also verified promising results for the methanolic fraction, without a complete clarification of the factors (cytokines, nitric oxide, ROS and enzymes) involved in the anti-inflammatory activity. In addition, the action of extracts produced from different parts of this vegetal specie has not been studied yet. Thus, this study aims the analysis of the factors (cytokines, nitric oxide, ROS and enzymes) involved in the anti-inflammatory activity of polar fractions from leaves and branches of *P. spruceanum*.

2. Material and methods

2.1. Collection, identification and processing of the plant materials

Leaves and thin branches of *P. spruceanum* were collected in July 2016 at the region of Lavras, Minas Gerais, Brazil (between coordinates 21º17’33.6”S and 44º59’15.1” W, 21º18’11.9”S and 44º59’18.8”W). This specie was identified by Prof. Dr. Vivette Appolinário Rodrigues Cabral from the Departamento de Ciências Florestais, Universidade Federal de Lavras. After botanical

identification, a voucher of *P*. *spruceanum* was deposited in the *Herbarium* of Universidade Federal de Lavras, under the code No 16399 HESAL. Plant materials were dried at room temperature until a constant weight, achieved and then powdered by a knife mill (MR Manesco, Brazil). The access was registered at SisGen, the Brazilian National System of Genetic Resource Management and Associated Traditional Knowledge under number A2B5290.

2.2. Preparation of the fractions

After fragmentation, powders of both materials (300.0 g) were subjected to ultrasonic-assisted extraction with 2 L of ethanol 95ºGL (Topázio, Brazil). After each cycle of 30 min, the mixture was filtered and the ethanol was recovered in a rotatory evaporator at ≤ 45 ºC under reduced pressure. This extraction process was repeated 10 times, resulting in the crude ethanol extract of leaves (46.8 g, 15.6% yield) and branches (28.2 g, 9.4% yield). A sample of each crude extract (20.0 g) was dissolved with 300 mL of methanol-water (1:1) and subjected to liquid-liquid partition with 150 mL hexane (Química Moderna, Brazil). After 4 repetitions of the partition process and recovery of extractive solvents, hexanic fractions from leaves (2.5 g, 12.6 % yield) and branches (2.3 g, 11.5%), as well as, hydromethanolic fraction (HM) from leaves (13.7 g, 68.5 % yield) and branches (18.1 g, 90.6%) were obtained.

2.3. Phytochemical composition

High-performance liquid chromatography coupled to electrospray ionization (ESI) tandem mass spectrometry and photodiode array detection was employed to identify phenolic compounds in the HM fractions. The analysis was performed on a UPLC Acquity chromatographer (Waters, USA) equipped with a CSH 130 C18 column (50 x 10 mm x 1.7 µm particle size) (Waters, USA). Milli-Q purified water with 0.1% methanoic acid and acetonitrile (ACN) with 0.1% methanoic acid were used as mobile phase. The flow rate was 0.3 ml/min and 4 µL of samples (1.0 mg/mL) were injected, with a linear gradient from 5% to 95% ACN in 10 min, held until 11 min. ESI ionization in negative and positive-ion modes were performed under the following conditions: Capillary voltage 3.5 kV, positive and negative ion modes, capillary temperature 320 °C, source voltage 5

kV, vaporizer temperature 320 °C, corona needle current 5 mA and sheath gas and nitrogen 27 psi. Analyses were run in full scan mode (100-2000 u). ESI-mass spectrometry (MS/MS) analyses were performed by a UPLC Acquity (Waters) with argon as the collision gas and collision energy set to 30 eV. The UV spectra were recorded from 190 to 450 nm. The flavonoids and tannins were identified by association of UV and mass spectrometric data.

The measurement of total flavonoids was made by the aluminum chloride ($AlCl_3$) colorimetric method according to Chang *et al.* 2002, with modifications. 100 µL of the extracts and fractions (5 mg/mL in ethanol 95%) were transferred to a 96-well plate and mixed with 40 µL of ethanol 95%, 4 µL of $AlCl_3$ (10% w/v), 4 µL of potassium acetate (1 mol/L) and 52 µL of water. The absorbance was read after incubation for 40 minutes using a microplate reader (Molecular Devices, E Max, USA) at 405 nm. The total flavonoids was quantified using a standard calibration curve of quercetin (2 – 45 µg/mL; r^2= 0.9992; y= 0.4490x + 0.0623). The experiment was made in triplicate and the results were expressed as mg of quercetin equivalent (QE) per g of sample (mgQE/g).

