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Original Paper

Humiria balsamifera extract inhibits nitric oxide and tumor necrosis factor production in LPS-stimulated macrophages

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Abstract

Humiria balsamifera is used in traditional medicine as anthelmintic, expectorant, to treat hepatitis, diarrhea, hemorrhoids; to cure chronic wounds; and to alleviate toothaches. This species occurs in Jurubatiba shoal, Rio de Janeiro state-Brazil, a rich region which offers a variety of promising bioactive product sources. The present study focuses on the chemical and pharmacological evaluation of *H. balsamifera*. The *n*-hexane, dichloromethane and ethyl acetate leaf fractions exhibited higher inhibitory potential on NO production. Friedelin (1), quercetin (2) and quercetin-3-α-*O*-arabinopyranoside (3) were isolated and characterized; the latter is described for the first time for *H. balsamifera*. Quercetin (2) showed the best inhibitory activity on NO production and moderate inhibition of TNF-α production. These results contribute to the knowledge of *Humiria balsamifera* as a source of anti-inflammatory compounds. Furthermore, the identification of the terpenes ß-amyrone, betulin, citronellol, eremophillene, dihydroactinolide and borneol, and the isolation of quercetin-3-α-*O*-arabinopyranoside are being reported for the first time for this species.

Key words: anti-inflammatory, Friedelin, Humiriaceae, quercetin-3-α-*O*-arabinopyranoside, TNF-α.

Resumo

Humiria balsamifera é utilizada na medicina tradicional como antihelmintico, expectorante, para tratar hepatite, diarreia, hemorroidas, na cura de feridas crônicas, e no alívio da dor de dente. Esta espécie ocorre na Restinga de Jurubatiba, no estado do Rio de Janeiro-Brasil, uma região rica que oferece uma variedade de fontes promissoras de produtos bioativos. O presente estudo foca na avaliação química e farmacológica da espécie *H. balsamifera*. As frações de folhas em n-hexano, diclorometano e acetato de etila apresentaram maior potencial inibitório na produção de NO. Friedelina (1), quercetina (2) e quercetina-3-*O*-α-arabinopiranosídeo (3) foram isolados e caracterizados; o último está sendo descrito pela primeira vez para *H. balsamifera*. A quercetina (2) apresentou a melhor atividade inibitória na produção de NO e moderada inibição da produção de TNF-α. Estes resultados contribuem para o conhecimento da espécie *Humiria balsamifera* como potencial fonte de substâncias anti-inflamatórias. E, ainda, a identificação dos terpenos ß-amirona, betulina, citronelol, eremofileno, dihidroactinolideo e borneol, e o isolamento da quercetina-3-α-*O*-arabinopiranosideo estão sendo descritos pela primeira vez para esta espécie.

Palavras-chave: anti-inflamatório, Friedelina, *Humiriaceae*, quercetina-3-α-*O*-arabinopiranosideo, TNF-α.

See supplementary material at <https://doi.org/10.6084/m9.figshare.16569393.v1>

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Introduction

nflammation is part of the survival strategy of the organism, however, there may be an excessive or inappropriate inflammatory response causing injuries to the body (Dinarello 2010). Steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) have been commonly used for treating inflammation. However, the use of these drugs are associated with several adverse effects (Harirforoosh *et al*. 2013; Ronchetti *et al.* 2018).

Considering the importance of plants as alternative sources for obtaining new anti-inflammatory bioactive constituents (Bernstein *et al*. 2018), this paper proposes to study *Humiria balsamifera* Aubl. This species also knows as "umiri-de-cheiro", "umiri-do-pará", "umirizeiro", "umiri" e "muréua" (Lorenzi & Matos 2008) occurs in the North, Northeast, Midwest and Southeast regions of Brazil (BFG 2018).

Humiria balsamifera is popularly used in the Amazon as a perfume and in the treatment of diseases such as hepatitis, diarrhea and hemorrhoids (Coelho-Ferreira 2009). Its use has also been described as anthelmintic, expectorant, healing action against toothache and chronic wounds (Lorenzi & Matos 2008). Considering some of these popular applications, such as for the treatment of hemorrhoids and toothache, it may be directly related to the modulation of the inflammatory process, so, the evaluation of this species in terms of its anti-inflammatory potential became relevant.

This species can be found in Jurubatiba shoal, RJ, Brazil. The shoal is a complex ecosystem in very delicate balance that have a typical flora, well adapted to characteristic conditions. The combination of several physical and chemical factors of this region, such as high temperature, salinity and high exposure to light (Cogliatti-Carvalho *et al.* 2001; Kelecom *et al*. 2002) makes it a promising source in the search for new bioactive products and evidencing the importance of its preservation.

Therefore, this study aims to investigate the chemical composition of this species, to evaluate the inhibitory activity on pro-inflammatory mediators produced by LPS-stimulated RAW 264.7 macrophages for the first time, and to isolate and identify compounds which can be related to this activity. This study intends to contribute to the knowledge related to the popular use of *H. balsamifera* associated with inflammation.

Materials and Methods

Chemicals and reagents

Lipopolysaccharide (*Escherichia coli* O111:B4) (LPS), dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), Griess reagent (*p*-aminobenzenosulphonamide 1% + 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid), N^G-monomethyl-L-arginine (L-NMMA), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and actinomycin D were purchased from Sigma-chemicals. Dulbecco Modified Eagle's Medium DMEM/F-12 and fetal bovine serum (FBS) were purchased from Gibco BRL®. The other chemicals used were: Triton X-100 (Vetec Chemical®), LDH KIT (Doles®), gentamicin (Invitrogen®), and murine recombinant TNF-α (Biosource®).

Plant material

The plant material was collected from: Rio de Janeiro, Brazil: Quissamã, Jurubatiba Shoal National Park, 22.19970°S, 41.4712°W, 20.IX.2010, fl., *T.U.P. Konno* (RFA33650), (University Federal Rio de Janeiro Herbarium). The botanical material was identified by the botanist T.U.P. Konno.

