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Pro-inflammatory activity of *Astronium fraxinifolium* Schott on Lipopolysaccharide-stimulated RAW 264.7 cells

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ABSTRACT

This study aimed to investigate the antioxidant and immunomodulating activities of ethanolic extract from the sapwood of *Astronium fraxinifolium* (EEAF) on Lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. The constituents of the EEAF were analyzed by high-performance liquid *chromatography* (HPLC). Antioxidant activity of EEAF was evaluated by its capacity of inhibiting the production of free radical *2,2'-diphenyl-1-picrylhydrazyl* and *2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid*. For the analysis of its immunomodulatory properties, Nitric oxide (NO), tumor necrosis factor alpha (TNF-α), and transforming growth factor beta (TGF-β) levels were determined in supernatants from LPS-stimulated RAW 264.7 cells after treatment with the EEAF at different concentrations. Expression for mRNA of Cyclooxygenase-2 (COX-2) and Inducible nitric oxide synthase (iNOS), and detection of COX-2 protein were also analyzed. Caffeic acid, quercetin, followed by orientin and ρ-coumaric acid, were identified in the extract by the HPLC technique. The EEAF showed poor antioxidant activity when compared to the reference standard. NO, expression of COX-2 mRNA and COX-2 protein were found in high levels when LPS-stimulated cells were treated with the EEAF. Moreover, increased levels of TNF-α and low secretion of TGF-β were demonstrated in supernatants from LPS-stimulated cells treated with EEAF at different concentrations. In opposition to many different types of medicinal plants, the EEAF demonstrated a powerful pro-inflammatory capacity.

INTRODUCTION

Astronium fraxinifolium Schott, popularly known as Gonçalves-alves, gonçaleiro, and aroeira-do-campo, belongs to the Anacardiaceae family and can be found on rocky and dry lands, with geographical distribution in the Brazilian Cerrado (Feitosa *et al*., 2011). Flowering occurs during the months of August and September. *Astronium fraxinifolium* is of great importance for the economy due to the high quality of timber production (Luna, 2012). There is a great advantage in its use because it is not at risk of extinction. Medicinal purposes for the plant include the treatment of ulcers and infections (Macedo and Ferreira, 2004). Antimicrobial properties, such as antibacterial (Montanari *et al*., 2012), antifungal (Bonifácio *et al*., 2015), and leishmanicidal activities (De Lima *et al*., 2014), have been demonstrated.

According to phytochemical studies, the plant leaves present (Z)-β-ocimene, (E)-β-ocimene, bicyclogermacrene, limonene, α-terpinolene, and viridiflorene (Montanari *et al*., 2012). Limonene bears anti-inflammatory and antioxidant activities (Złotek *et al*., 2016). Alpha-terpinolene is antifungal and leishmanicidal (Ramos *et al*., 2014). With respect to the anti-inflammatory properties of *A. fraxinifolium*, scientific knowledge is still poor and needs to be better elucidated.

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Inflammation is a response of the immune system against invasive stimuli, infection, or tissue damage. It is a complex process regulated by various chemical mediators and immune cells (Guo *et al*., 2016). Macrophages are essential for achieving homeostasis, through the process of phagocytosis and production of chemokines, cytokines, and lipid mediators (Arulselvan *et al*., 2016). Lipopolysaccharide (LPS) is a component of Gram-negative bacteria outer cell membrane that induces macrophages to produce proinflammatory mediators, including iNOS, Cyclooxygenase-2 (COX-2), tumor necrosis factor alpha (TNF-α), and Nitric oxide (NO) (Lawrence *et al*., 2002), and Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Singh *et al*., 2017). LPS-stimulated macrophages have been widely used to provide an *in vitro* inflammatory environment, which contributes, for instance, to study the role of anti-inflammatory compounds (Dong *et al*., 2017). Taking into account the limited scientific knowledge regarding the immunomodulatory properties of *A. fraxinifolium,* the present study aimed to evaluate its immunomodulating and antioxidant activities on LPS-stimulated RAW 264.7 cells.

MATERIAL AND METHODS

Plant material

Sapwood from *A. fraxinifolium Schott* (register at SISGEN A166486, 04/24/2018) was collected in the city of Lavras da Mangabeira (06º45′12″S 38º57′52″W 239 m), Ceara, Brazil. A voucher specimen (54265) was deposited at the Herbarium Prisco Bezerra, Department of Biology, Universidade Federal do Ceará, Brazil.