The measurement of total condensed tannins was made by the vanillin - hydrochloric acid method according to Perez *et al.* 1999, with modifications. 40 *µ*L of the extracts and fractions (5 mg/mL in ethanol 95%) were transferred to a 96-well plate and were added 200 µL of vanillin-HCl (vanillin 4%:hydrochloric acid 8% 1:1 in methanol). The absorbance was measured after incubation for 20 minutes using a microplate reader (Molecular Devices, E Max, USA) at 490 nm. The total tannins was quantified using a standard calibration curve of catechin (2 – 45 µg/mL; r^2= 0.9921; y= 0.0014x - 0.0280). The experiment was made in triplicate and the results were expressed as mg of catechin equivalent (CE) per g of sample (mgCE/g).

2.4. Reagents and antibodies

Lipopolysaccharide (LPS) (*Escherichia coli* O111:B4), human IFN-γ, 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) and sulforhodamine B were purchased from Sigma (St-Louis, MO, USA). Antibodies specific for cyclooxygenase 2 (COX-2), 488 inducible nitric oxide synthase (iNOS) and Alexa Fluor 488 were purchased from Abcam (Cambridge, MA, USA).

2.5. Cell culture

Murine macrophages J774A.1 (ATCC TIB-67™) were cultured in RPMI-1640 Medium containing L-glutamine (0.3 g/L) (Sigma, USA), 10% (v/v) fetal bovine serum (FBS) and 60 µg/L gentamicin (Sigma, USA), and maintained in a humidified incubator at 37 ºC and 5% CO_2.

2.6. Cytotoxicity

Cells were distributed in 96-well microtiter plate using a density of 5 x 10^5 cell/well (final volume 100 µL) and after they were incubated for 4 h. Cells were treated with the fractions dissolved in 2% DMSO, at concentrations ranging from 1.00 to 0.12 mg/mL. Cell viability was evaluated using the sulforhodamine B assay (SRB) (Skehan et al. 1990). Absorbances were measured using a microplate reader (Molecular Devices, E Max, USA) at 490 nm and the percentage of viable cells was calculated over untreated cells. The experiment was performed in triplicate.

2.7. Cytokines, nitric oxide, N-acetylglucosaminidase and matrix-metalloproteinase (MMP-9)

Cells (5 x 10^5 cell/well, final volume 100 µL) were distributed in 96-well plate and after incubation for 4 h, they were treated or not with the fractions dissolved in 2% DMSO (0.50, 0.25 and 0.12 mg/mL) and stimulated or not with LPS (10 µg/mL) and IFN-γ (100 ng/mL). After incubation for 24 h, 48 h or 72 h, the supernatant was removed and stored at -80 ºC for analysis of cytokines, nitric oxide, N-acetylglucosaminidase (NAG) and metalloproteinase (MMP-9) activity. The experiment was performed in quadruplicate.

Cytokines (IL-12, IL-6, IL-10, TNF-α and IFN-γ) were quantified using the Cytometric Bead Array Mouse Inflammation CBA Kit (BD Biosciences, San Jose, CA) performed according to the manufacturer's instructions and the data were acquired on a BD FACS Calibur flow cytometer and processed by FCAP Software Array.

Nitric oxide was analyzed indirectly by the quantification of nitrite and nitrato by the Griess reaction method (Green et al., 1982). Nitrite concentrations

were determined by extrapolation from standard curve, constructed using various concentrations of sodium nitrite, and the results were expressed as nanomolar (nmol/L). Absorbance values were measured using a microplate reader (Molecular Devices, E Max, USA) at 570 nm.

NAG activity was analyzed by the quantification of *p*-nitrophenol produced by hydrolysis of 4-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma, USA) (Shimada et al., 2015). Absorbance values were measured by a microplate reader (Molecular Devices, E Max, USA) at 450 nm. NAG activity was calculated considering *p*-nitrophenol amount (nmol), incubation time (min) and volume in each well (mL). The results were expressed as U/L.

MMP-9 activity was analyzed by zimography (Hu and Beeton, 2010). After measured by Bradford (Bio-Rad), equal amounts of proteins were resolved by electrophoresis in polyacrylamide gel containing SDS 8% (SDS-PAGE) and gelatin at a final concentration of 0.1 %. After electrophoresis, the gels were washed twice for 30 min in 2.5% of Triton X-100 to remove SDS. Gels were incubated in buffer substrate (1 M Tris-HCl pH 7.4; 0.1% $CaCl_2$ and 1.17% NaCl) for 18 h. Gels were stained with Coomassie brilliant-blue and distained with aqueous solution with 4% methanol and 8% acetic acid for active bands visualization. Semi-quantitative analysis of the MMP-9 protein bands was performed using Image J Software (NIH, Bethesda, MD, USA) and the results were expressed as percentage in relation to untreated cells.

2.8. Intracellular reactive oxygen species (ROS)

Intracellular ROS were measured by 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma, USA). Cells (5×10^5 cells/well, final volume 100 µL) were treated with fractions and LPS+IFN-γ as previously described and incubated for 24 h. After, the cells were removed, incubated with 0.5 mM DCFH-DA for 30 min at 37 °C and analyzed by BD FACS Calibur flow cytometer. The date were analyzed by FlowJo software (Tree Star, San Carlos, CA, USA).

