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Vicenin-2, a Potential Anti-inflammatory Constituent of *Urtica circularis*

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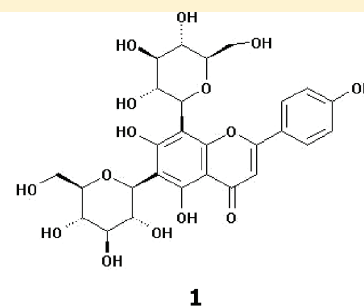
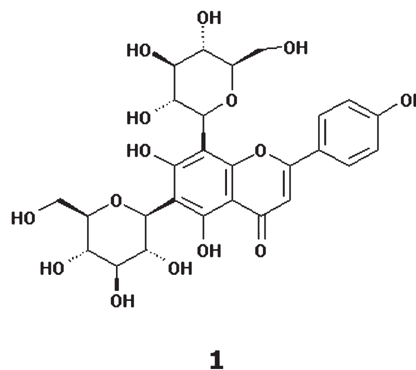
ABSTRACT: Vicenin-2 (**1**), a flavonoid glycoside, was isolated and identified from an ethanol extract of the aerial parts of *Urtica circularis*. This crude extract was found to possess significant anti-inflammatory activity in a carrageenan-induced rat hind paw edema model (41.5% inhibition at a dose of 300 mg/kg; ip). The effects of **1** on several inflammatory mediators were investigated. In cultured murine macrophages, this compound modified LPS-induced total nitrite and TNF-α production, in addition to the LPS-induced translocation of the nuclear factor NF-κB.



Inflammation is associated with the physiopathology of various clinical conditions. Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed medicines due to their high efficacy in the treatment of pain, fever, and inflammation. However, the use of NSAIDs is associated with the occurrence of adverse effects.[1] It has been shown that many plant-derived substances play a relevant role in the process of development of new strategies to treat complaints related with inflammation and pain.[2,3]

Urtica circularis (Hicken) Sorarú (Urticaceae) is known by the common names "caá poropí", "ortiga", and "urtiginha miúda".[4,5] It is an Argentinean native herb, also distributed in Paraguay, Uruguay, and Brazil.[4,6,7] This annual plant, 20−60 cm high,[4] can be found in different types of habitats such as waste land, roadsides, and river banks. The genus name "*Urtica*" derives from the Latin verb "urere", which means to burn, because of its urticant hairs.[8] *U. circularis* is used popularly as an astringent and diuretic, to promote fertility, as a "hepatic" (root decoction), against cough, to lose weight, as an antirheumatic and anti-inflammatory agent (in alcohol for muscular pain),[5] against diarrhea (decoction), and to eliminate dandruff and avoid hair loss. The leaves of this plant are considered edible and are eaten in salads.[6] The roots of *U. dioica* and *U. urens* are used in Europe for self-treatment of benign prostate hyperplasia.[9] There are numerous reports about the use of *U. dioica* for the treatment of arthritic pain.[10] Also, *U. dioica* and *U. macrorrhiza* have shown antioxidant activity[11] as well as anti-inflammatory and analgesic effects in in vitro assays and animal models.[12,13] Apart from one previous report,[14] no other biological, phytochemical, or pre-clinical studies on *U. circularis* have been published. The aim of this study was to evaluate the anti-inflammatory activity of the ethanol extract of *U. circularis* in two animal models and to identify an active compound. This is the first report of vicenin-2 (**1**), a major component of the ethanol extract, in showing activity on nitric oxide (NO) production, tumor necrosis factor (TNF-α) release, and regulation of NF-κB in macrophages activated with lipopolysaccharide (LPS).



The anti-inflammatory activity of the ethanol extract of *U. circularis* was assayed in two experimental models used in the search for new anti-inflammatory drugs from natural products, carrageenan-induced paw edema in rats and 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced ear edema in mice. The carrageenan-induced paw edema model in rats represents inflammation during the early acute stages. The ethanol extract of

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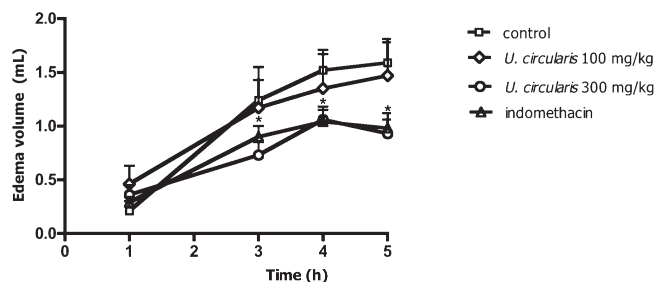

Figure 1. Effect of the ethanol extract of *U. circularis* in the carrageenan-induced paw edema test. Each value represents the means ± SEM of results from six rats. Statistical differences from a control group were determined by a Bonferroni test (*$p < 0.01$ versus control group).