Preparation of the crude extracts

The plant material was properly separated into leaves and bark and then dried in an oven with air circulation for 72 h at 45 ºC. Dried leaves (224 g) and bark (66.6 g) were macerated at room temperature with ethanol for 7 days, and the resulting extractive alcoholic solutions were subsequently evaporated under vacuum to provide the extracts (39.80 g and 3.55 g, respectively). Fresh leaves (50 g) were also extracted with boiling distilled water (2 L) for 15 min. This material was then subjected to a second extraction step at low temperature (40 °C) for 30 min with magnetic stirring. The aqueous extract (AqEx, 4.2 g) was lyophilized for quantification (Santos *et al*. 2020).

Fractions obtained by liquid-liquid partition

The leaf ethanol extract (LE) was suspended (36.25 g) in MeOH:H_2O (9:1, 250 mL) and initially submitted to partition with *n*-hexane (HEX) (4 × 150 mL, 4.39 g). The separated aqueous MeOH layer was evaporated under reduced pressure and then resuspended in water. The aqueous solution was extracted by increasing polarity solvents, as

follows: dichloromethane (DCL) (4 × 150 mL, 0.35 g), ethyl acetate (EtOAc) (4 × 150 mL, 2.13 g) and butanol (BuOH) (5 × 150 mL, 3.41 g) (Santos *et al*. 2020). The CH_2Cl_2:H_2O mixture led to forming an emulsion which reduced according to dichloromethane renewal. The final residual emulsion was separated and named as the residual dichloromethane fraction (RDCL, 0.35 g). The ethyl acetate fraction spontaneously precipitated resulting in ethyl acetate supernatant (EtOAc Sup.) and precipitate (EtOAc Ppt., 107.4 mg). The partitions (hexane, dichloromethane, ethyl acetate and butanol) were dried under reduced pressure, while the aqueous residue was freezed and liophylized. All samples were resuspended in DMSO (20 mg/mL) for later biologicals assays.

General experimental conditions

GC-MS

The fractions were analyzed by GC-MS in a gas chromatograph (Shimadzu 2010) with a GCMS-QP2010 interface with electronic ionization energy of 70 eV, split ratio 1/20; mass range of *m/z* 30–500 D, and a scanning time of 1 s. We used a RTx-5MS column (30 m × 0.25 mm × 0.25 µm film; Restek Corporation, USA). The following conditions were adopted: helium as the carrier gas (1.1 mL min^{-1}); temperature setting from 60 to 280 °C (15 °C min^{-1}); injector temperature (250 °C) and interface (300 °C). The samples were prepared by adding 200 µL MSTFA in 2 mg of fraction. Each sample was dissolved in 2 ml of chloroform after 15 minutes, and 1 µl was then injected with an autosampler. Compound identification was based on comparing the mass spectra and the NIST spectrometer data bank together with a literature data comparison.

NMR

1D (^{1}H-, APT) and 2D (HSQC, HMBC, COSY) NMR spectra acquisitions were performed in a Varian Inova 500 or Varian Mercury-VX 400 (500 or 400 MHz for ^{1}H and 125 MHz for ^{13}C NMR) NMR spectrometer using $CDCl_3$ or CD_3OH as deuterated solvents. Chemical shifts (δ) were referenced to internal TMS standards (δ=0,^{1}H), being expressed in ppm units and the coupling constants (*J*) in Hertz (Hz).

HPLC-DAD

HPLC-DAD analysis was performed using a 250 × 4.6 mm NUCLEOSIL® C18 5 µm column (SHIMADZU® LC-20AT). The adopted mobile phase was (A): H_2O adjusted to pH 3.0 by H_3PO_4, and (B): CH_3CN, with a gradient elution constituted by acetonitrile from 0 to 18% over 10 min, 18–20% over 20 min, 20–21% over 40 min, 21–22% over 22 min, 22–100% over 50 min, 100–0% over 55 min, and 0% over 60 min at a flow rate of 1 mL/min. The detection was registered in a diode array detector (DAD) (Shimadzu SPD -M20A). The samples were injected at 2 mg/mL in acetonitrile with a volume injection of 20 µL by an autosampler (Shimadzu SIL-20A).

Isolation of the compounds

Compound 1 (Sub-fraction 65–71) (11.4 mg) was isolated from the HEX fraction. The hexane fraction (2.07 g) was resuspended in a minimun amount of ethyl acetate and, then, a portion of silica gel (8.12 g) was added into this solution. The mixture was taken to the rotary evaporator until dry and produce a pastille that was later applied in the top of an open column chromatography (CC) (silica gel, 40–63 micras SILICYCLE®) (h = 60 cm; i.d. = 3.4 cm). It was adopted the following elution gradient protocol: dichloromethane:hexane (1:9) (3:7) (1:1); ethyl acetate:hexane (1:9), (1:4), (3:7), (2:3), (1:1), (3:2), (7:3), (4:1), (9:1) to pure ethyl acetate, methanol:ethyl acetate (1:9), (1:4), (3:7), (2:3), (1:1), (3:2), (7:3), (4:1), (9:1) to pure methanol. The samples (1–390) (20 mL) were grouped according TLC comparison, using a combination of HEX:DCL and HEX:EtOAc as eluents in different proportions. Sulfuric anisaldehyde spray under heating was adopted as the chromogenic reagent (Wagner & Bladt 1996). Sub-fractions of 65 to 71 with high purity were eluted at the proportion of ethyl acetate:hexane (1:9), and were grouped for further spectroscopic analysis.