The plant material (1,000 g) was dried in an *oven with forced air circulation* for a period of 48 hours at 65°C. Ethanolic extract was obtained by macerating the plant material with absolute ethanol for 7 days at room temperature. After the concentration of the material on a rotary evaporator under reduced pressure at 40°C, the final extract (yield of 1.8%) was submitted to phytochemical analysis.

Phenol quantification

Phenolic compounds in the ethanolic extract from the sapwood of *A. fraxinifolium* (EEAF) were quantified by the Folin–Ciocalteau technique (Singleton *et al*., 1965). The assay was performed in triplicate, using gallic acid (Sigma, USA) as the standard reference.

Flavonoids quantification

Flavonoid content in the EEAF was also quantified (Vennat *et al*., 1992). Quercetin (Sigma, USA) was used as the reference standard.

Tannin quantification

Tannin content was determined by the Folin–Denis colorimetric method (Galvão *et al*., 2018). Tannic acid (Sigma, USA) was used as the reference standard.

High-performance liquid chromatography (HPLC)

Chromatographic profile of the EEAF was done by the High-Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD) on a Prominence high-performance liquid *chromatography (HPLC)* AutoSampler (SIL-20A) (Shimadzu, Kyoto, Japan), equipped with a Shimadzu LC-20AT pumps connected to a DGU degasser 20A5 with a CBM 20A integrator, SPD-M20A diode array detector, and LC solutions in software 1.2 SP1. EEAF and reference standards was injected into a Phenomenex C18-reverse-phase column (4.6 mm × 250 mm). The elution was performed using mobile phases A (ultrapure water containing 2% acetic acid, pH 3.0) and B (acetonitrile). Fifty microliters of the EEAF (15 mg/ml) were injected at a solvent flow rate of 0.6 ml/min. Reference standards were tested at 0.025–0.300 mg/ml. Quantification of the compounds was performed by analyzing the peaks and their retention time (t_R), considering the optical reading for gallic acid (at 254 nm), catechin (at 280 nm), ρ-cumaric, acid and caffeic acid (at 325 nm), quercetin (at 366 nm), rutin, luteolin, and orientin (at 366 nm). The chromatographic procedures were done in triplicate. The limit of detection and the limit of quantification were calculated according to Boligon *et al*. (2015).

Evaluation of the antioxidant activity

Inhibition of 2,2'-diphenyl-1-picrylhydrazyl (DPPH) activity

Analysis of the antioxidant activity of the EEAF extract was done by the *2,2'-diphenyl-1-picrylhydrazyl* (DPPH) radical scavenging method (Brand-Williams *et al*., 1995), modified. The results were expressed according to the following formula: Inhibition percentage (%) = [Abs (DPPH) − Abs (sample) / Abs (DPPH)] × 100, using quercetin as the reference standard. The assays were run in sextuplicate. Abs (DPPH) corresponded to the absorbance of the DPPH solution; Abs (sample) corresponded to the absorbance of the solution containing the EEAF extract at a determined concentration.

Inhibition of 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) activity

Anti-oxidant activity of the EEAF extract was also tested by the *2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid* (*ABTS*) method (Miller and Rice-Evans, 1997), modified. The percentage of inhibition was calculated according to the following formula: Inhibition percentage (%) = $[(OD_{ABTS} - OD_{extract}) / OD_{ABTS}] \times 100$. The optical density OD_{ABTS} corresponded to the optical density of the *ABTS* solution. The $OD_{extract}$ corresponded to the optical density of the solution containing the EEAF extract at a particular concentration. The assays were carried out in triplicate.

Evaluation of EEAF cytotoxicity

The 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide *(MTT)* tetrazolium *assay* (Rodrigues *et al*., 2019) was used to evaluate the viability of *RAW 264.7 cells (BCRJ, Brazil)* after treatment with the EEAF. RAW 264.7 cells (5×10^5 cells/ml) were seeded into 96-well culture microplates in Dulbecco's Modified Eagle Medium (DMEM) (LGC, Brazil) supplemented with 10% heat-inactivated fetal bovine serum (LGC, Brazil) and gentamicin (50 µg/ml, Sigma, USA), before incubation overnight at 37°C and 5% CO_2. After this period, the cells were treated with different concentrations of EEAF (15.63–500.00 µg/ml). After 24 hours incubation, the supernatant was discarded and the adherent cells were washed with phosphate-buffered saline (PBS). Subsequently, a new supplemented DMEM medium was pipetted into the wells, followed

by the addition of (MTT, Sigma, USA) at 500 µg/ml in PBS. After a 4 hours-incubation at 37°C and 5% CO_2, the supernatant was discarded and 100 µl of 100% dimethylsulfoxide (DMSO, Sigma, USA) were added to the plates. The plates were vigorously shaken for 15 minutes and the optical density was read at 570 nm wavelength using an Enzyme-linked immunosorbent assay (ELISA) plate reader. The assays were performed in triplicate.