2.9. COX-2 expression

COX-2 expression was analyzed by Western blot. Cells (7×10^6 cells/well, final volume 2 mL, in 6-well plate) were treated with the leaves fraction (0.50 mg/mL) and LPS+IFN-γ as previously described and incubated for

24 h. Then, the cells were removed and the nuclear proteins were extracted using lysis buffer (0.22% NaCl, 0,15% Tris, 0.12% sodium deoxycholate, 0.25% Triton-X100 and 2.5% protease Inhibitor Cocktail, Sigma, USA). After measured by Bradford (Bio-Rad), equal amounts of proteins were fractionated by NuPAGE gel (Thermo Fisher Scientific) in MOPS running buffer and then transferred to PVDF membrane (Bio-Rad). After incubation with antibodies, bands of interest were revealed by chemiluminescence (Invitrogen).

2.10. iNOS expression

iNOS expression were analyzed by immunofluorescence. Cells (1×10^5 cells/well, final volume 100 µL, in chamber slides) were treated with the leaves fraction (0.50 mg/mL) and LPS+IFN-γ as previously described and incubated for 24 h. For the analysis of iNOS expression, the macrophages were fixed in 4.0% paraformaldehyde and permeabilized with 0.5% Triton X-100, followed by blocking with 1% BSA. The treated macrophages were stained with mouse anti-iNOS monoclonal antibody (1:100) overnight at 4°C. After the incubation, wells were washed with PBS and incubated again at room temperature with Alexa Fluor 488 rabbit anti-mouse (1:200) for 2 h. The wells were washed and stained with DAPI (0.2 µg/mL) and washed again. The slide was mounted using Fluoromount solution (Sigma, USA). Images were generated and captured by Zeiss LSM780 confocal microscope using a 63x objective. Fluorescence was quantified by Image J Software (NIH, Bethesda, MD, USA).

2.11. Statistical analysis

The results are presented as mean ± standard error mean (SEM). The data were evaluated by one-way analysis of variance (ANOVA) followed by Dunn's test, using GraphPad Prism software. P values less than 0.05 ($p < 0.05$) were considered as indicative of significance.

3. Results and discussion

3.1. Phytochemical composition

Phytochemical analysis allowed the identification of main compounds of the hydromethanolic fractions of leaves and branches from *P. spruceanum*, being found one tannin (procyanidin B) and six flavonoids (catechin,

isoquercitrin, quercetin-3-*O*-glucuronide, rutin, quercitrin and kaempferol-3-*O*-rhamnoside) (Table 1). Both fractions had a similar chromatographic profile, with differences observed in the proportion of the peaks and the presence of kaempferol-3-*O*-rhamnoside that is present only in the fraction from leaves (Table 1). The majority of these phenolic compounds has already been identified in the *P. spruceanum* leaves (Amparo et al., 2018), but it is the first time that isoquercitrin and rutin are reported in this specie.

Table 1

Phenolic compounds identified by means of UV and LC-DAD-ESI/MS analysis of hydromethanolic fractions from leaves and branches of *Protium spruceanum.*

Rt (min)	**Compound**	**UV** (nm)	**MS/MS** **$[M - H]^{-1}$** (*m/z*)	**MS** **$[M + H]^{+1}$** (*m/z*)	**References**
1.90	Procyanidin B[l,b]	279	577.3 (407.1; 289.2; 245.0; 205.1; 137.1; 124.9)	579.4 (291.4)	Callemien and Colin, 2008; Amparo et al., 2018
2.02	Catechin[l,b]	279	289.1 (245.1; 177.0; 165.0; 137.0; 124.9; 109.1)	291.4	Callemien and Colin, 2008;Amparo et al., 2018
2.64	Isoquercitrin[l,b]	260; 350	462.6 (300.1; 274.2; 254.8; 179.0; 151.0)	465.2 (303.2)	Brito et al., 2014
2.78	Quecetin-3-*O*-glucuronide[l,b]	268; 352	477.2 (273.1; 257.2; 178.6; 150.6)	479.3 (303.2)	Fabre et al., 2001; Amparo et al., 2018
2.78	Rutin[l,b]	270; 350	609.5 (445.3; 300.5; 255.2; 228.1)	611.4 (303.1)	Engels et al., 2012
3.08	Quercitrin[l,b]	264; 348	447.3 (301.8; 273.2; 257.1; 151.2)	449.4 (303.3)	Fabre et al., 2001; Amparo et al., 2018
3.41	Kaempferol-3-*O*-rhamnosíde[l]	263; 342	431.3 (285.0; 255.2; 238.9; 227.1; 212.8; 178.9; 163.2; 151.0)	433.3 (287.2)	Fabre et al., 2001; Amparo et al., 2018

Rt: Retention time; l: identified in the leaves fraction; b: identified in the branches fraction.

