SUPPLEMENTARY MATERIAL

SHORT COMUNICATION

Cecropia hololeuca: A NEW SOURCE OF COMPOUNDS WITH POTENTIAL ANTI-INFLAMMATORY ACTION

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ABSTRACT

Our objective is to investigate the phytochemical components, antioxidant capacity and in vitro and in vivo anti-inflammatory action from *Cecropia hololeuca* bark aqueous extract (AECh). The chemical characterization of AECh was performed through CE-UV, FTIR and NMR Spectroscopy. In vitro assays were performed with the AECh on murine macrophages J774A.1 cells in order to analyse cell viability, NO, TNF-α and IL-1β productions and the in vivo anti-inflammatory potential in acute carrageenan paw oedema in mice. The AECh showed a decrease in the production of NO, TNF-α and IL-1β, without altering the cell viability and reduction of the paw thickness in the 2nd, 3rd and 4th hour. The extract presented 72% free radical scavenging, 0.60% flavonoid content and showed the presence of gallic acid, caffeic acid and catechin as major constituents. The *C. hololeuca* bark extract showed important antioxidant and anti-inflammatory activity, emphasizing the industrial and pharmacological potential of this plant.

Keywords: *Cecropia hololeuca*, phenolic compounds, anti-inflammatory, cytotoxicity.

3. Experimental

Plant material

The barks from *Cecropia hololeuca* Miquel were collected in the Teófilo Otoni city (Minas Gerais state, Brazil) on May, 2018, taxonomically identified and deposited in the collection from UFVJM herbarium in the Diamantina city (Exsiccates - HDJF 5495).

Plant Extraction from Cecropia hololeuca barks

The barks (1.0 kg) of *Cecropia hololeuca* were dried at room temperature, ground and extracted with H_2O using an ultrasonic bath (UNIQUE®) for 20 minutes for three times. After evaporation under reduced pressure, the aqueous extract of *C. hololeuca* barks (AECh) was submitted to CE-UV, FTIR and ^{1}H NMR analysis and biological assays.

^{1}H NMR, CE-UV and FTIR analysis

The ^{1}H NMR (500 MHz; D_2O) spectra from aqueous extract were obtained on the Varian Inova 500® Spectrometer.

In the electrophoretic analysis, an Agilent Technologies Model 7100 CE device was equipped with a high voltage source (± 30 kV), a Photodiode Array Detector (PDA), a temperature control inside the cartridge by air passage and Agilent ChemStation - Rev. B.04.03 (Model 7100) Data Control, Acquisition and Data Processing Program. Capillary without melted silica coating (Polymicro Technologies) with a total length of 48.5 cm in diameter. The operating parameters of the CE were: detection at 254 nm, capillary at 25ºC and applied voltage of +20 kV. The buffer used was 100 mmol/L of Tris-HCl, pH 8.5. The new capillary was activated and conditioned on the first day of use by washing with a pressure of 1000 mbar with 1.0 mol/L NaOH solution for 30 minutes followed by 10 minutes of water. At the beginning of the analysis, the capillary was conditioned for 5 minutes NaOH 1 mol/L, followed by 5 minutes water and 10 minutes electrolyte, injection: 50 mbar during 5s, preconditions between analyses: 2 min. The cleaning conditioning between the runnings was 5 minutes with the running electrolyte.

The spectra obtained in the Infrared Region (FTIR) were recorded in a Bruker ALPHAFT-IR MB102 spectrometer in the region of 4000-400 cm^{-1} using the Attenuated Total Reflectance (ATR) technique. The electrophoretic and spectroscopic analyses were done at Department of Chemistry from Federal University of Juiz de Fora.

Evaluation of chemical and pharmacological potential from Cecropia hololeuca

Antioxidant potential: To evaluate the antioxidant potential, *in vitro* photocolorimetric method of free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) described by Mensor et al. (2001).