Plant activity on LPS-activated cells

RAW 264.7 cells (5×10^5 cells/ml) were incubated with LPS from *Escherichia coli* O128:B12 (Sigma, USA) at 1 µg/ml in a 24-well culture plate during 24 hours at 37°C and 5% CO_2. Nontoxic concentrations of EEAF (31.25, 62.5, and 125 µg/ml) and controls (0.1% DMSO and 4.0 µg/ml dexamethasone, Sigma, USA) were added to the wells. After 24 hours, the cells or their supernatants were collected and analyzed.

NO measurement

NO measurement was indirectly assessed by the amount of nitrite in the cell culture supernatant using Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediaminedihydrochloride), according to the procedure described by Rodrigues *et al*. (2019). Sodium nitrite (Sigma, USA) was used as the reference standard.

TNF-α and transforming growth factor beta (TGF-β levels) measurement

Quantification of TNF-α and transforming growth factor beta (TGF-β) levels in cell supernatants was performed by using sandwich-enzyme immunoassays, according to the manufacturer's recommendations (Novex®, Life Technologies, USA).

mRNA for COX-2, iNOS, and TGF-β

Expression of mRNAs for COX-2, iNOS, and TGF-β was evaluated by a reverse transcriptase-polymerase chain reaction technique. Extraction of RNA from LPS-stimulated RAW 264.7 cells after treatment with the EEAF (31.25, 62.5, and 125 µg/ml) was done using Trizol (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Total RNA (0.5 µg) was reversely transcribed into complementary DNA (cDNA) with the aid of the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), according to the manufacturer's recommendations. The sequences of nucleotides for designing target-specific primers were obtained from NCBI and analyzed by the OligoPerfect™ Designer software (Thermo Fisher Scientific, USA). The following primers were used: COX-2, F: 5′-AGAAGGAAAT GGCTGCAGAA-3′ and R: 5′-GCTCGGCTTCCAGTATTGAG-3′; iNOS, F: 5′-CACCTTGGAGTTCACCCAGT-3′ and 5′-ACC ACTCGTACTTGGGATGC-3′; TGF-β, 5′-TTGCTTCAGCTCCA CAGAGA-3′ and 5′-TGGTTGTAGAGGGCAAGGAC-3′; β-actin (housekeeping gene), F: 5′-AGCCATGTACGTAGCCATCC-3′ and R: 5′-CTCTCAGCTGTGGTGGTGAA-3′. The number of amplification cycles was adjusted according to the target in order to obtain an exponential increase of the amplicons for each biomarker. The annealing temperature and the number of cycles used for the amplification of each target are described in Table 1. Reactions were performed using an 2720 Thermal Cycler (Applied Biosystems, USA). The amplicons were submitted to electrophoresis on a 2.5% agarose gel followed by staining with SYBR Safe stain (Invitrogen, USA). The bands were visualized by a ChemiDoc™ MP Imaging System (BIO-RAD, EUA) and their intensity was analyzed with the Image Lab™ software (version 5.1, BIO-RAD, USA).

Table 1. Target molecules and their optimized annealing temperature and the number of amplification cycles employed in the PCR amplification.

Target	Annealing temperature (°C)	Amplification cycles
COX-2	60°C	25
iNOS	55°C	30
TGF-β	60°C	25
β-actin	60°C	25

COX-2 detection by the western blot technique

After treatment with the plant material or with the reference drug (4.0 µg/ml dexamethasone, Sigma, USA), LPS-stimulated RAW 264.7 cells were lysed using Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma, USA), which was supplemented with protease inhibitor cocktail P8340 (Sigma, USA) at 1:100 (v/v), phenylmethylsulfonyl fluoride (Sigma, USA) at 2 mM and sodium orthovanadate (Sigma, USA) at 1 mM. Quantification of total proteins was done by the DC™ (detergent compatible) protein assay kit (Bio-Rad Laboratories, USA). Cell proteins (20 µg per well) were electrophoresed on an 8% sodium dodecyl sulfate-polyacrylamide gel, under a voltage of 125 V for 1 hour and 20 minutes at room temperature. The separated polypeptides were blotted onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, USA), under a constant amperage of 400 mA, at 4°C for 2 hours. After blocking the membranes with Tris solution containing 5% skim milk and 5% bovine serum albumin (BSA) (Sigma, USA) overnight, they were incubated (2 hours) with primary rabbit antibody to COX-2 (Santa Cruz Biotechnology®, USA) at a dilution of 1:500 in 5% BSA. Anti-β-actin antibody (Cell Signaling Technology, USA) was used as an internal reference standard. After the washing step (five times with Tris-buffered solution containing 1% Tween 20), and incubated with the secondary peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology, USA), at 1:3000 dilution in 5% BSA for 2 hours. For the chemiluminescence detection, luminol solution containing H_2O_2 (Amersham ECL™ Prime reagent) was used. The membranes were photographed by a ChemiDoc™ MP Imaging System (BIO-RAD, USA) and the Image Lab™ software (version 5.1, BIO-RAD, USA) was used for the image acquisition and edition.