Although the chromatographic profile appeared to be similar, the leaves fraction showed a total flavonoid content higher than the branches fraction (22.2 ± 2.2 and 6.2 ± 0.9 mgQE/g, respectively). On the other hand, the branches fraction presented a total content of condensed tannin higher than the leaves fraction (518.1 ± 17.0 and 300.0 ± 28.6 mgCE/g, respectively). Variations in the concentration and distribution of secondary metabolites between vegetable parts are often reported and occur due to differences in metabolism of plant organs (Chunlong et al., 2008).

3.2. Cytotoxicity and cytokines

The fractions at concentrations 0.12, 0.25 and 0.50 mg/mL showed no cytotoxic effect for J774A.1 macrophages, with cell viability above 70% (Fig. S1, supplementary material) (ISO, 2009). Thus, these concentrations were selected for the anti-inflammatory activity tests.

The *in vitro* stimulation of macrophages with LPS+IFN-γ induce the M1-type polarization, a phenotype characterized by phagocytic activity and production of pro-inflammatory cytokines and mediators as nitric oxide (Mosser and Edwards, 2008; Su, Aldawsari and Amiji, 2016). This profile of inflammatory response was observed in all analysis of this present study.

However, the treatment with the fractions increased the production of the IL-10 and IL-6 cytokines when compared with the untreated cells (Fig. 1.a and b). IL-10 is an immunomodulatory cytokine that inhibits the production of pro-inflammatory cytokines and chemokines, being considered a potent anti-inflammatory cytokine (Saxena, et al., 2015). On the other hand, IL-6 is a pleiotropic cytokine that can execute pro-inflammatory and anti-inflammatory functions (Scheller et al., 2011). In this sense, IL-6, as well as IL-10, can induce M2-type macrophages polarization, which is a regulator phenotype characterized by production of angiogenic and immunomodulatory cytokines, and it is recognized as an anti-inflammatory macrophage, essential for the resolution and not exacerbation of the inflammatory process (Chen et al., 2018b; Mosser and Edwards, 2008; Sanmarco et al., 2017).

