

Identification of Ellagic Acid Derivatives in Methanolic Extracts from *Qualea* Species

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The methanolic extract from the barks of the medicinal plant *Qualea parviflora* (Vochysiaceae) was fractionated by column chromatography over silica gel followed by gel permeation over Sephadex LH-20 to give 3,3'-di-*O*-methylellagic acid-4-*O*- β -D-glucopyranoside (**1**), 3-*O*-methylellagic acid-4'-*O*- α -L-rhamnopyranoside (**2**), 3,3',4-tri-*O*-methylellagic acid-4'-*O*- β -D-glucopyranoside (**3**), and 3,3'-di-*O*-methylellagic acid (**4**), together with triterpenes and saponins. We also performed comparative analyses among this species and *Q. grandiflora* and *Q. multiflora* using high-pressure liquid chromatography. The biological assays showed that, when compared to the standard ellagic acid, compounds **1–4** are less cytotoxic but have a lower capacity of stimulating murine peritoneal macrophages to release nitric oxide and tumoural- α necrose factor.

Key words: *Qualea*, Ellagic Acid Derivatives, Liquid Chromatography

Introduction

Qualea parviflora, *Q. grandiflora* and *Q. multiflora* (Vochysiaceae) are trees found on the American Continent and are used as antiseptic, astringent, antidiarrheal and against gastrointestinal disorders (Grandi *et al.*, 1989; Septímio, 1994; Hiruma-Lima *et al.*, 2006). Previous studies on *Qualea* species afforded fatty acids and polysaccharides in the seeds (Mayworm and Salatino, 2002; Mayworm *et al.*, 2000) besides triterpenes and saponins in the barks (Nasser *et al.*, 2006).

As part of our ongoing research on bioactive compounds from Brazilian plants for the treatment of tropical diseases, we have started to work on natural products which can be triggered with the participation of the immunological system. Macrophages are cells of the innate immunological system which have several functions, such as the removal of foreign bodies, the cytotoxicity of tumoural cells, the presentation of antigens and the activation of T-cells with production of cytokines (Adams and Hamilton, 1984).

The activation of macrophages is a key process of the innate immunity for the initiation and propagation of defensive reactions against pathogens. When activated by pathological stimuli or injuries, the macrophages liberate pro-inflammatory cytokines and pro-inflammatory mediators such as tumoural- α necrose factor (TNF- α) and NO. NO is a highly soluble radical, formed through the oxidation of the nitrogen atom of the amino acid L-arginine by means of the inducible nitric oxide enzyme (iNOS) (Nathan, 1992). Both the production of NO and the activation of macrophages are regulated by liberated cytokines which have autocrine function, such as TNF- α , and a paracrine function, such as interferon- γ (IFN- γ) (Kovallousky *et al.*, 2000).

In the present paper we report the isolation of ellagic acid derivatives from the methanolic extract of barks of *Q. parviflora*, as well as the identification of these compounds in *Q. grandiflora* and *Q. multiflora* using high-pressure liquid chromatography analyses (HPLC-UV-PDA). We also

evaluated the immunological activities of the isolated compounds **1–4** comparatively to that of ellagic acid (EA).

Experimental

Chemicals

Silica gel 60 (Merck, Darmstadt, Germany) for thin layer chromatography (TLC) was used. Acetonitrile (ACN) and trifluoroacetic acid (TFA), HPLC grade, were purchased from Mallinckrodt Chemical (Phillipsburg, USA). [²H₆]-Dimethyl sulfoxide (DMSO-*d*₆) from Merck was also used. Highly purified water from a Milli-Q RG water purification system from Millipore (Bedford, USA) was used in all procedures. Ellagic acid (EA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solvents used for Sephadex LH-20 chromatography and TLC were of analytical reagent grade from Merck.

Instrumentation

The HPLC analyses were performed using a chromatographic system consisting of a Jasco Liquid Chromatograph (Tokyo, Japan) with a PU-2089 quaternary solvent pump, a MD-2010 photodiode array detector (Jasco) and a Rheodyne 7725 sample injector with a 20 μ l sample loop (Rheodyne, Cotati, CA, USA). EZChrom Elite Data System software was used for both the operation of detector and for data processing.