Statistical analysis

Data were expressed as mean ± standard error of the mean and analyzed by Student's *t*-test or analysis of variance, followed by Tukey's post-test. All analyses were performed using GraphPad Prism version 7.0 for Windows (GraphPad Software, San Diego, CA). The values considered statistically significant presented a value of $p \leq 0.05$.

RESULTS AND DISCUSSION

Spectrophotometric analysis of total phenols, flavonoids, and tannins was performed in the EEAF. Amounts equivalent to 60.00 ± 1.538 mg of total phenols, 1.159 ± 0.851 mg of flavonoids, and 48.94 ± 0.037 mg of tannins were found in

100 g of EEAF. According to Silva *et al*. (2010), *Astronium* sp. comprises flavonoids, proanthocyanidins such as profisetinidine and prorobinetinidine, tannins and lignins. The authors suggested that the condensed and hydrolyzable flavonoids and tannins found in the genus *Astronium* are responsible for certain characteristics such as astringency, and complexing with macromolecules, such as proteins, polysaccharides, and metal ions.

Phenols (e.g., gallic acid, caffeic acid, coumaric acid, rosmarinic acid) are produced as secondary metabolites and may present antioxidant and antimicrobial activities (Lin *et al*., 2016).

Figure 1 demonstrates the HPLC profile of the EEAF extract. Molecules from eight peaks were identified and quantified according to known concentrations of the standard solutions and their retention time (t_R), that is, caffeic acid (t_R = 23.11 minutes, peak 3, 5.12 ± 0.01 mg/ml) and quercetin (t_R = 39.91 minutes, peak 7, 2.19 ± 0.01 mg/ml), followed by orientin (t_R = 32.15 minutes, peak 5, 1.16 ± 0.03 mg/ml) and ρ-coumaric acid (t_R = 29.86 minutes, peak 4, 1.15 ± 0.01 mg/ml) (Table 2). The constituents of the extract were similar to those found by Martins *et al*. (2018) in the extract from the bark of *A. fraxinifolium*. Caffeic acid may present several biological properties, such as antioxidant and antimicrobial activities (Magnani *et al*., 2014), and anti-inflammatory activity by inhibiting the COX-2 enzyme (Erdemli *et al*., 2015). Quercetin is a flavonoid that bears antioxidant, anti-inflammatory, antibacterial, antiviral, gastroprotective, immunomodulatory, neuroprotection functions and is used in the treatment of obesity and cardiovascular diseases (Li *et al*., 2016). Luteolin, quercetin, and caffeic acid exert their immunomodulating properties by inhibiting nuclear factor kappa B, with consequent decrease of interleukin (IL)-1β and TNF-α production (Juman *et al*., 2012).

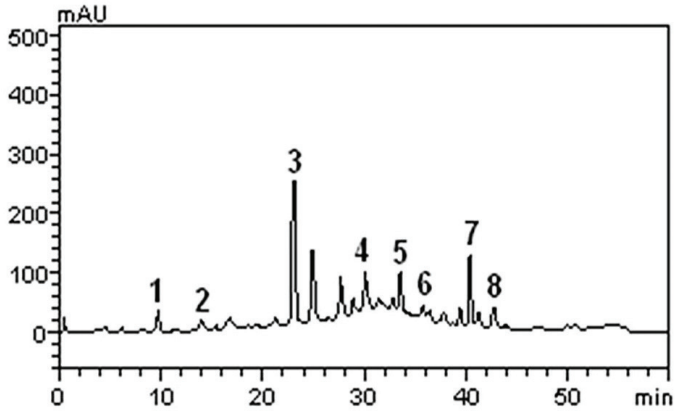

Figure 1. Chromatographic profile of the EEFA in HPLC-DAD. Peak 1: gallic acid, peak 2: catechin, peak 3: caffeic acid, peak 4: ρ-coumaric acid, peak 5: orientin, peak 6: rutin, peak 7: quercetin, and peak 8: luteolin.