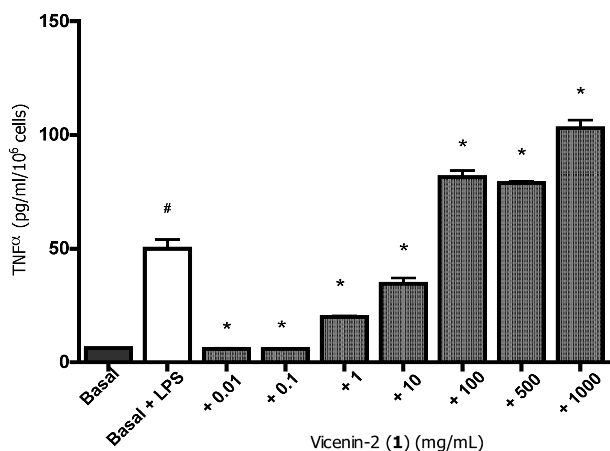

Figure 2. Effect of **1** on TNF-α concentration. Results are expressed as means ± SEM of three experiments made in triplicate. Basal: cells without treatment; basal + LPS: cells previously treated with LPS. The statistical differences between basal + LPS and LPS + **1** were determined by ANOVA followed by Dunnet's test (*$p < 0.01$). The statistical differences between basal cells and basal + LPS were determined by Student's *t*-test (#$p < 0.01$).

U. circularis, at 300 mg/kg, showed a significant inhibition of paw edema starting 3 h after carrageenan administration (41% inhibition), maintaining the response for 5 h. Indomethacin (10 mg/kg) produced a maximum inhibition of 38.4% at 5 h.

In contrast, when the plant extract was administrated topically, it was not effective in inhibiting TPA-induced mouse ear edema development at 0.8, 2, and 5 mg/ear, although indomethacin (1.0 mg/ear) attenuated the inflammation response (inhibition of 87.3%) in a significant manner.

Taking into account the anti-inflammatory activity observed for the plant crude extract, a phytochemical analysis procedure was performed. The total phenolic content, expressed as gallic acid equivalents per gram, was 7.5 GAE/g of ethanolic extract. The chemical composition of this extract was analyzed by HPLC,[15] and a major component was found to be the flavonoid glycoside vicenin-2 (**1**) (t_R: 22.08 min). The isolation of **1** was conducted by different chromatographic methods. When the chromatogram was observed at 254 and 360 nm, no other significant peaks were found. The isolated compound was identified by spectroscopy (UV−vis, ^{1}HNMR, MS) and by comparison with spectroscopic literature data.[16−18]

Beneficial properties have been reported for **1** as an anti-oxidant[19] agent and as an antihepatotoxic agent against CCl_4- and galactosamine-induced cytotoxicity in primary cultured rat hepatocytes.[20] This compound has shown, in addition, significant trypanocidal activity.[21] Also, a *Passiflora edulis* fraction, composed of isoorientin, vicenin-2 (**1**), and spinosin, showed anti-inflammatory activity by inhibiting leukocytes and neutrophils.[22]

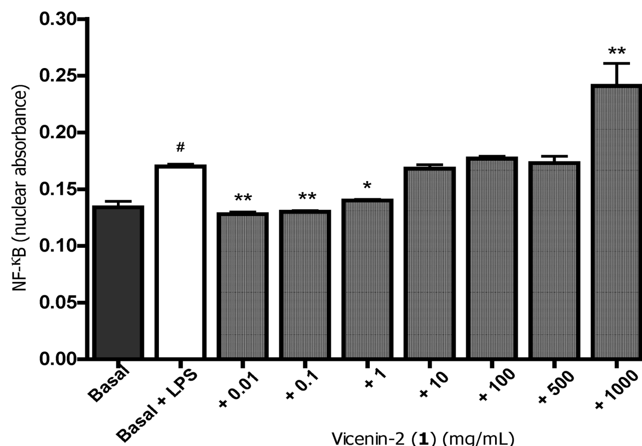

Figure 3. Effect of **1** on NF-κB translocation. Results are expressed as means ± SEM of three experiments made in triplicate. Basal: cells without treatment; basal + LPS: cells previously treated with LPS. Statistical differences between basal + LPS and LPS + **1** were determined by ANOVA followed by Dunnet's test (**$p < 0.01$; *$p < 0.05$). Statistical differences between basal cells and basal + LPS were determined by Student's *t*-test (#$p < 0.01$).

The pro-inflammatory cytokine TNF-α and the reactive free radical NO synthesized by inducible nitric oxide synthase (iNOS) are important macrophage-derived inflammatory mediators and are also reported to be involved in the development of inflammatory diseases. Thus, the effect on the excessive productions of TNF-α and NO can be employed as criteria to evaluate the anti-inflammatory activity of test compounds. In this study, three inflammatory parameters were evaluated using macrophages incubated with and without LPS (basal values) and in the absence or presence of different concentrations of **1** (0.01 to 1000 μg/mL).

The results obtained for the TNF-α assay are shown in Figure 2. LPS increased the TNF-α basal level significantly (about 700%). Compound **1** decreased significantly LPS-stimulated TNF-α in a manner inversely related to concentration. Low concentrations of **1** (0.01, 0.1, 1, and 10 μg/mL) decreased TNF-α with respect to basal values. Concentrations of 100, 500, and 1000 μg/mL of **1** increased (62−100%) TNF-α in a significant manner, in comparison with the group of cells treated with LPS but without treatment with this compound.