Compounds 2 (Sub-fraction 105-107) (4.3 mg) and 3 (Sub-fraction 96) (4.5 mg) were isolated from EtOAc Ppt. (101.1 mg) by a CC (silica C18, 40–63 µm, SILICYCLE®) (h = 38 cm; i.d. = 1.7 cm) by adopting a gradient elution varying from water, methanol:water (1:9), (1:4), (2:3), (3:2), (4:1), methanol and methanol:acetonitrile (1:1). The samples (1–115) (20 mL) were grouped according to TLC comparison using a combination of butanol:acetic acid:water as eluent in different proportions. Furthermore, 2-aminoethyl diphenylborinate/polyethylene glycol 400 were used as chromogenic reagents for detection (Wagner & Bladt 1996). Sub-fractions 105 to 107 (compound 2) and 96 (compound 3) with high purities eluted at methanol:water (3:2) and (4:1), respectively, were concentrated and quantified.

Biological assay

Inhibition of NO production by LPS-stimulated macrophages

The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (ATCC), grown at 37 °C and 5% CO_2 in DMEM/F-12 supplemented with 10% FBS. Macrophages (10^5 cells/mL) were seeded in 96-well plates and incubated for 24 h under 5% CO_2 37 °C. Cells were treated with samples (0.8, 4, 20 and 100 µg/mL) and stimulated with 1 µg/mL LPS. Culture supernatants were collected for NO and TNF-α assays after 24 h (TNF-α assay as described below). Nitrite concentration was determined as an indicator of NO production, according to the Griess test (Griess 1879). Absorbance was spectrophotometrically measured at 540 nm. Nitrite concentration was calculated by comparison with a sodium nitrite standard curve.

Cytoxicity

The cytotoxic effects of samples on RAW 264.7 stimulated with LPS were determined using the lactate dehydrogenase (LDH) release assay (for extacts and fractions) and using the MTT assay (for isolated compounds). In both cases, the cytotoxicity percentage was calculated in relation to the negative control (untreated and LPS-stimulated macrophages), and to the positive control (stimulated macrophages treated with 1% (v/v) Triton X-100).

LDH assay

Cytoplasmic enzyme lactate dehydrogenase (LDH) release was determined using 50 µL of culture supernatant collected at the end of the assay (Muzitano *et al*. 2006). The LDH amount was colorimetrically verified using a commercial kit (Doles®).

MTT assay

3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide (MTT) (Mosmann 1983) was used to evaluate the viability of the cells exposed to the isolated substances. The adherent macrophages remaining were treated with 10 µL of MTT (5 mg/mL), and further incubated for 2 h at 37 °C and 5% CO_2. The viable cells reduced the MTT into formazan crystals, which were dissolved in HCl (4 mM) in isopropanol. This reduction was spectrophotometrically estimated at 570 nm to determine cell viability.

TNF-α assay

TNF-α was measured by a L929 fibroblast bioassay (Shiau *et al.* 2001), based on the sensitivity of L929 cells to the cytotoxic effect of TNF-α. First, L929 cells were seeded at a density of 2.5×10^5 cells/mL in a 96-well plate and incubated for 24 h in DMEM/F-12 with 10% FBS and 20 µg/mL of gentamicin at 37 °C and 5% CO_2. The resulting cell monolayers were treated with the macrophage culture supernatants in the presence of actinomycin D (2 µg/mL) after 24 h of incubation. L929 viability was determined by the MTT method (Mosmann 1983). The reading was performed at 570 nm on a spectrophotometer plate. The cell viability percentage was determined using the culture supernatant of non-stimulated macrophages (negative control TNF-α production) and the culture supernatant of stimulated macrophages (positive control TNF-α production) as controls. A standard curve with recombinant mouse TNF-α was used to measure the TNF-α concentration found in the samples.

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean±standard error (M±SEM). The results were representative of three independent experiments. Statistical analyses were performed by one-way ANOVA followed by a Tukey's post-test. The results were considered statistically significant for $p < 0.05$. The IC_{50} was determined by non-linear regression based on the results of the concentration-response curve.

Results and Discussion

Extracts and fractions analysis by chromatographic techniques and corresponding activities

No significant differences ($P > 0.05$) were observed between the leaf and bark ethanolic extracts, nor for the AqEx (decoction), concerning NO and TNF-α inhibition, as well as cytotoxicity in LPS-stimulated RAW 264.7 cells (IC_{50} >100 µg/mL). The HEX, DCL and EtOAc Ppt leaf fractions exhibited the highest NO inhibition, with IC_{50} of 9.26±0.83, 15.43±0.63 and 19.34±0.70 µg/mL, respectively. The DCL fraction and EtOAc Ppt. displayed the greatest promising TNF-α inhibition, with IC_{50} equal to 44.92±0.84 and 52.52 ± 0.96 µg/mL, respectively. Among the most active fractions, a cytotoxic effect was also verified (78.79±1.03 µg/ml) for the DCL fraction, indicating that the activity described above is probably non-specific (Tab. 1).

Table 1 – Effect of the extracts and fractions of *Humiria balsamifera* on the inhibition of NO and TNF-α production, as well as cytotoxicity in LPS-stimulated RAW 264.7 macrophage cells.

Humiria balsamifera	**Inhibitory NO production (IC_{50}, µg/mL)**	**Inhibitory TNF-α production (IC_{50}, µg/mL)**	**Cytotoxicity (IC_{50}, µg/mL)**
Extracts			
Bark extract (BE)	> 100[a]	> 100[a]	> 100[a]
Leaf extract (LE)	> 100[a]	> 100[a]	> 100[a]
Aqueous extract (AqEx)	> 100[a]	> 100[a]	> 100[a]
Fractions			
Hexane fraction (HEX)	9.26 ± 0.83[c]	> 100[a]	> 100[a]
Dichloromethane fraction (DCL)	15.43 ± 0.63[d]	44.92 ± 0.84[b]	78.79 ± 1.03[b]
Residual dichloromethane (RDCL)	> 100[a]	> 100[a]	> 100[a]
Ethyl acetate supernatant (EtOAc Sup.)	> 100[a]	> 100[a]	> 100[a]
Ethyl acetate precipitate (EtOAc Ppt.)	19.34 ± 0.70[d]	52.52 ± 0.96[c]	> 100[a]
Butanol fraction (BuOH)	95.50 ± 0.65[b]	> 100[a]	> 100[a]
Aqueous fraction (AQ)	> 100[a]	> 100[a]	> 100[a]

IC_{50}, half maximal inhibitory concentration. Means followed by different letters in columns differ by the Tukey test ($P < 0.05$).