Table 2. Compounds obtained from EEAF after separation by HPLC chromatography.

Peaks	Compounds	Ethanolic extract from the sapwood	LOD	LOQ
		mg/ml	µg/ml	µg/ml
1	Gallic acid	0.74 ± 0.02	0.009	0.034
2	Catechin	0.39 ± 0.03	0.023	0.076
3	Caffeic acid	5.12 ± 0.01	0.027	0.089
4	ρ-coumaric acid	1.15 ± 0.01	0.015	0.047
5	Orientin	1.16 ± 0.03	0.011	0.036
6	Rutin	0.37 ± 0.01	0.018	0.061
7	Quercetin	2.19 ± 0.01	0.013	0.047
8	Luteolin	0.72 ± 0.02	0.007	0.023

LOD = limit of detection, LOQ = limit of quantification.

First of all, we have tested the antioxidant activity of the EEAF. The 50% inhibitory capacity of free radicals of the EEAF was equivalent to 132.9 ± 0.877 µg/ml for DPPH and 176.7 ± 11.20 µg/ml for ABTS. The reference standard, that is, quercetin, was significantly more active than the *A. fraxinifolium* extract. The EEAF did not demonstrate a relevant antioxidant activity, differently from the results obtained by Martins *et al*. (2018). A large amount of EEAF would be necessary to neutralize the free radicals DPPH and ABTS in comparison to quercetin, the reference standard used in the experiments. Although quercetin was one of the constituents identified in the extract, it does not seem that it exerts a meaningful antioxidant activity in the EEAF, probably due to its low concentration in the extract.

Before testing the activity of the EEAF in the *in vitro* cell assays, cytotoxic analysis of the extract was done. The results revealed that concentrations equivalent to 500 µg/ml of EEAF on RAW 264.7 cells were toxic (Fig. 2). For this reason, concentrations lower than the mentioned ones were used in the following assays.

For NO measurement, it was observed that the mean concentrations of nitric oxide in supernatants from LPS-stimulated cells treated with EEAF at 31.25 µg/ml, 62.5 µg/ml, and 125 µg/ml were 11.83 µM, 14.31 µM, and 13.15 µM, respectively. No statistically significant difference was found among the groups. The EEAF did not promote any nitrite production in unstimulated cells when treated with the highest concentration of the extract. Therefore, the EEAF did not interfere in NO levels (Fig. 3). In regard to mRNA for iNOS enzyme, a reduction of its expression

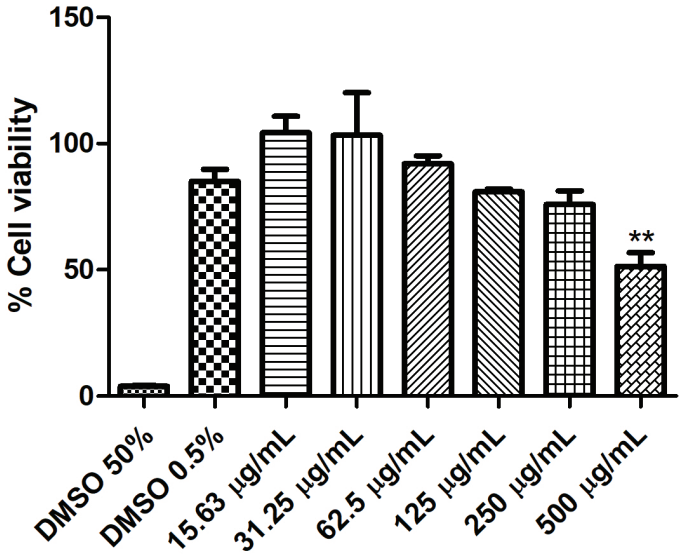

Figure 2. Cell viability assay using the MTT assay. RAW 264.7 cells were treated with EEAF at different concentrations (15.63 µg/ml; 31.25 µg/ml; 62.5 µg/ml; 125 µg/ml; 250 µg/ml; 500 µg/ml). The control group was cultured in the presence of 0.5% of DMSO. $^{**}p < 0.01$ versus Control (Student's *t*-test)

was observed but without statistical significance (Fig. 4A). The pathways for the generation of oxygen and nitrogen species depend on the NADPH oxidase and iNOS inducible enzymes, respectively. Recently, it was demonstrated that NO presents paradoxal properties. NO has a powerful antimicrobial activity when it reacts with superoxide ion forming peroxynitrite; on the other hand, it is required for normal cardiovascular function. Therefore, it means that in order to achieve an adequate antioxidant activity, it is necessary that only NADPH oxidase activity be diminished (Sui *et al*., 2019).