Briefly, 20 μg/mL of the methanolic solution of the diluted samples was added to 220 μg/mL of a free radical methanolic DPPH solution. After 30 minutes of reaction, the reading was carried out at 517 nm using a UV-Vis spectrophotometer (Shimadzu UV 1601). All readings were performed in triplicate and the average of the obtained data was then calculated. After that, the percentage of the antioxidant activity of the AECh was estimated, by the following formula:

AA = 100 - [(Aa-Ab) x 100]/Ac (**1**)

Where AA = percentage of antioxidant activity; Aa = absorbance of the sample; Ab = white absorbance; Ac = control absorbance.

Total flavonoid content: Total flavonoid content was subsequently determined using the adapted Dowd method (Arvouet-Grand et al. 1994). Briefly, 500 μL of 2% $AlCl_3$ in methanol was mixed with the same volume of sample solution (100 μL of AECh to 50 mL of distilled water). Thus, the absorbance at 425 nm was read after ten minutes against a blank, consisting of a solution (500 μL) of methanol with 500 μL of $AlCl_3$. In this case, the total flavonoid content was determined using a standard rutin. Finally, the total content of flavonoids was expressed as %, considering the dry extract content of the extracts.

Cell culture: Stock solutions of the AECh, at 1 mg/mL, were made from sterile RPMI and stored at -80 °C until use. J774A.1 cells line murine macrophages were maintained in culture bottles containing supplemented RPMI-1640 medium (1% non-essential amino acids, 100 μg/mL streptomycin and penicillin, and 5% foetal bovine serum) in a humid atmosphere of 5% CO_2 at 37 °C. After reaching confluence the bottles were scraped and the cells plated in 96-well plates at the concentration of $5x10^4$/mL cells. J774A.1 cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C in the presence of the AECh at the concentrations of 200, 50 and 10 μg/mL for one hour and subsequently stimulated with LPS (1 μg/mL) and IFN-γ (0.9 ng/mL) at 10% of the culture volume for 6 or 48 hours. After 6 hours of culture, the supernatant was withdrawn for the TNF-α and IL-1β dosage. After 48 hours of culture, the supernatant was withdrawn for the NO dosage. For cell viability evaluation, the cells were incubated in 96-well plates in the concentration of $5x10^4$ cells/mL, in a humidified atmosphere of 5% CO^2 at 37 °C in the presence of the AECh at the concentrations of 200, 50 and 10 μg/mL for 48 hours.

Cell viability evaluation by the MTT assay from *Cecropia hololeuca* extract: Cell viability was measured using the MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay in culture of not stimulated cells. After 48 hours of culture the supernatant was removed and the cells incubated with 100 μL of supplemented RPMI and 10 μL of MTT (5 mg/mL) for 4 hours in a humidified atmosphere of 5% CO_2 at 37 °C. After this time the supernatant was

withdrawn from the wells without any change in the precipitate. The formed formazan crystals were then dissolved by the addition of 100 µL of DMSO in each well. The complete solubilisation was done by a slight agitation of the plates. The optical density was measured at 560 nm (EZ Read 200, Biochrom) and the viability (%) obtained by the formula ((X1 / X2) * 100), considering X1 and X2 the mean optical density in wells of treated cells and cells untreated, respectively.

Evaluation of inflammatory mediator's production NO, TNF-α and IL-1β: The 48 hour supernatants from the stimulated cell cultures were analysed for quantification of nitrites by the Griess method. Aliquots of the supernatants were plated with 1% sulphanilamide and 0.1% N-(1-naphthylethylenediamine). The nitrite production was quantified by comparison to a standard curve with different concentrations of NaNO2. The optical density was measured at a wavelength of 540 nm (EZ Read 200, Biochrom). NO production (%) was obtained by the formula ((X1/X2) * 100), considering X1 and X2 the average NO production in the wells of treated cells and untreated cells, respectively. The 6 hours culture supernatants' were submitted to the measurement of cytokines (IL-1β and TNF-α) by the Immunoenzymatic method (ELISA). ELISA plates were sensitized with the capture antibody, diluted in a specific buffer according to the manufacturer's instructions (BD Biosciences, Pharmigen, San Diego, USA). They were incubated overnight and then washed with PBS-Tween four times, blocked with PBS-foetal serum for 1 hour. After the blockade of 01 hour, the plates were washed again and the cytokine samples and standards added into the wells of the plates. Plates were incubated for 24 hours at 4 °C. After the incubation period, they were washed and the second biotinylated antibody was added. The plate was incubated for 1 hour at room temperature and washed for another 4 times. The enzyme conjugate was added and the plates were incubated for another hour. After incubation, the reaction was revealed by the addition of the substrate and finally blocked with 2N sulphuric acid. The reading was done on a microplate reader (EZ Read 200, Biochrom) at 450 nm. Quantities of cytokines were calculated from the standard curves obtained by the different concentrations of the respective recombinants for IL-1β and TNF-α (BD Biosciences, Pharmigen, San Diego, USA).