The NMR spectra in DMSO-*d*₆ were obtained using a Varian INOVA-500 spectrometer (Palo Alto, USA), operating at 500 MHz for ¹H NMR and 126 MHz for ¹³C NMR. Chemical shifts are given in δ (ppm) using tetramethylsilane (TMS) as internal standard.

ESI-IT mass spectra were obtained in the positive ion mode on a Micromass Q-TOF mass spectrometer (Manchester, UK). Typical ESI-IT-MS conditions were as follows: source temperature, 100 °C; desolvation temperature, 100 °C; capillary voltage, 3.0 kV; cone voltage, 40 V. Sample solutions in MeOH were introduced into the electrospray source using a syringe pump at a flow rate of 8 μ l min⁻¹.

Preparation of the methanolic extract

Barks of *Q. parviflora* were collected at Ypê Garden (Cerrado region), Porto Nacional City

(Tocantins State, TO), Brazil by Adriane R. Duarte; barks of *Q. grandiflora* were collected at street Pedro Afonso (Tocantins State, TO), Brazil by Cristiano Borges Pereira; barks of *Q. multiflora* were collected at Ypê Garden (Cerrado region), Porto Nacional City (Tocantins State, TO), Brazil by Dr. Hiruma-Lima. Voucher specimen were identified by Dr. S. F. Lolis, UNITINS, Porto Nacional City, Brazil and deposited, respectively, under Nos. 9226, 3379 and 4158 at UNITINS herbarium.

The air-dried and powdered barks (500 g) of *Q. parviflora*, *Q. grandiflora* and *Q. multiflora* were exhaustively extracted (three times) with chloroform and methanol (48 h, 4 l each) at room temperature. Solvents were evaporated at 60 °C under reduced pressure and afforded the CHCl₃ extract (1.7, 1.8 and 1.7 g) and MeOH extract (4.6, 5.4 and 5.1 g), respectively. This procedure was repeated several times in order to obtain material for preparative separations and biological assays.

Isolation and identification of compounds **1–4** from *Q. parviflora*

To isolate and purify the compounds, the MeOH extract of *Q. parviflora* (8.0 g) was fractionated by CC (silica gel, 160.0 g, CHCl₃/MeOH gradient) followed by repeated purification on Sephadex LH-20 (3 cm \times 40 cm, MeOH, isocratic) and by silica gel CC (silica 60H, 20.0 g, CHCl₃/MeOH gradient) to give compounds **1** (12 mg), **2** (8 mg), **3** (10 mg) and **4** (16 mg). The compounds were identified by instrumental analysis using MS and ¹H and ¹³C NMR.

Compound 1: ¹H NMR (500 MHz, DMSO-*d*₆): δ = 7.51 (1H, s, H-5'), 7.80 (1H, s, H-5), 4.08 (3H, s, 3-OCH₃), 4.04 (3H, s, 3'-OCH₃), 5.12 (1H, d, H-1'', *J* = 7.5 Hz), 3.37 (1H, H-2''), 3.35 (1H, H-3''), 3.23 (1H, H-4''), 3.42 (1H, H-5''), 3.70 (2H, H-6''). – ¹³C NMR (126 MHz, DMSO-*d*₆) δ = 114.3 (C-1), 140.9 (C-2), 141.8 (C-3), 151.4 (C-4), 111.9 (C-5), 112.8 (C-6), 158.4 (C-7), 111.8 (C-1'), 141.6 (C-2'), 140.4 (C-3'), 153.0 (C-4'), 111.8 (C-5'), 110.6 (C-6'), 158.5 (C-7'), 61.6 (3-OCH₃), 60.9 (3'-OCH₃), 101.4 (C-1''), 73.3 (C-2''), 76.5 (C-3''), 69.5 (C-4''), 77.3 (C-5''), 60.6 (C-6''). – ESI-IT-MS (positive ion mode, 40 V): *m/z* = 515.0805 [M+Na]⁺ (100), calcd. 515.0801. Compound **1** was identified as 3,3'-di-*O*-methyl ellagic acid-4-*O*- β -D-glucopyranoside (Nawwar *et al.*, 1982; Pakulski and Budzianowski, 1996).