These results are being described for the first time to extracts and fractions from this species, adding an important pharmacological interest.

The following crude extracts EBC, EBF and AqEx were evaluated by HPLC-DAD and showed similar chromatographic profiles (Fig. 1), with compounds in T_R less than 15.0 min (a - T_R 10.2 min and b - T_R 14.5 min) with UV suggestive of phenolic compounds such as bergenin (approx. 215 and 270 nm) (Qin *et al*. 2010), already described for this specie (Silva *et al.* 2004). There were also observed compounds with T_R between 20.0 and 27.0 min (highlighting c - T_R 24.9 min) showing to be compatible to flavonoid derivatives (approx. 205, 254 and 354 nm) (De Rijke *et al*. 2006). These results can justify the fact that the described extracts showed similar behavior when evaluated in NO and TNF-α inhibition, and in cytotoxicity test in LPS-stimulated RAW 264.7 cells. The ethyl acetate supernatant (EtOAc Sup.) when analyzed by HPLC-DAD also showed bergenin (T_R 14.5 min) and the same flavonoid derivatives (T_R 20.0 to 27.0 min) in a considerable higher amount compared to the extracts. While in the ethyl acetate precipitate (EtOAc Ppt.) it was observed two major compounds with T_R 14.5 (a) and 24.9 min (b) (Fig. 1), compatible to bergenin and a flavonoid derivative, respectively.

The analysis of the hexane fraction by GC-MS showed, based on the characteristic mass spectrum, the majority presence of hexadecanoic acid, tetratetracontane and terpenes derivatives (betulin, friedelin, ß-amyrone) (Figure S1, available on supplementary material <https://doi.org/10.6084/m9.figshare.16569393.v1>; Tab. 2).

In the dichloromethane fraction, other major compounds were identified in addition to friedelin and hexadecanoic acid (Figure S2, available on supplementary material <https://doi.org/10.6084/m9.figshare.16569393.v1>; Tab. 3). Among these compounds, the presence of some terpenes is highlighted, such as citronellol, eremophillene, dihydroactinolide and borneol, the last as the majority compound of this fraction (38.57% peak purity). This factor may be relevant to justify the observed activity since this compound has already been described for its anti-inflammatory potential (Zhong *et al*. 2014; Zou *et al*. 2017). In addition, the ability of borneol to increase cell uptake of other substances is also reported, which would lead to increased apoptosis in tumor cells (Su *et al.* 2013). This could contribute to understand the cytotoxic profile presented by this fraction against Raw 264.7 macrophages, which could be associated with an increase in cellular uptake of the other compounds

present in this fraction.

To sum up, the EtOAc Sup. and the HEX fractions showed to be the most actives, with less citotoxicity, which are in accordance with the literature, that has been described the anti-inflammatory potential of some of the derivatives found in those fractions, such as the hexadecanoic acid (Aparna *et al.* 2012), phenolic (Gao *et al*. 2015; Maleki *et al*. 2019) and terpenes derivatives, including betulin (Reyes *et al*. 2006; Liu *et al*. 2019), friedelin (Jin *et al*. 2018) and amyrone (Almeida *et al*. 2015). Some of the identified compounds have already been described for species from the same family Humiriaceae, such