Carrageenan induced paw oedema: Female mice from BALB/c strain were obtained from *Centro de Bioterismo-CEBIO* of the Federal University of Minas Gerais. The experimental procedure was approved by the Committee on Ethics in the Use of Animals - CEUA-Mucuri from Federal University of the Jequitinhonha and Mucuri Valleys (protocol 002R/2018). The procedures were done according to Reis et al. (2013). The mice were weighed and divided into 5 groups (n=5 animals per group): Group I: Induced with carrageenan and treated with PBS; Group II: Induced with carrageenan and treated with dexamethasone (0.5 mg/kg) diluted in PBS; Group III: Induced with carrageenan and treated with AECh (500 mg/kg) diluted in PBS; Group IV: Induced with carrageenan and treated with AECh (250 mg/kg) diluted in PBS; Group

V: Induced with carrageenan and treated with AECh (100 mg/kg) diluted in PBS. The paws were measured with caliper (0h). Dexamethasone treated animals were used as a positive treatment. Thirty minutes before the induction of the oedema, they were treated by gavage (100 μL) with PBS, dexamethasone (0.5 mg/kg) or AECh (500, 250 or 100 mg/kg). Carrageenan (2.5%) was dissolved in PBS and 20 μL was injected into the left paw, and 20 μL PBS into the right paw, of the whole group. The right and left paws were measured 1, 2, 3 and 4 hours after carrageenan injection, and the differences between the paws were calculated. The oedema induced in the paws of the mice by carrageenan was determined by the formula: [Paw oedema (mm)] = [measured paw injected carrageenan (mm) - paw measure of PBS (mm)].

Statistical analysis: The results presented are representative of three independent experiments and are shown as mean ± standard error. The difference significance was analysed using Student's t-test, Mann-Whitney, One-way ANOVA or Kruskal-Wallis when appropriate. The differences were considered significant when $p < 0.05$.

Figures:

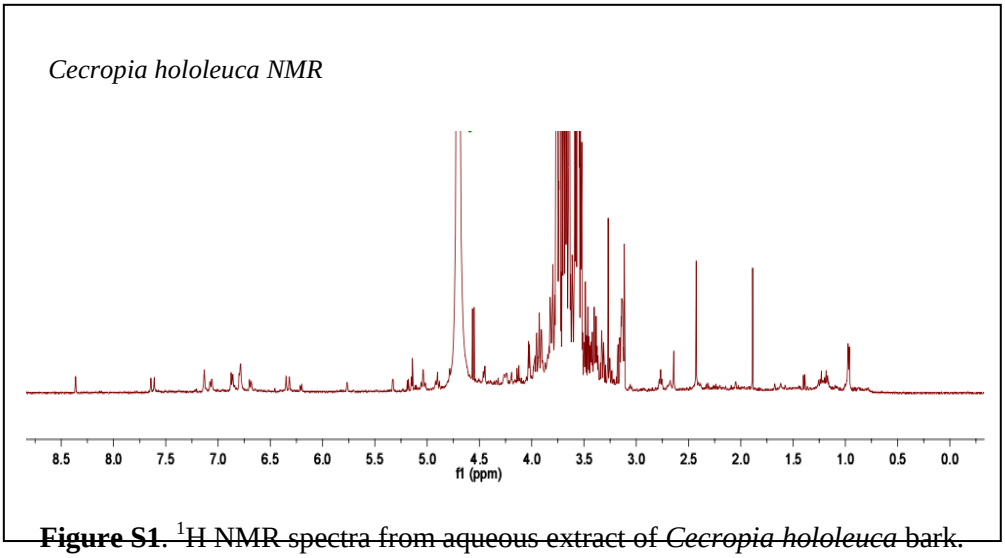

Figure S1. ^{1}H NMR spectra from aqueous extract of *Cecropia hololeuca* bark.