Compound 2: ^1H NMR (500 MHz, DMSO- d_6): δ = 7.48 (1H, s, H-5), 7.68 (1H, s, H-5'), 4.01 (3H, s, 3-OCH₃), 5.45 (1H, d, H-1'', J = 1.5 Hz), 3.98 (1H, dd, J = 1.5 Hz and 3.5 Hz), 3.82 (1H, dd, J = 3.5 Hz and 9.0 Hz), 3.34 (1H, t, J = 9.0 Hz and 9.5 Hz), 3.53 (1H, dq, J = 9.5 Hz and 6.0 Hz), 1.14 (1H, d, J = 6.0 Hz). – ^{13}C NMR (126 MHz, DMSO- d_6): δ = 111.4 (C-1), 136.3 (C-2), 140.1 (C-3), 152.6 (C-4), 111.3 (C-5), 111.3 (C-6), 158.7 (C-7), 114.4 (C-1'), 114.4 (C-2'), 141.9 (C-3'), 146.7 (C-4'), 112.0 (C-5'), 113.1 (C-6'), 158.8 (C-7'), 60.9 (3-MeO), 100.0 (C-1''), 69.0 (C-2''), 70.1 (C-3''), 71.8 (C-4''), 69.8 (C-5''), 17.8 (C-6''). – ESI-IT-MS (positive ion mode, 40 V): m/z = 463.0742 [M+H]⁺ (100), calcd. 463.0876. Compound **2** was identified as 3-*O*-methylellagic acid-4'-*O*- α -L-rhamnopyranoside (Yazaki and Hillis, 1976; Guo and Yang, 2005).

Compound 3: ^1H NMR (500 MHz, DMSO- d_6): δ = 7.67 (1H, s, H-5), 7.84 (1H, s, H-5'), 4.09 (3H, s, 3'-OCH₃), 4.04 (3H, s, 3-OCH₃), 4.00 (3H, s, 4-OCH₃), 5.43 (1H, d, H-1'', J = 6.5 Hz), 3.37 (1H, H-2''), 3.30 (1H, H-3''), 3.34 (1H, H-4''), 3.28 (1H, H-5''), 3.70 (2H, H-6''). – ^{13}C NMR (126 MHz, DMSO- d_6): δ = 112.9 (C-1), 141.2 (C-2), 141.0 (C-3), 154.4 (C-4), 107.7 (C-5), 112.7 (C-6), 158.2 (C-7), 113.7 (C-1'), 141.3 (C-2'), 141.8 (C-3'), 151.9 (C-4'), 112.4 (C-5'), 112.1 (C-6'), 158.4 (C-7'), 61.4 (3-OCH₃), 56.8 (4-OCH₃), 61.7 (3'-OCH₃), 101.3 (C-1''), 73.3 (C-2''), 76.5 (C-3''), 69.5 (C-4''), 77.3 (C-5''), 60.6 (C-6''). – ESI-IT-MS (positive ion mode, 40 V): m/z = 507.1133 [M+H]⁺ (100), calcd. 507.1139. Compound **3** was identified as 3,3',4-tri-*O*-methylellagic acid-4'-*O*- β -D-glucopyranoside (Li *et al.*, 1999).

Compound 4: ^1H NMR (500 MHz, DMSO- d_6): δ = 7.51 (1H, s, H-5 and H-5'), 4.05 (3H, s, 3-OCH₃ and 3'-OCH₃). – ^{13}C NMR (126 MHz, DMSO- d_6): δ = 111.6 (C-1 and C-1'), 141.2 (C-2 and C-2'), 140.3 (C-3 and C-3'), 152.3 (C-4 and C-4'), 111.5 (C-5 and C-5'), 112.1 (C-6 and C-6'), 158.5 (C-7 and C-7'), 61.0 (3-OCH₃ and 3'-OCH₃). – ESI-IT-MS (positive ion mode, 40 V): m/z = 331.0454 [M+H]⁺ (100), calcd. 331.0826. Compound **4** was identified as 3,3'-di-*O*-methylellagic acid (Nawwar *et al.*, 1982; Pakulski and Budzianowski, 1996; Sato, 1987).