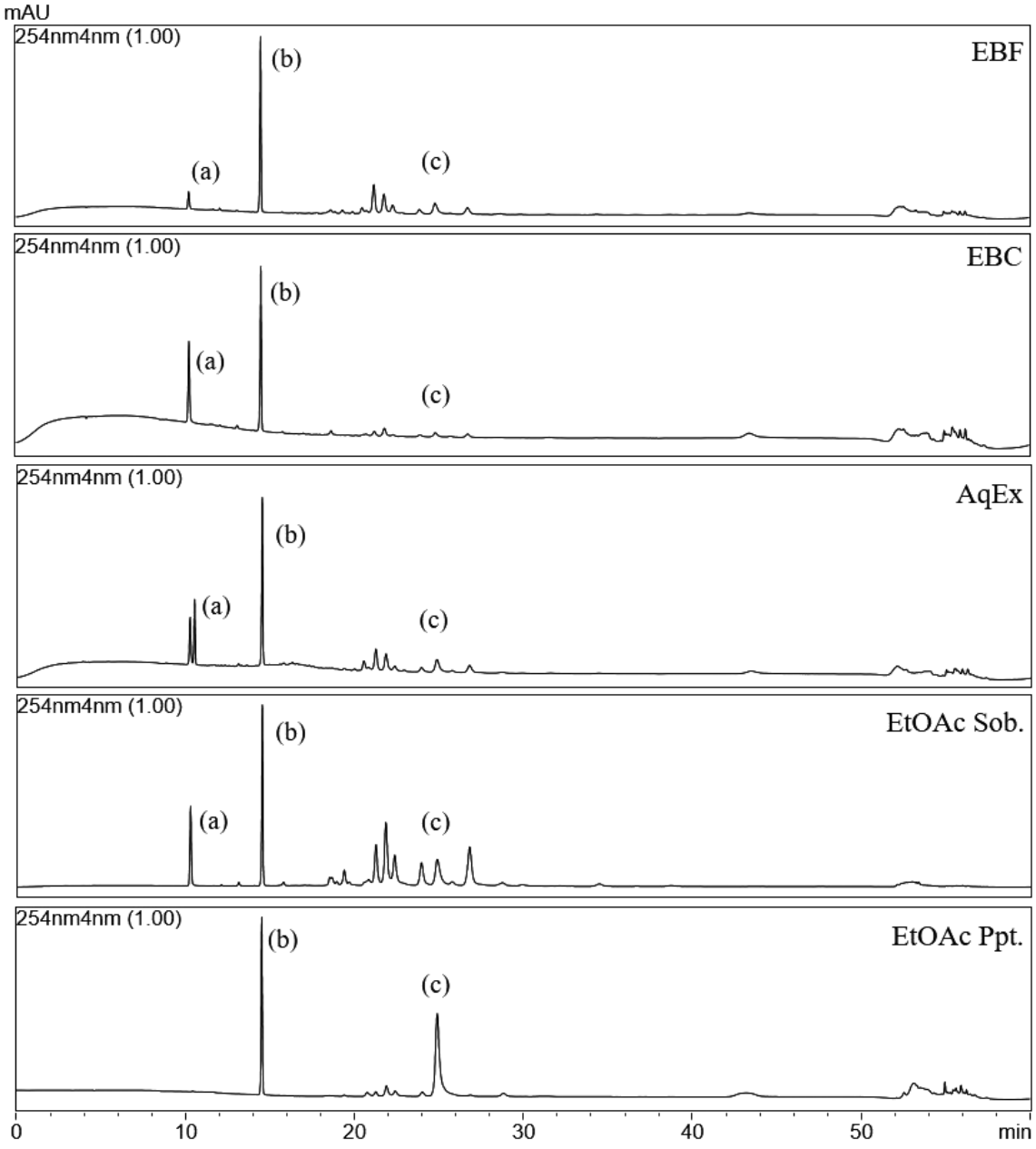

Figure 1 – HPLC chromatogram (254 nm) of EBF, EBC, AqEx extracts and EtOAc Sob. and EtOAc Ppt. fractions of *Humiria balsamifera*. [a = T_R 10.2 min; b = T_R 14.5 min; c = T_R 24.9 min. UV spectra (a) and (b) approx. 215 and 270 nm and (c) approx. 205, 254 and 354 nm].

Table 2 – Identified compounds in the hexane fraction of *Humiria balsamifera* by GC-MS.

Nº	RT[a]	Peak purity (%)[b]	Proposed Compound
1	5.24	3.01	4-Methyl-4-[(trimethylsilyl)oxy]-2-pentanone
2	15.88	1.28	Methyl ester of 2-hydroxy-2-methyl-butyric acid
3	17.69	0.81	Not detected
4	**17.89**	**10.76**	**Hexadecanoic acid, trimethylsilyl ester**
5	23.44	1.46	Not detected
6	**24.97**	**28.08**	**Tetratetracontane**
7	25.34	1.19	3,7,11,15-Tetramethyl-hexadecanol, trimethylsilyl ether
8	26.90	2.34	Not detected
9	27.78	1.08	Propane, 2-methoxy-2-methyl
10	**31.13**	**4.42**	**3-α-(Trimethylsiloxy)cholest-5-ene**
11	**31.53**	**10.08**	**β-amyrone**
12	**32.25**	**7.36**	**Not detected**
13	**32.39**	**10.25**	**Betulin**
14	**35.71**	**17.88**	**Friedelin**

[a] = Retention time in minutes; [b] = Calculated as relative area; majority compounds highlighted in bold.

as bergenin in *Sacoglottis gabonensis* (Ogan 1971), and hexadecanoic acid, friedelin, betulin and bergenin identified in *Endopleura uchi* (Marx *et al.* 2002; Abreu *et al.* 2013), being bergenin associated to the anti-inflammatory activitiy of the plant (Nunomura *et al*. 2009). It is important to highlight that it is the first time that tetratetracontane, ß-amyrone, betulin, citronellol, eremophillene, dihydroactinolide and borneol are being reported for this species and also for the genus *Humiria*.

Compound identification

Compound 1 (85%) presented by GC-MS *m/z* 426 [M^+] is compatible with a triterpene skeleton (Figure S3, available on supplementary material <https://doi.org/10.6084/m9.figshare.16569393.v1>). The loss of a methyl group is shown by the fragment *m/z* 411. Other important fragmentations were observed at *m/z* 341, 273, 205 and 123, assigned to the A, B, C and D rings, respectively (Shiojima *et al*. 1992). ^{1}H-NMR (500 MHz, $CDCl_3$) showed signals between 0.72 and 2.49 ppm characteristic of the triterpene friedelin, which had already been earlier isolated from *H. balsamifera* (Silva *et al.* 2004). The following methyl hydrogens were observed: δ_H 0.82-0.90 (3H, m, H-23); 0.72 (3H, s, H-24); 0.82-0,90 (3H, m, H-25); 1.01 (3H, s, H-26); 1.05 (3H, s, H-27); 1.18 (3H, s, H-28); 1.00 (3H, s, H-29); and 0.95 (3H, s, H-30). This analysis also indicated the presence of metilenic hydrogens between δ_H 1.25-2.40, and metinic hydrogens δ_H 2.24 (1H, m, H-4), 1.39 (1H, m, H-8), 1.54 (1H, m, H-10), and 1.56 (1H, m, H-18). HSQC (500 MHz, $CDCl_3$) indicated a characteristic correlation of a friedelane ring, a H-23 methyl group with C-23 in the most protected region of the spectra (δc 6.9) due to effect caused by the carbonyl group (C-3, δc 213.1). HMBC (500 MHz, $CDCl_3$) showed seven quaternary carbons δ_C 213.1 (C-3), 42.0 (C-5), 37.6 (C-9), 39.7 (C-13), 38.3 (C-14), 29.9 (C-17), 28.2 (C-20). Characteristic correlations to the friedelan ring as H-23/C-3 and H-4/C-3 were also observed in this spectrum. The correlation between the H-4 and H-23 was verified in COSY spectrum (500 MHz, $CDCl_3$). These data are in agreement with literature (Queiroga *et al*. 2000; Almeida *et al*. 2011).