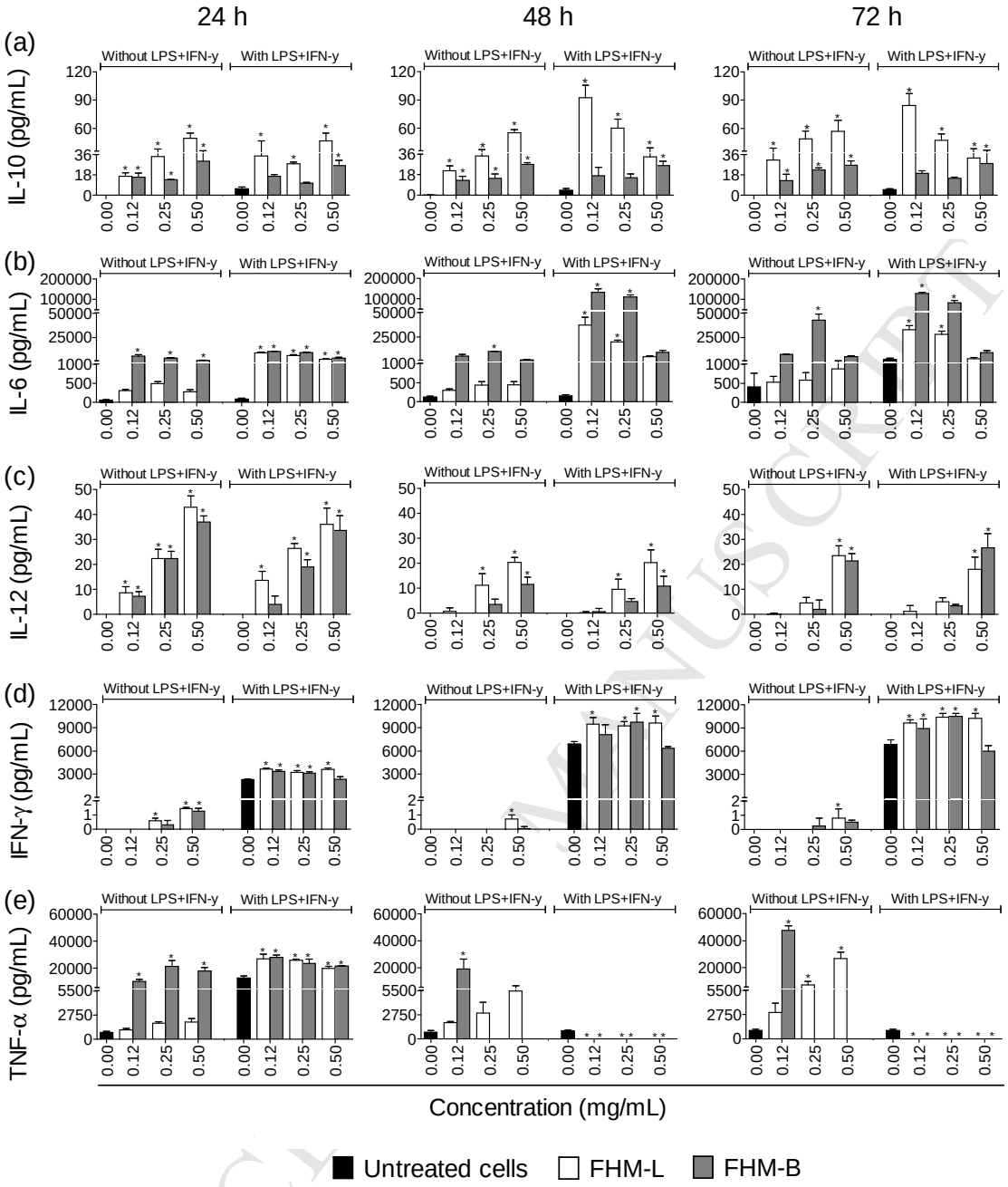

Fig. 1. Quantification of cytokines (a) IL-10, (b) IL-6, (c) IL-12, (d) IFN-γ and (e) TNF-α in J774A.1 macrophages stimulated or not with LPS+IFN-γ and treated with hydrometanolic fraction from leaves (FHM-L) or branches (FHM-B) of *Protium spruceanum* for 24 h, 48 h or 72 h. All data are expressed as mean and standard deviation of experiments in quadruplicates. *p<0.05 *versus* untreated cells.

The treatment with the fractions also induced the production of the pro-inflammatory cytokines (IL-12, IFN-γ and TNF-α) in relation to the untreated cells (Fig. 1.c, d and e). However, since extracts induced the increase of IL-10,

cytokine balance determination is also important in understanding their effects on inflammation (ratio of pro-inflammatory cytokine versus IL-10 immunomodulatory cytokine) (Dodoo et al., 2002; Neto et al., 2018).

The analysis of the cytokine balance showed that both fractions at 24 hours of treatment induced the increase of the IL-12/IL-10 ratio when compared with the untreated cells. However, at 48 and 72 hours, the leaves had this effect only at the highest concentration (0.50 mg/mL) (Fig. 2.a). This better result of the leaves fraction is associated to its IL-10 results, where IL-10 levels were higher than those obtained from branches fraction (Fig. 1.a). In general, the treatment with the fractions reduced the ratios IFN-γ/IL-10 and TNF-α/IL-10, with better results for longer times of treatment, indicating a better response over time (Fig. 2.b and c).