HPLC-UV-PDA analyses

Sample preparation

MeOH extracts of the barks of *Q. parviflora*, *Q. grandiflora* and *Q. multiflora* (10 mg) were dis-

solved in methanol (4.0 ml) and applied to a Sep-Pak RP18 cartridge, preconditioned with methanol (2 × 100 ml), to remove lipidic material. The MeOH extracts and compounds **1–4** were filtered through a 0.45 μm polytetrafluoroethylene (PTFE) membrane filter and aliquots submitted to HPLC-UV-PDA.

Chromatographic analyses of the *Qualea* species

The analytical column was a Phenomenex Luna RP18 column (250 × 4.6 mm I. D. × 5 μm , Torrance, CA, USA), equipped with a Phenomenex security guard (4.0 × 2.0 mm); the mobile phase composition used was: water (eluent A) and acetonitrile (eluent B), both with 0.05% of TFA. Absorption was measured at 254 nm. The mobile phases were degassed, the flow rate of the mobile phase was 1.0 ml min⁻¹ and the injection volume was 20 μl . The experiments were performed at room temperature.

The chromatographic fingerprint of the three *Qualea* species was established using as standards compounds **1–4** isolated from *Q. parviflora* and comparing their retention times and UV spectra with those observed in the chromatographic runs of *Q. multiflora* and *Q. grandiflora*.

Animals

Swiss mice (6–8 weeks old, weighing 18–25 g), supplied by Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, Araraquara, SP, Brazil, were maintained in a polycarbonate box [at (23 ± 1) °C, (55 ± 5)% humidity, 10–18 circulations/h and a 12 h light/dark cycle] with water and food available *ad libitum*. These experiments were accomplished in agreement with the Committee of Animal Ethics, with the protocol 06/2005.

Peritoneal exsudate cells (PEC)

Thioglycollate elicited PEC were harvested from Swiss mice by using 5.0 ml of sterile PBS, pH 7.4. The cells were washed twice by centrifugation at 200 × g for 5 min at 4 °C and resuspended in an appropriate medium for each test.

Assessment of cellular viability

For the cellular viability assay it was used the method based on the capacity of the viable cells to cleave the tetrazolic ring present in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), due to the action of dehydrogenase

enzymes present in the active mitochondria, forming formazan crystals (Mosmann, 1983).

The cells were resuspended in RPMI-1640 medium containing 5% fetal bovine serum, 100 UI ml⁻¹ penicillin, 100 UI ml⁻¹ streptomycin and 50 mM 1-mercaptoethanol for an adjustment to the concentration of 5 · 10⁶ cells ml⁻¹. 100 µl of the suspension and 100 µl of the extract were added to each well of the plaque, where the peritoneal macrophages were incubated for 24 h. MTT was added (100 µl) and the plaque was incubated for 3 h at 37 °C with 5% CO₂. Absorbance were measured in a UV/visible spectrophotometer at 540 nm with a reference filter of 620 nm. The culture medium and cells were used as a negative control (NC) and represented 100% of viability of the macrophages.

NO measurement

The cells were resuspended in RPMI-1640 complete medium and adjusted to 5 · 10⁶ cells ml⁻¹. 100 µl of the suspension were placed into each well of 96-well plates and incubated with 100 µl of the extracts. The plates were incubated 24 h at 37 °C, 7.5% CO₂. Lipopolysaccharide from *Escherichia coli* O111:B4 (LPS) was used as a positive control. The nitrite concentrations were indirectly measured by a quantitative colorimetric assay using the Griess reagent system: 50.0 µl aliquots of extracts were added to 50.0 µl of Griess reagent (1% sulphanimide, 0.1% naphthylethylenediamine and 3% H₃PO₄) and incubated at room temperature for 10 min, and the absorbance was measured at 540 nm in an ELISA reader (Multiskan Ascent Labsystems). Samples in triplicate were assayed in four experiments and reported as µmol NO/5 · 10⁵ cells ± SD quantified from the standard curve (Green *et al.*, 1982).