Compound 2 (91%): HPLC/UV-DAD: t_R 47.06 min and $\lambda_{máx}$ 201, 254 and 369 nm in the UV spectra (characteristic of flavonoids) (Fig. 2). ^{1}H-NMR (500 MHz, CD_3OD): 6.16 (1H, s H-6), 6.37 (1H, s, H-8), 7.72 (1H, m, H-2'), 6.89 (1H, d, J = 8.5 Hz, H-5'), and 7.62 (1H, d, J = 8.5 Hz, H-6'). HSQC (500 MHz, $CDCl_3$) analysis allowed

Table 3 – Identified compounds in the dichloromethane fraction of *Humiria balsamifera* by GC-MS.

Nº	RT[a]	Peak purity[b] (%)	Proposed Compound
1	12.40	0.99	3-Octenoic acid, trimethylsilyl ester
2	**12.45**	**3.06**	**Dihydroactinolide**
3	13.22	0.63	1,1-Dimethoxypropane
4	14.15	0.92	Trimethylsilyl 2-oxopentanoate
5	14.72	0.69	Not detected
6	14.88	1.83	1-Cyclopentyl-1-trimethylsilyloxyethane
7	15.01	1.89	Not detected
8	15.09	1.68	Not detected
9	**15.18**	**3.92**	**4.β.H,5.α.-Eremophil-1(10)-ene, 11-(trimethylsiloxy)**
10	15.28	5.92	Not detected
11	15.37	0.91	2,5-Dimethylhexane-2,5-dihydroperoxide
12	**15.44**	**38.57**	**Borneol, tert-butyldimethylsilyl ether**
13	15.63	1.01	Not detected
14	15.83	3.41	Not detected
15	**15.91**	**6.39**	**β-Citronellol, trimethylsilyl ether**
16	16.18	1.08	(Methoxymethyl)trimethylsilane
17	16.47	2.64	E-11-Tetradecenol,trimethylsilyl ether
18	16.55	0.57	2-(2-Methoxypropoxy)-1-propanol
19	16.66	0.45	Cyclopropanecarboxylic acid
20	17.69	1.99	Not detected
21	**17.89**	**6.78**	**Hexadecanoic acid, trimethylsilyl ester**
22	25.89	0.80	Not detected
23	**35.70**	**13.67**	**Friedelin**

[a] = Retention time in minutes; [b] = Calculated as relative area; majority (> 3.0%) identified compounds highlighted in bold.

to observe δ_C 98.0 (C-6) and 92.9 (C-8) coupling to δ_H 6.16 (H-6) and 6.37 (H-8), referring to the A ring. In addition, the δ_C 114.6 (C-5'), 120.1 (C-6') and 147.2 (C-2') coupling to 6.89 (H-5') and 7.62 (H-2',6'), respectively, relative to B ring. HMBC (500 MHz, $CDCl_3$) showed nine quaternary carbons δ_C 147.2 (C-2), 175.5 (C-4), 161.0 (C-5), 164.8 (C-7), 156.8 (C-9), 102.9 (C-10), 122.6 (C-1'), 147.2 (C-3') and 144.8 (C-4'). COSY (500 MHz, $CDCl_3$) analysis allowed to observe the H-5' and H-6' correlation of the B ring. These data are characteristic of flavonol quercetin (Fossen *et al.* 1998; Saaby *et al.* 2009), which had already been isolated earlier from *H. balsamifera* (Silva *et al.* 2004).

Compound 3 (70%): HPLC/UV-DAD 25.49 min and $\lambda_{máx}$ 202, 255 and 355 nm in the UV spectra (Fig. 3). Spectroscopy data showed signals characteristic of quercetin-3-*O*-α-arabinopyranoside (Wollenweber *et al.* 1997; Ahmadu *et al.* 2007), described for the first time for this species. The ^{1}H-NMR (500 MHz, CD_3OD) displayed characteristic signals of the aglycone quercetin δ_H 6.21 (1H, d, *J* = 2.0, H-6), 6.41 (1H, d, *J* = 2.0, H-8), 7.74 (1H, d, *J* = 2.2, H-2'), 6.90 (1H, d, *J* = 8.5, H-5'), 7.57 (1H, dd, *J* = 2.2 and 8.5, H-6') and sugar signals δ_H 5.10 (1H, d, *J* = 6.7, H-1''), 3.90 (1H, dd, *J* = 6.6 and 8.5, H-2''), 3.65 (1H, dd, *J* = 3.1 and 8.4, H-3''), 3.83 (1H, s, *J* = 8.5, H-4''), 3.80 (1H, d, *J* = 3.5, H-5a''), and 3.43