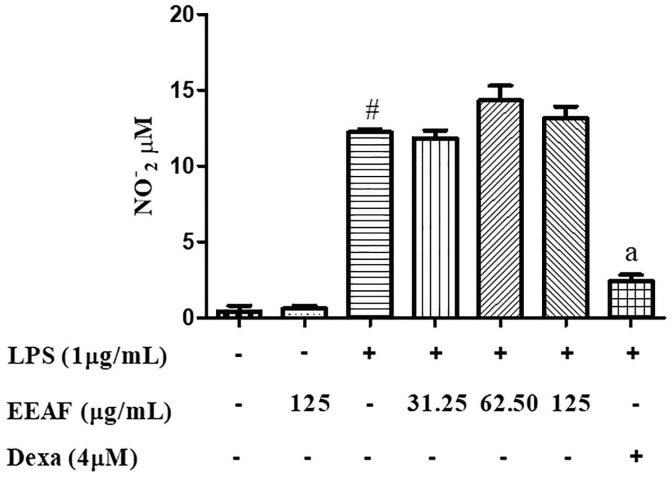

Figure 3. Nitrite (NO_2^-) quantification in supernatants from LPS-stimulated RAW 264.7 cell submitted to different types of treatment. Controls: cell in 0.1% DMSO; LPS-stimulated cells; LPS-stimulated cells treated with 4 µM dexamethasone; LPS-stimulated cells treated with EEAF at 31.25 µg/ml; 62.5 µg/ml; 125 µg/ml. a $p < 0.001$ versus LPS-stimulated cells; # $p < 0.001$ versus Control (Tukey's test).

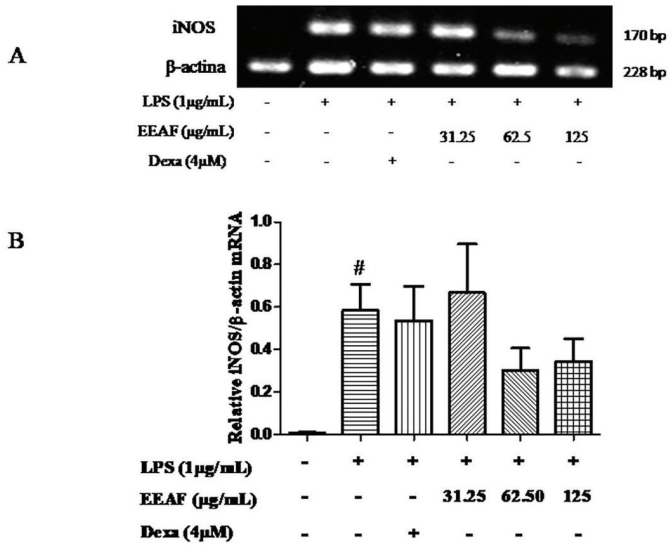

Figure 4. Expression of iNOS mRNA from LPS-stimulated RAW 264.7 cells treated with different concentrations of EEAF. Controls: Cells in 0.1% DMSO; LPS-stimulated cells; LPS-stimulated cells treated with 4 µM dexamethasone; LPS-stimulated cells treated with EEAF at 31.25 µg/ml; 62.5 µg/ml; 125 µg/ml. # $p < 0.001$ versus Control (Tukey's test).

In respect to cytokine analysis, as demonstrated in Figure 5, all the concentrations of the EEAF induced a significant secretion of TNF-α in comparison to LPS-stimulated cells without treatment ($p < 0.001$; Tukey's test). The highest levels of the cytokine were found in the group treated with EEAF at 125 µg/ml ($p < 0.001$).

TNF-α is a cytokine that acts as an endogenous mediator, being able to stimulate IL-6 and IL-1β secretion. The cytokines present synergistic effects among them, which amplifies the signaling pathway of inflammation (Ying *et al*., 2013). Interestingly, phenolic compounds may be classified from no action to strong activity in inhibiting TNF production (Grigore, 2017). It is important to remind that to inhibit TNF is an important target, mainly in inflammatory diseases such as arthritis (Henriques *et al*., 2016). Nonetheless, we have found increased levels of TNF-α in all groups treated with the EEAF in comparison to the untreated LPS-stimulated group. *Allium sativum* (garlic acid) has also the ability to stimulate the production of TNF-α by macrophages (Sung *et al*., 2015).