TNF-α measurement

The TNF-α cytokine released in the supernatant of a PEC culture was quantified by an immunoenzymatic assay (ELISA). The 96-well plates were coated overnight at room temperature with 100 µl/well of a purified rat anti-mouse cytokine capture antibody (anti- TNF-α) at 4 µg ml⁻¹ (Kit DuoSet®, R&D Systems, Minneapolis, MN, USA) in sodium bicarbonate buffer, pH 7.2. Plates were washed three times with 0.01 M phosphate-buffered (pH 7.2) saline (PBS) containing 0.05% Tween 20 (PBS-T). Plates were blocked with 300 µl/well of

1% BSA in PBS at room temperature for 60 min, and washed three times with PBS-T. Standard murine cytokines (BD Biosciences Pharmingen, San Diego, CA) or samples (supernatant of macrophage culture) were diluted in RPMI-1640 medium and aliquots of 100 µl were added to the appropriate wells. Plates were incubated at room temperature for 120 min, washed three times with PBS-T, and 100 µl of biotinylated goat anti-mouse cytokine (TNF-α) detection monoclonal antibody at 200 ng ml⁻¹ diluted in BSA-PBS were added to each well. Plates were incubated at room temperature for 120 min, and washed three times with PBS-T. 50 µl of streptavidin-horseradish peroxidase conjugate diluted 1:200 in BSA-PBS were added to each well, and plates were incubated at room temperature for 20 min. Plates were then washed three times with PBS-T and 100 µl of substrate [10 mM citric phosphate buffer, pH 5.5, containing 0.4 mM tetramethylbenzidine (Sigma Chemicals Co.) and 1.2 mM H₂O₂] were added to each well. The reaction was stopped by adding 50 µl of 2 N H₂SO₄. Absorbance was read at 450 nm on a microplate reader (Multiskan Ascent Labsystems), and cytokine concentrations were calculated by a curve of known concentrations of the TNF-α standard. Results were expressed in pg ml⁻¹.

Statistical analysis

The results are expressed as means ± SD of five experiments. One-way ANOVA with Tukey post test was performed using GraphPad InStat (San Diego, California, US) with the level of significance set at *p* < 0.05.

Results and Discussion

Isolation and identification of compounds 1–4

A portion of the MeOH extract of *Q. parviflora* was fractionated successively by CC over silica gel followed by gel permeation over Sephadex LH-20 to give the ellagic acid derivatives **1–4** (Fig. 1), identified by instrumental analysis using MS and ¹H and ¹³C NMR, as described in the Experimental section.

The purified compounds were used to characterize the chromatographic fingerprint of the methanolic extract using HPLC-UV-PDA. Under optimum analytical conditions the retention time for compounds **1–4** were, respectively, 12.7, 16.3, 19.6 and 25.9 min.

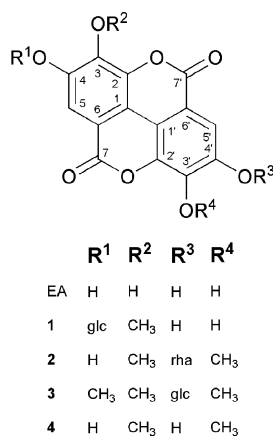


Fig. 1. Chemical structure of EA and compounds 1–4 isolated from *Qualea parviflora*.

The chromatographic profiles of *Q. grandiflora* and *Q. multiflora* were also investigated. Despite they are roughly similar, we observed that *Q. parviflora* produces a larger number of secondary metabolites than *Q. grandiflora* and *Q. multiflora*. The chromatographic profiles revealed a striking difference between these three species: in *Q. parviflora* the peak corresponding to compound 4 is the most intense, whereas in *Q. grandiflora* and *Q. multiflora* compound 1 presents the major peak. Compounds 1, 2 and 4 occur in all three species, whereas 3 could not be detected in *Q. multiflora*.

MTT assay

In this assay the macrophages were exposed to compounds 1–4 and to EA for an incubation period of 24 h in a CO₂ chamber at the temperature of 37 °C.