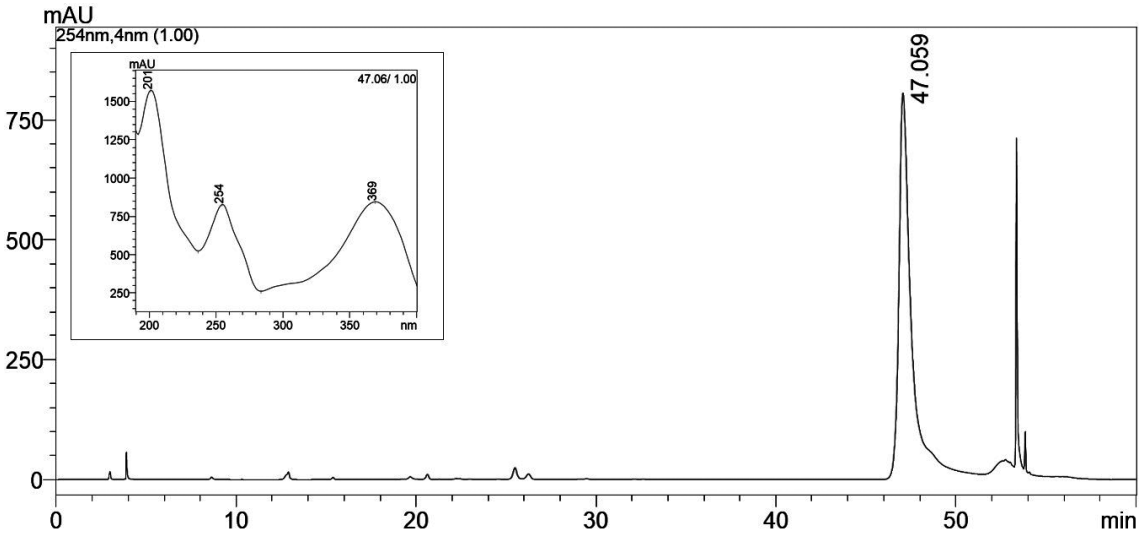

Figure 2 – HPLC Chromatogram (254 nm) and UV spectra of the compound 2 (quercetin).

(1H, d, *J* = 10.8, H-5b'). APT (100 MHz, CD_3OD) data indicated the presence of nine methine carbons δ_C 100.3 (C-6) and 95.1 (C-8) of ring A, 116.3 (C-5'), 123.1 (C-6') e 117.5 (C-2') of ring B and 104.4 (C-1''), 72.8 (C-2''), 69.1 (C-3''), and 74.0 (C-4''), and one methylene carbon 67.0 (C-5'') of sugar portion. It was possible to identify 10 quaternary carbons from HMBC (500 MHz, CD_3OD), namely: 158.2 (C-2), 135.2 (C-3), 179.7 (C-4), 162.4 (C-5), 166.6 (C-7), 158.2 (C-9), 105.0 (C-10), 122.8 (C-1'), 145.8 (C-3') and 149.7 (C-4'). This spectra alowed to confirm the existence of a glycoside moiety attached to the quercetin through the OH of C-3 of the C ring by the H-1'' (5.10 ppm) and C-3 (135.2 ppm) correlation and enable confirmation of this structure by other observed correlations.

NO and TNF-α inhibition and cytotoxicity of isolated compounds in LPS-stimulated RAW 264.7 cells

Quercetin (2) presented the highest potential in NO (IC_{50} 4.58±0.95 µg/mL) and TNF-α (IC_{50} 33.25±0.88 µg/mL) inhibition. In addition, this compound was more active than the L-*N*MMA inhibitor and showed no cytotoxic effect up to a concentration of 20 µg/mL, in which excellent inhibitory activity was observed for NO production

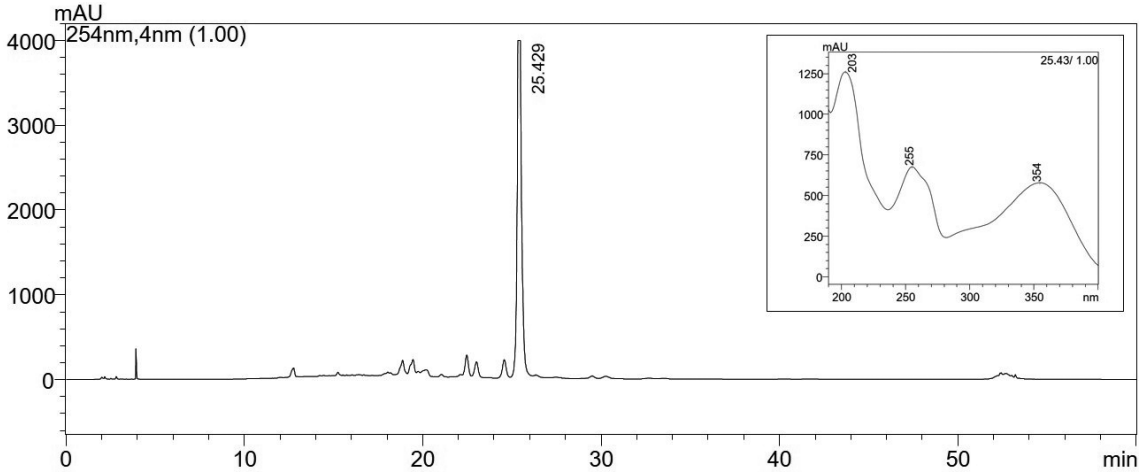

Figure 3 – HPLC Chromatogram (254 nm) and UV spectra of the compound 3 (quercetin-3-*O*-α-arabinopyranoside).

(84.87±1.47%). Friedelin (1) and quercetin-3-*O*-α-arabinopyranosyl (3) demonstrated no significant NO and TNF-α inhibition (IC_{50} > 100 µg/mL). Besides, we observed low cytotoxicity for compounds 1 and 3 (Tab. 4).

Friedelin (1) has already been briefly described in the literature for its anti-inflammatory activity. Friedelin isolated from the stems of *Heritiera littoralis* Aiton. (Malvaceae) exhibited IC_{50} > 100 µM (approximately 42.67 µg/mL) in the NO inhibition (Tewtrakul *et al.* 2010), a result which can be considered compatible with the observed in this study (at 100 µg/mL; 24.23 ± 2.41% inhibition). This substance was isolated from the leaves and twigs of *Acer mandshuricum* Maxim. (Aceraceae), being evaluated at 100 nM (approximately 0.04 µg/mL) and inhibiting 23.5% of TNF-α release (Ding *et al.* 2010). A slightly reduced TNF-α inhibition percentage (14.98 ± 0.20%) was verified in this study at 0.8 µg/mL and, in addition to that, it was also observed that even with the increase of the evaluated concentrations, no increase in the inhibitory effect was noted, suggesting that it may be the maximum effect caused by friedelin.