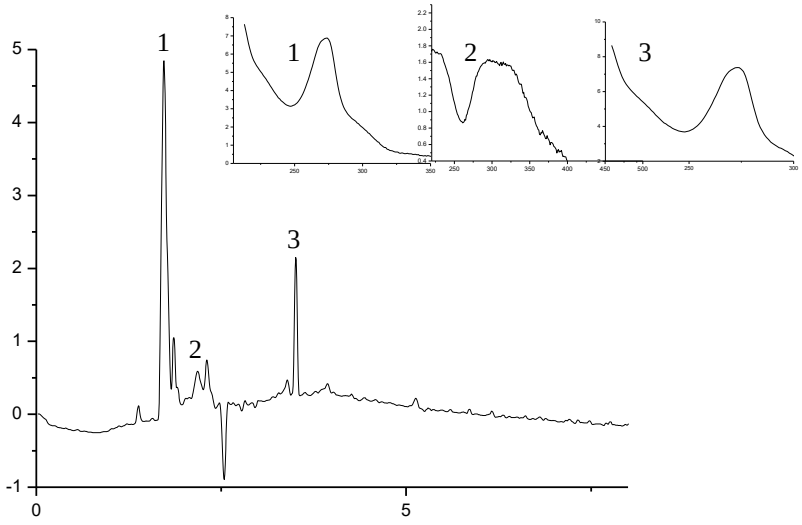

Figure S2. Electropherogram and UV spectras obtained by CE-UV analysis from three major compounds from aqueous extract of *Cecropia hololeuca* bark.

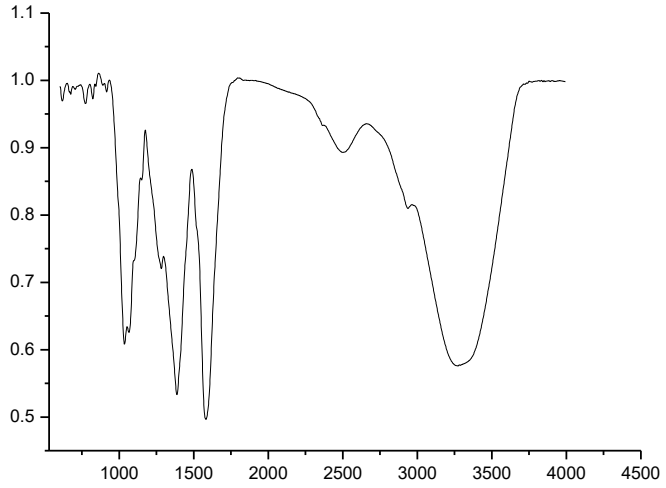

Figure S3.

FTIR spectra from aqueous extract of *Cecropia hololeuca* bark.