The results referring to the cellular viability of the murine peritoneal macrophages showed that at 12.5 µg ml⁻¹ EA they presented viability of (31.0 ± 6.8)% while its derivatives presented the following viabilities: 1, (98.9 ± 1.0)%; 2, (85.8 ± 4.7)%; 3, (98.7 ± 1.2)%; 4, (52.1 ± 3.4)% (Fig. 2). Only EA and 4 presented statistical difference ($p < 0.001$) when compared to the negative control. These results indicate that substitution of the OH group in compounds 1–4 leads to a reduction of the cytotoxicity when compared to EA. Therefore, this fact suggests that the presence of the OH group at positions 3 and 3' could be responsible for the cytotoxicity presented by EA. In fact, Constantinou *et al.* (1995) have reported on structure-

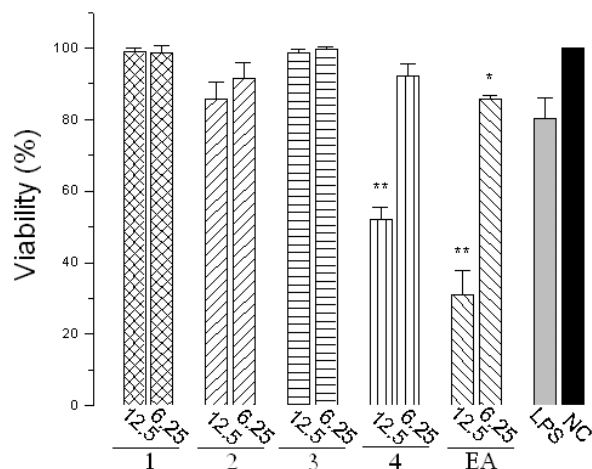


Fig. 2. Viability of the peritoneal macrophages when exposed to EA and compounds 1–4 at 6.25 and 12.5 µg ml⁻¹. Cells in culture medium (RPMI-1640) were used as a negative control (NC) and represent 100% of viability of the macrophages. Cells incubated with lipopolysaccharide from *Escherichia coli* 0111:B4 (LPS) were used as a positive control. ** $p < 0.001$, * $p < 0.01$ in comparison with NC.

activity studies that recognized the 3,3'-hydroxy groups and the lactone groups as the most essential elements for the topoisomerase inhibitory action of plant phenolics.

At lower concentration (6.25 µg ml⁻¹) the cytotoxic effect of EA and its derivatives 1–4 reduced: EA, (85.7 ± 1.2)%; 1, (98.8 ± 2.0)%; 2, (91.6 ± 4.4)%; 3, (99.6 ± 0.6)%; 4, (92.3 ± 3.3)% (Fig. 2), thus displaying a dose-response effect on the cellular viability. Only EA presented statistical difference when compared to the negative control ($p < 0.01$).

NO and TNF-α measurement

The macrophage is one of the principal phagocytes cells of the immunological system, being capable of secreting more than 100 types of substances. Part of its effectiveness is due to the production of nitric oxide (NO), hydrogen peroxide (H₂O₂) and pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Keil *et al.*, 1999). The liberation of toxic mediators by the activated macrophage often causes tissue damages, but it is important in the defense of the host because it allows the destruction of large extracellular pathogens, which could not be otherwise phagocyted.

The production of NO is due to, among other factors, the autocrine stimulation of macrophages by cytokine TNF- α (Kovallousky *et al.*, 2000). Considering the intrinsic relationship between those inflammatory mediators, it is expected that the stimuli capable of inducing the production of NO will also be capable of stimulating the production of the TNF- α cytokine. This fact could be observed in the investigation of the isolated acidic polysaccharide of *Phellinus linteus*, that stimulated the murine peritoneal macrophages by liberation of NO and TNF- α (Kim *et al.*, 2003).

Literature indicates that ellagic acid has antioxidant activity, acting as scavenger of radicals such as NO and superoxide anion and that the antioxidant/pro-oxidant activity of phenolic compounds is dependent on such factors as metal-reducing potential, chelating behaviour, pH value, and solubility characteristics; besides, many antioxidants can also exhibit pro-oxidant behaviour under certain conditions (Decker, 1997).

The determination of NO was performed using the Griess reagent (Malinsk *et al.*, 1996). The results demonstrate that EA stimulated the production of NO by the murine peritoneal macrophages in a significant manner. At the concentration of 6.25 mg ml⁻¹, EA stimulated the liberation of (30.3 \pm 2.2) μ mol of NO/5 \cdot 10⁵ cells, while the results referring to the positive control LPS was (66.5 \pm 4.5) μ mol of NO/5 \cdot 10⁵ cells (Fig. 3). EA showed as significant difference compared to both the positive control (LPS) (p < 0.001), as well as the negative control (cells) (p < 0.001). On the other hand, substances 1–4 did not stimulate the production of NO (p > 0.05). Assays were also performed in order to determine the inhibition of NO in LPS-stimulated macrophages. Neither EA nor compounds 1–4 presented inhibiting activity for the liberation of this mediator (data not shown).