Quercetin (2), as well as friedelin (1), already earlier isolated from *H. balsamifera* (Silva *et al.* 2004), has been widely described in the literature as having anti-inflammatory potential. Quercetin at 20 and 100 µg/mL was able to inhibit approximately 85% of NO production (IC_{50} 4.58 ± 0.95 µg/mL) in this study. These data agree with the literature since this compound inhibited between 75–80% of NO production at 20 µM (6.04 µg/mL) and 100 µM (30.2 µg/mL) (Manjeet & Ghosh 1999; Shen *et al.* 2002; Ho *et al.* 2017) showing IC_{50} 4.08 µg/mL (Wang *et al.* 2019). Quercetin was also described for having the ability to inhibit the release of TNF-α, presenting IC_{50} < 200 µg/mL (667 µM) (Calixto *et al.* 2004). A recent study evaluated the activity of quercetin from 6.25 to 25 µM in the release

Table 4 – Effect of the isolated compounds on the inhibition of nitric oxide, TNF-α production and cytotoxicity in LPS-stimulated RAW 264.7 macrophage cells.

Humiria balsamifera	**NO[a] Inhibition (% of control)**	**TNF-α[b] Inhibition (% of control)**	**Cytotoxicity[c] (% of control)**
Friedelin			
0.8 µg/mL	13.65 ± 0.13***	14.98 ± 0.20**	8.82 ± 3.47***
4 µg/mL	19.67 ± 4.65***	11.85 ± 0.75*	10.55 ± 0.04***
20 µg/mL	18.96 ± 3.17***	12.98 ± 0.68*	6.39 ± 1.43***
100 µg/mL	24.23 ± 2.41***	13.75 ± 5.57**	3.70 ± 1.70***
Quercetin			
0.8 µg/mL	15.26 ± 3.08*	7.73 ± 0.33	0.15 ± 3.84***
4 µg/mL	39.84 ± 0.50***	11.76 ± 0.54*	0.00 ± 8.31***
20 µg/mL	84.87 ± 1.47***	19.25 ± 0.98***	0.00 ± 0.31***
100 µg/mL	84.73 ± 0.53***	95.46 ± 1.46***	78.92 ± 1.66**
Quercetin-3-O-α-arabinopyranoside			
0.8 µg/mL	0.00 ± 0.40	8.62 ± 1.66	4.36 ± 1.88***
4 µg/mL	24.36 ± 2.01***	6.27 ± 3.39	2.08 ± 2.87***
20 µg/mL	27.71 ± 0.27***	9.64 ± 2.38	2.42 ± 0.74***
100 µg/mL	32.35 ± 3.13***	12.84 ± 1.63*	12.09 ± 0.49***

[a] = samples were different from the positive control MΦ + LPS (0.00 ± 0.40%), except Quercetin-3-O-α-arabinopyranoside at 0.8 µg/mL; [b] = samples were different from the positive control MΦ + LPS (0.00 ± 0.27%), except Quercetin at 0.8 µg/mL and Quercetin-3-O-α-arabinopyranoside at 0.8-20 µg/mL; [c] = all samples were different from the positive control Triton (100 ± 0.01%); *** = P < 0.001; ** = P < 0.01; * = P < 0.05. L-*N*MMA at 20 µg/mL (51.36 ± 0.54% NO inhibition).

of TNF-α, showing no relevant effect on the production of TNF-α at the tested concentrations (Lee *et al.* 2018).

Quercetin-3-*O*-α-arabinopyranoside (3) is herein described for the first time in *H. balsamifera* extracts. The non-expressive effects of this compound on NO and TNF-α production compared with the correspondent aglicone quercetin agree with literature data. Quercetin-3-*O*-α-arabinopyranoside isolated from guava leaves (Kim *et al.* 2015) and *Acer tegmentosum* Maxim. (Aceraceae) (Lee *et al.* 2014) evaluated at 10–100 μM demonstrated no significant NO and TNF-α inhibition in RAW 264.7 cells. This compound similarly isolated from *Psidium acutangulum* DC. (Myrtaceae) did not inhibit NO when tested at 50 μg/mL (Houël *et al.* 2016). A higher inhibitory activity of pro-inflammatory mediators for quercetin (2) compared to quercetin-3-*O*-α-arabinopyranoside (3) was also observed in the present study. Therefore, it can be suggested that glycosylation leads to an increase in the polarity and molecular size, and hence may alter the interaction with the target molecule and its passage through the phospholipidic membrane, modifying the possible activity on intracellular sites.

Other activities previously described in the literature for these compounds such as analgesic, antipyretic, antimicrobial, and wound healing (Antonisamy *et al.* 2011; Maalik *et al*. 2014; Hatahet *et al.* 2016; Noundou *et al.* 2016; Sa *et al.* 2017; Özbilgin *et al*. 2018; Singh *et al.* 2018) may contribute to justify the use of this species in traditional medicine to treat hepatitis, diarrhea, hemorrhoids, in curing chronic wounds, and alleviating toothaches.

In summary, this study describes the NO and TNF-α inhibition by *H. balsamifera*, the isolation of quercetin-3-*O*-α-arabinopyranoside and identification of terpenes ß-amyrone, betulin, citronellol, eremophillene, dihydroactinolide and borneol for the first time in this species. Thus, promising results for a species of Jurubatiba shoal are demonstrated in this work, evidencing this region as being a relevant source in the search for new bioactive products. These aspects reinforce the importance of biodiversity preservation of Jurubatiba shoal. The results also strengthen the proposal that inhibition of NO and TNF-α production might be a useful screening strategy in searching for new anti-inflammatory compounds, especially by using medicinal plant extracts.

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