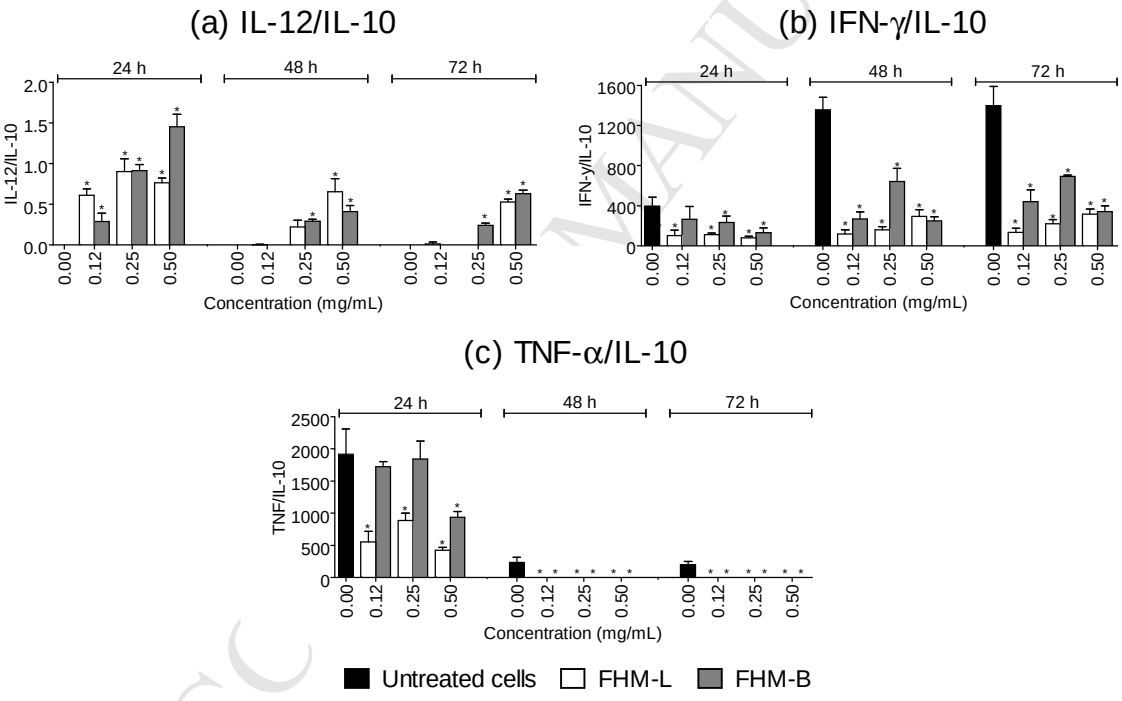

Fig. 2. Analysis of the cytokine balance in J774A.1 macrophages stimulated with LPS+IFN-γ, treated with hydrometanolic fraction from leaves (FHM-L) and branches (FHM-B) of *Protium spruceanum* for 24 h, 48 h and 72 h. All data are expressed as mean and standard deviation of experiments in quadruplicates. **p*<0.05 *versus* untreated cells.

The excess of IFN-γ is associated to several inflammatory diseases and is involved at cell death by the increase of apoptotic mediators (Lee et al.,

2017). TNF-α mediates the accumulation of immune cells at the site of inflammation, being an inducer of the inflammatory response, and the excess of this cytokine is associated with the pathophysiology of various diseases (Duque and Descoteaux, 2014).

Therefore, the cytokines results showed an immunomodulatory activity of the polar fractions from leaves and branches of *P. spruceanum*. They were able to increase production of IL-6 and IL-10 and could control the inflammatory response, where the leaves fraction presented the most interesting results.

3.3. Nitric oxide and ROS

The stimulation of macrophages with LPS+IFN-γ induced the increase of nitric oxide (NO) levels in the supernatant and this effect is time dependent. However, the tested fractions, at all concentrations and treatment times, reduced NO production by stimulated macrophages (Fig. 3.a).

The production of nitric oxide is essential in the regulation of physiological processes, host defense, inflammation and immunity and its pro-inflammatory effects include vasodilation, edema, cytotoxicity and the mediation of cytokine-dependent processes that can lead to tissue destruction (Abramson et al., 2001). NO reacts with ROS (O_2^-) and generates peroxynitrite ($ONOO^-$), a highly toxic anionic radical that is also a substrate for the COX-2 enzyme, responsible for the production of pro-inflammatory prostaglandins (Syahida et al., 2006).

The evaluation of ROS was performed only at 24 hours of treatment, since the results of cytokines (Fig. 1 and 2) and nitric oxide (Fig. 3.a) indicated that the fractions have already generated effects at this time. In addition, the response of LPS stimulated macrophages to produce ROS is faster than the production of cytokines (Kasahara et al., 2011).

The treatment with all concentrations of leaves fraction reduced the intracellular amount of ROS in the cells stimulated with LPS+IFN-γ. However, the branches fraction presented similar results only at high concentrations (Fig. 3.b).

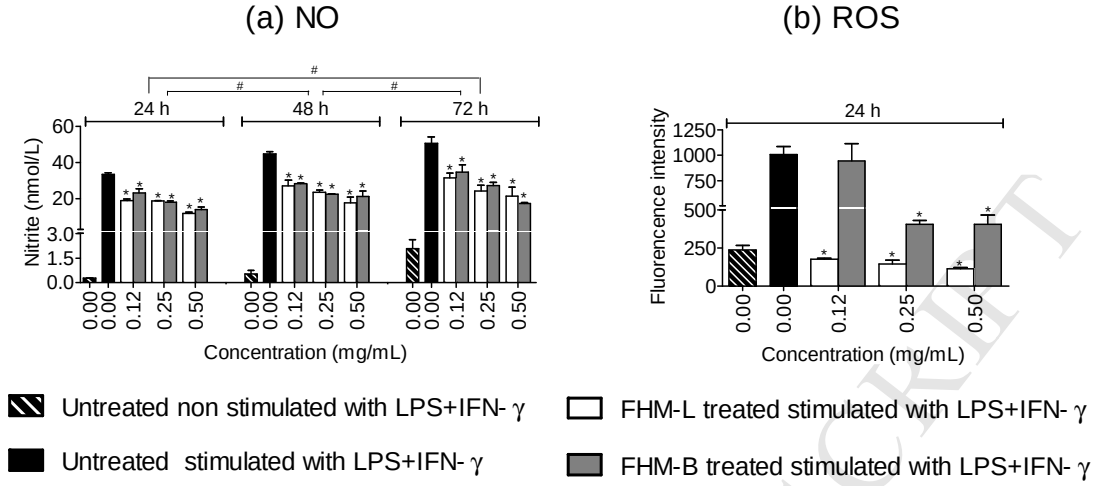

Fig. 3. Analysis of (a) nitric oxide (NO) and (b) reactive oxygen species (ROS) in J774A.1 macrophages stimulated or not with LPS+IFN-γ, treated with hydrometanolic fraction from leaves (FHM-L) or branches (FHM-B) of *Protium spruceanum* for 24 h, 48 h or 72 h. All data are expressed as mean and standard deviation of experiments in quadruplicates. **p*<0.05 *versus* untreated cells stimulated with LPS+IFN-γ. $^{\#}p$<0.05 between the groups with different treatment time.