The liberation of cytokine TNF- α was also determined in this study. TNF- α is one of the best studied cytokines, since it is a multifunctional molecule originally described for its ability to destroy *in vivo* transplanted tumours (Carswell *et al.*, 1975). This cytokine stimulates the mononuclear phagocytes to secrete chemokines that contribute to the inflammatory response. Several specific actions of TNF- α can contribute to its lethal actions at extremely high concentrations. TNF- α reduces the tissue perfusion by means of relaxing the tonus of smooth vascular muscles, acting directly on the

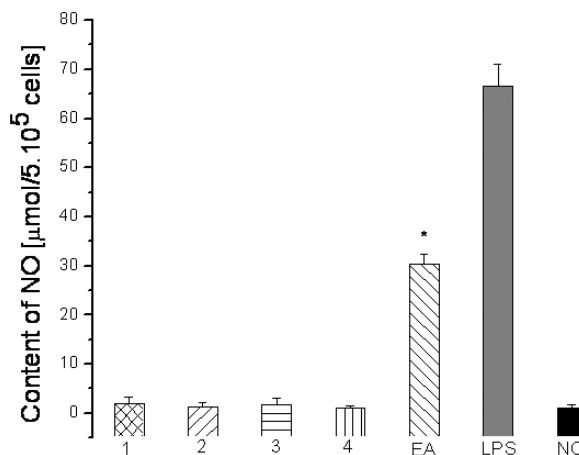


Fig. 3. Production of nitric oxide in peritoneal macrophages exposed to EA and compounds 1–4 at 6.25 μ g ml⁻¹. Cells incubated with lipopolysaccharide from *Escherichia coli* 0111:B4 (LPS) were used as a positive control and cells in culture medium (RPMI-1640) as a negative control (NC). * p < 0.001 in comparison with NC.

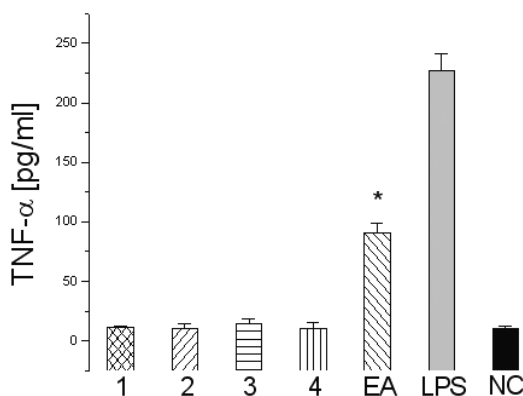


Fig. 4. Production of TNF- α in a culture of peritoneal macrophages when exposed to EA and compounds 1–4 at 6.25 μ g ml⁻¹. Cells incubated with lipopolysaccharide from *Escherichia coli* 0111:B4 (LPS) were used as a positive control and cells in culture medium (RPMI-1640) as a negative control (NC). * p < 0.001 in comparison with NC.

cells of smooth muscles and also in the production of vasodilators such as NO, through the induction of NOS.

The present results indicate that EA stimulated the production of TNF- α , thus reinforcing the correlation between the synthesis of TNF- α and NO. At the concentration of 6.25 μ g ml⁻¹ EA induced the production of (90.3 \pm 8.5) pg of TNF- α ml⁻¹,

while the positive control stimulated the production of (226.6 ± 14.4) pg of TNF- α ml $^{-1}$ (Fig. 4), with statistical difference both in relation to the positive and negative controls, of $p < 0.001$. On the other hand, ellagic acid derivatives **1–4** did not stimulate the murine peritoneal macrophages to produce TNF- α , $p > 0.05$.

In conclusion, EA presented a significant cytotoxic activity and has a stimulating effect on the immunological system. Conversely, ellagic acid derivatives **1–4** showed lower cytotoxicity and lower production of mediators by peritoneal macrophages,

thus suggesting that hydroxy groups could be the responsible factor for the biological activity of EA.

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