A significant decrease of TGF-β was found when stimulated cells were treated with the EEAF at 31.25 µg/ml ($p < 0.01$), at 62.5 µg/ml and 125 µg/ml ($p < 0.001$), in comparison to those untreated (Fig. 6). TGF-β is an important immunomodulatory cytokine that has pleiotropic activity, acting on angiogenesis and in the repair phase of the inflammation (Poniatowski *et al*., 2015).

The treatment of LPS-stimulated RAW 264.7 cells with the EEAF at 62.5 µg/ml promoted an increase in COX-2 mRNA expression when compared to LPS-stimulated cells without treatment ($p < 0.05$, Fig. 7). Increased levels of COX-2 protein were also found in the LPS-stimulated cells treated with EEAF at 31.25 µg/ml when compared to LPS-stimulated group without treatment ($p < 0.05$, Fig. 8). COX-2 enzyme may be inducible by IL-1 and TNF and converts arachidonic acid to prostaglandins in macrophages and other cells leading to vasodilation, vascular

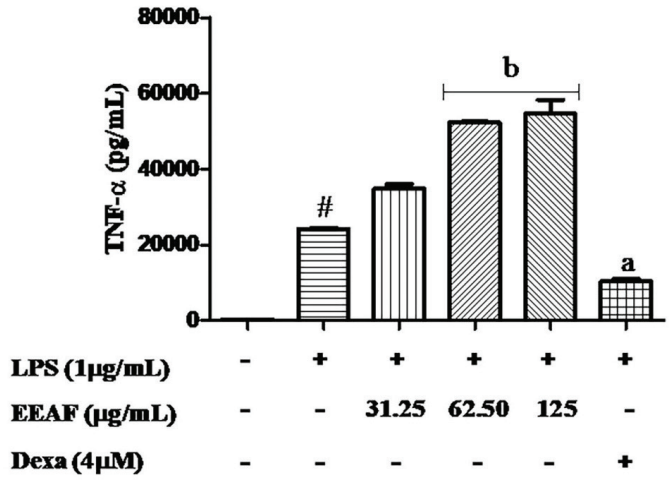

Figure 5. TNF-α measurement by ELISA in supernatants from LPS-stimulated RAW 264.7 cells treated with different concentrations of EEAF. Controls: cell in 0.1% DMSO; LPS-stimulated cells; LPS-stimulated cells treated with 4 µM dexamethasone; LPS-stimulated cells treated with EEAF at 31.25 µg/ml; 62.5 µg/ml; 125 µg/ml. a $p < 0.001$ versus LPS-stimulated cells; b $p < 0.001$ versus EEAF at 31.25 µg/ml; # $p < 0.001$ versus Control (Tukey's test).

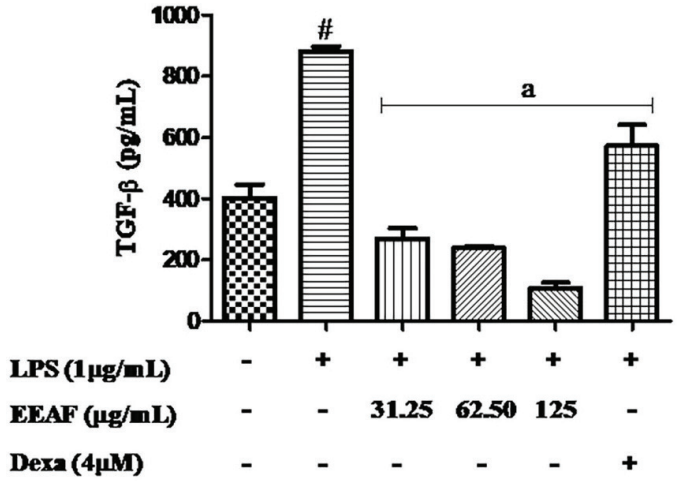

Figure 6. TGF-β measurement by ELISA in supernatants from LPS-stimulated RAW 264.7 cells treated with different concentrations of EEAF. Controls: cell in 0.1% DMSO; LPS-stimulated cells; LPS-stimulated cells treated with 4 µM dexamethasone; LPS-stimulated cells treated with EEAF at 31.25 µg/ml; 62.5 µg/ml; 125 µg/ml. a $p < 0.01$ versus LPS-stimulated cells; # $p < 0.001$ versus Control (Tukey's test).