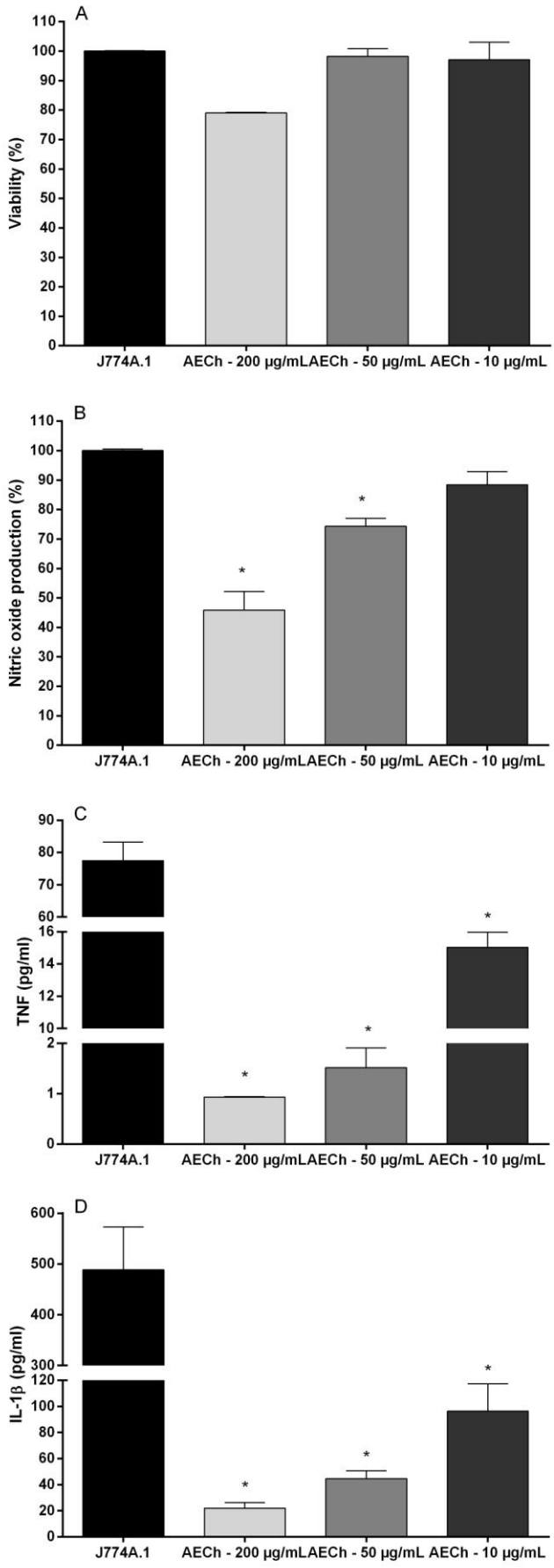

Figure S4. J774A.1 cells were stimulated or not with LPS+IFN-γ and treated or not with 200, 50 or 10 µg/mL of the aqueous extract from *Cecropia hololeuca* bark (AECh). (**A**) Cell viability (%). (**B**) Nitric oxide

(NO) production (%). (**C**) TNF-α production. (**D**) IL-1β production. *p <0.05 vs. control. The columns represent Mean ± SEM. J774A.1 = cells not treated with extract.

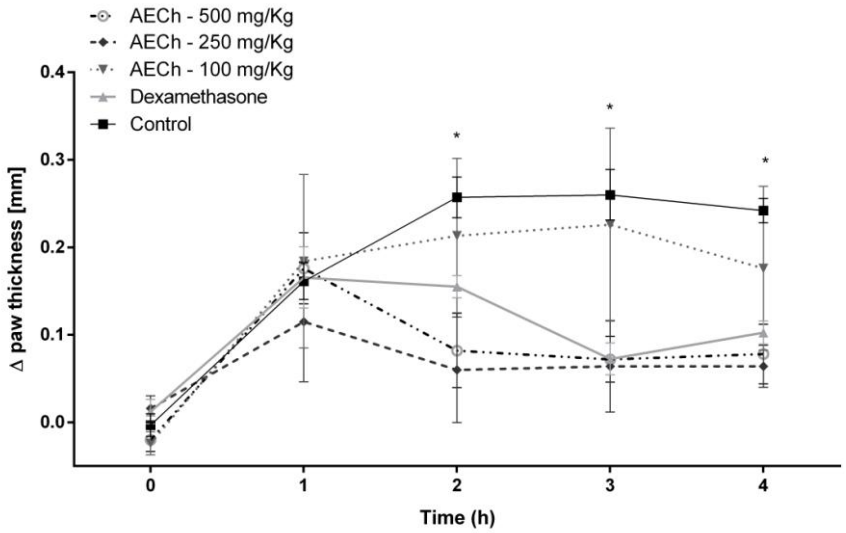

Figure S5. Effect of the administration of aqueous extract from *Cecropia hololeuca* bark (AECh) at concentrations 500, 250 or 100 mg/kg, or dexamethasone (0.5 mg/kg), on mice paw inflammation. Symbols and vertical lines indicate mean ± SEM of the variation in the paw thickness [mm]. * p<0.05 when compared the control group (not treated mice) to Dexamethasone, AECh 250 mg/kg or AECh 500 mg/kg.

References

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