ROS are key signaling molecules that play an important role in the progression of inflammatory disorders and their excess cause endothelial dysfunction and tissue injury ending with death cell (Mittal et al., 2014).

The results of reduction of NO and ROS are associated with the increase in IL-10 induced by fractions (Fig. 1), cytokine that inhibit pathways of synthesis of these pro-inflammatory mediators (Cunha, Moncada and Liew, 1992; Dokka et al., 2001). Therefore, NO and ROS reduction is related to the anti-inflammatory potential of *P. spruceanum*.

3.4. NAG and MMP-9 activity and COX-2 and iNOS expression

NAG and MMP-9 activity and COX-2 and iNOS expression were evaluated only by the treatment with leaves fraction, which showed the best results on the previous tests: high levels of IL-10 (Fig. 1.a) and consequently, better results in immunomodulation, with reduction of TNF-α/IL-10 and IFN-γ/IL-10 ratio (Fig.2) and ROS (Fig.3.b) at all concentrations since the lowest

treatment time (24 h) (Fig. 2), results different from those obtained from branches fraction. The analysis of NAG showed that the treatment with the fractions reduced the activity of this enzyme when compared to untreated cells (Fig. 4.a). This reduction effect can be attributed to the production of IL-10 (Fig. 1) as well, since this cytokine inhibits the macrophages activation and consequently the NAG enzyme secretion by activated macrophages (Berlato et al., 2002). Thus, the NAG activity suppression is associated with an anti-inflammatory action (Moura et al., 2011; Negrão et al., 2010; Pinho-Ribeiro et al., 2016).

The analysis of MMP-9 activity showed that the treatment with the fractions reduced its activity in relation to untreated cells (Fig. 4.b). MMP-9 is secreted by macrophages and regulates the leukocyte migration, promoting extracellular matrix remodeling and its inhibition is associated with anti-inflammatory activity of several phenolic compounds (Huang et al., 2012). MMP-9 can be modulated by ROS and NO and the inhibition of these factors by antioxidants has been shown to decrease MMP-9 expression and activity (O'Sullivan et al., 2014; Poitevin et al., 2008). Thus, MMP-9 inhibition is correlated with the reduction of NO and ROS generated by the treatment with the fractions (Fig. 3).

Treatment with the fraction also inhibited the COX-2 expression in relation to the untreated cells stimulated with LPS + IFN-γ (Fig. 4.c). Cyclooxygenases (COX) are enzymes responsible for the production of prostaglandins, an arachidonic acid-derived lipid autacoids essential in generating the inflammatory response. The COX-1 isoform is constitutively expressed in most cells and COX-2 is induced by inflammatory stimuli, hormones and growth factors. This enzyme is a common target of anti-inflammatory drugs (Ricciotti and Fitzgerald, 2011).

The iNOS expression also was inhibited by the treatment with the fraction in relation to the untreated cells stimulated with LPS + IFN-γ (Fig. 4.d). This result is connected with the reduction of NO generated by the treatment with the fractions (Fig. 3.a). iNOS is the isoform of NO synthases expressed in many cell types in response to agents as LPS and cytokines and this enzyme contributes to the pathophysiology of several inflammatory diseases (Förstermann and Sessa, 2012).