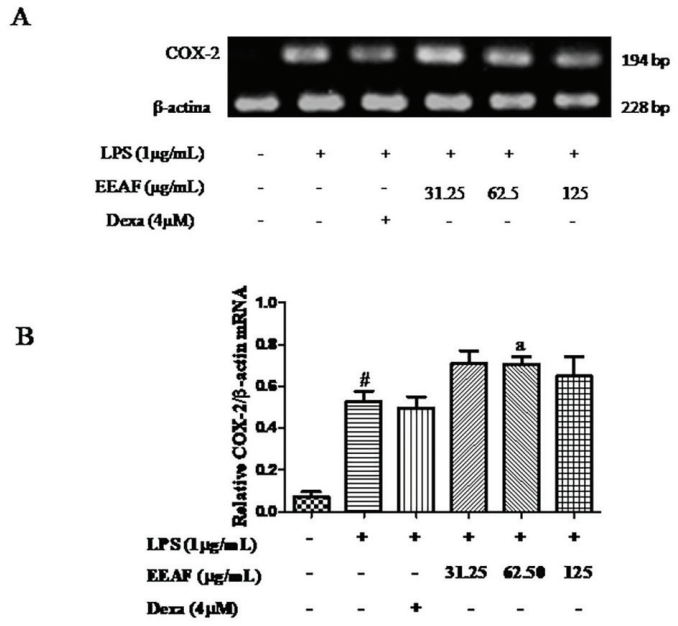

Figure 7. Expression of COX-2 mRNA from LPS-stimulated RAW 264.7 cells treated with different concentrations of EEAF. Controls: Cells in 0.1% DMSO; LPS-stimulated cells; LPS-stimulated cells treated with 4 µM dexamethasone; LPS-stimulated cells treated with EEAF at 31.25 µg/ml; 62.5 µg/ml; 125 µg/ml. a $p < 0.05$ versus LPS-stimulated cells; # $p < 0.001$ versus Control (Student's *t*-test).

permeability, fever, hyperalgesia, but paradoxically may produce lipid mediators with anti-inflammatory activity (Chen, 2010).

To increase the inflammatory response may be of great importance when the organism is chronically infected (Wang *et al*., 2016). Our present work was able to demonstrate that the EEAF makes macrophage more efficient in the production of inflammatory mediators which was evidenced by the increase in TNF-α, COX-2, and a decrease in TGF-β. These results could

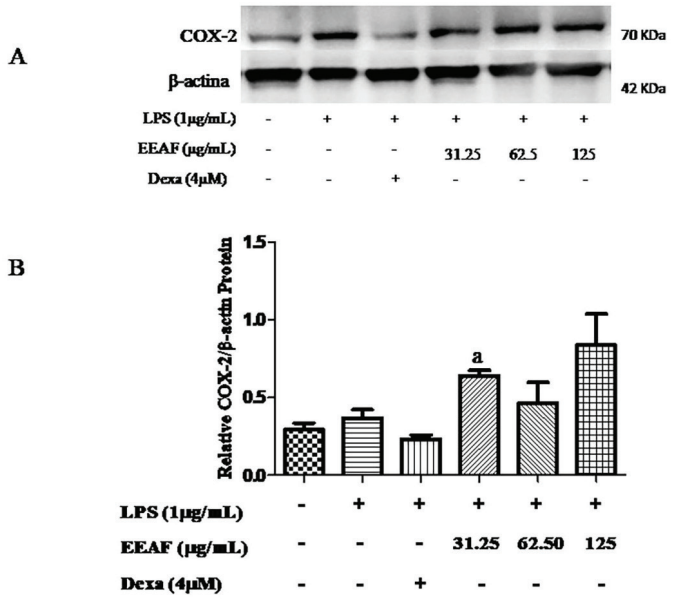

Figure 8. COX-2 measurement by Western blot technique in LPS-stimulated RAW 264.7 cells treated with different concentrations of EEAF. Controls: Cells in 0.1% DMSO; LPS-stimulated cells; LPS-stimulated cells treated with 4 µM dexamethasone; LPS-stimulated cells treated with EEAF at 31.25 µg/ml; 62.5 µg/ml; 125 µg/ml. a $p < 0.05$ vs LPS-stimulated cells; # $p < 0.001$ versus Control (Student's *t*-test).

probably explain the reason for the potent leishmanicidal activity presented by *A. fraxinifolium*, demonstrated by *in vitro* and *in vivo* assays (De Lima *et al*., 2014). This is the first report which clearly demonstrates the proinflammatory activity of *A. fraxinifolium* sapwood.

CONCLUSION

In conclusion, in opposition to many different types of medicinal plants that show anti-inflammatory properties, the ethanolic extract from the sapwood of *A. fraxinifolium* presents a powerful pro-inflammatory capacity. This property may be extremely relevant in regard to the treatment of chronic infectious diseases based on medicinal plants. For this reason, studies are being conducted in order to evaluate the proinflammatory properties of EEAF in cells infected with *Leishmania*.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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