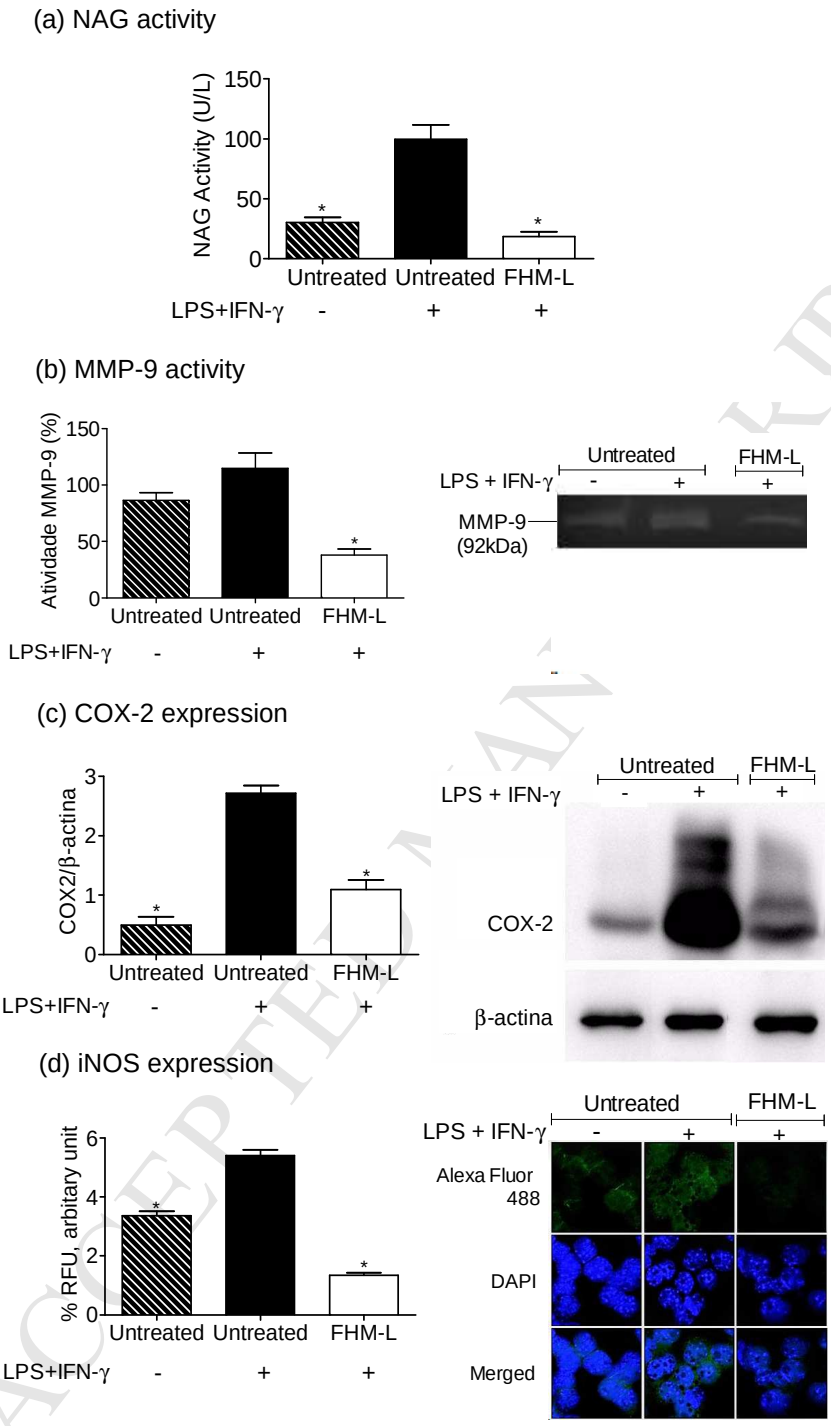

Fig. 4. Analysis of (a) N-acetylglucosaminidase (NAG), (b) matrix-metalloproteinase activity (MMP-9), (c) cyclooxygenase (COX-2) and (d) inducible nitric oxide synthase (iNOS) in J774A.1 macrophages stimulated or not with LPS+IFN-γ, treated with hydrometanic fraction from leaves (FHM-L) 0.50 mg/mL of *Protium spruceanum* for 24 h. All data are expressed as mean

and standard deviation of experiments in quadruplicates. *p<0.05 *versus* untreated cells stimulated with LPS+IFN-γ.

Therefore, the reduction of NAG, MMP-9, COX-2 and iNOS enzymes is also associated with the anti-inflammatory action of *P. spruceanum*, such as the immunomodulation.

In general, the leaves fraction showed the best results and it can be hypothesized that its content of total flavonoids higher than those of the branches fraction could be responsible for the anti-inflammatory activity, since the identified flavonoids catechin, rutin, quercitrin, isoquercitrin and kaempferol-3-*O*-rhamnoside activity has already been reported in the literature (Camuesco et al., 2004; Chen et al., 2001; Chung et al., 2015; Li et al., 2016; Murakami et al., 2015; Silva et al., 2017).

Flavonoids are considered potential candidates for new anti-inflammatory drugs and their activity involve the inhibition of the synthesis of different pro-inflammatory mediators, such as NO and cytokines, inhibition of enzymes activities and transcription factors (Serafini et al., 2010).

4. Conclusion

In the current study, the mode of action underlying of the *P. spruceanum* as an anti-inflammatory agent has been indicated for the first time. The hydromethanolic fractions from leaves and branches of *P. spruceanum* present an immunomodulatory effect, with increase of IL-10 production. In addition, the anti-inflammatory activity of *P. spruceanum* reported by traditional medicine can be attributed to the reduction of NO and ROS production and the inhibition of NAG, MMP-9, COX-2 and iNOS enzymes.

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