Compound **1** (0.01 to 1000 μg/mL) was also studied on NF-κB translocation in LPS-stimulated macrophages. As shown in Figure 3, LPS increased significantly NF-κB nuclear translocation in comparison with basal cells. At low concentrations (00.1, 0.1, and 1 μg/mL), **1** decreased the translocation induced by LPS. Some concentrations of **1** (10, 100, and 500 μg/mL) did not exert any effect, but 1000 μg/mL **1** increased NF-κB translocation significantly.

Total nitrite release by macrophages, an index of NO production, was determined using Griess reagent.[23] LPS increased significantly NO (around 200%) with respect to basal cells (Figure 4). Compound **1** reduced the effect of LPS on nitric oxide inversely according to concentration. Low concentrations

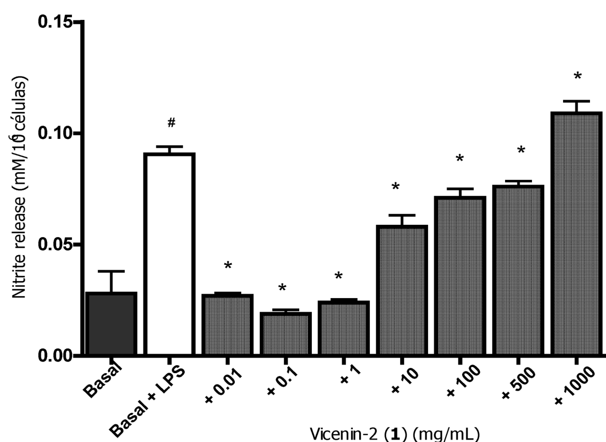

Figure 4. Effect of **1** on nitrite release. Results are expressed as means ± SEM of three experiments made in triplicate. Basal: cells without treatment; basal + LPS: cells previously treated with LPS. The statistical differences between basal + LPS and LPS + **1** were determined by ANOVA followed by Dunnet's test (*$p < 0.01$). The statistical differences between basal cells and basal + LPS were determined by Student's *t*-test (#$p < 0.01$).

(0.01, 0.1, and 1 μg/mL) decreased NO (around 70%) to basal values (no significant differences were observed with basal values), but high concentrations (10, 100, and 500 μg/mL) decreased NO only 16%, and at 1000 μg/mL, NO increased.

Activated macrophages are recognized widely as cells that play an important role in the inflammatory processes. After stimulation with LPS, macrophages secrete several pro-inflammatory products such as TNF-α, interleukins, and NO. A number of transcriptor factor families, including NF-kB, activator protein 1 (AP-1), and others, have been considered as critical regulators of gene expression in the setting of the inflammatory process. Although these factors play an essential beneficial role in normal physiology, inappropriate regulation of NF-kB activity and sustained production of pro-inflammatory cytokines and NO have been implicated in the pathogenesis of several diseases.

Compound **1** exerted a biphasic effect not only on NO but also on TNF-α levels in a model of murine macrophages activated with LPS. Low concentrations of **1** decreased both NO and TNF-α, suggesting an anti-inflammatory action. In high concentrations **1** exerted a pro-inflammatory activity, related not only to TNF-α production but also to NO induction. LPS causes NF-κB nuclear translocation to induce iNOS, which synthesizes NO from L-arginine.[24] Low concentrations of **1** suppressed the LPS-triggered activation of NF-κB, and this effect was related to a decreased nitrite release. Nevertheless, at concentrations at which **1** did not modify NF-κB translocation, a low nitrite release was still observed. Since LPS activates other routes to enhance iNOS gene expression such as mitogen-activated protein kinases (MAPKs), it could be suggested that **1** modifies not only NF-κB but also other pathways.

LPS produces an activation of the cytokine cascade, resulting in increased levels of TNF-α. It is known that the increased activity of NF-κB is also involved in the transcriptional activation of TNF-α genes, although regulation at the post-transcriptional level also has been implicated. TNF-α signal transduction pathways are complex and are still not fully understood;[25] however, the effect of **1** on the transcription factor NF-κB could explain, at least in part, the observed variations in TNF-α levels. NF-κB and TNF-α represent targets for new types of treatment in order to inhibit the inflammatory response in instances where this process becomes chronic or deregulated, so **1** may turn out to represent a new class of anti-inflammatory agent or adjuvant therapy in order to enhance the efficacy of other anti-inflammatory agents.

The effects observed in this study showed a hormetic pattern since low concentrations of **1** decreased NO and TNF-α levels and diminished NF-κB translocation, while high concentrations of this flavonoid glycoside produced the opposite effect in inducing NF-κB translocation. Many compounds isolated from plants and transcription factors have been involved in hormetic responses that have been recognized as integral to the normal physiological function of cells and organisms, representing an adaptive mechanism.[26]

■ EXPERIMENTAL SECTION

General Experimental Procedures. Proton nuclear magnetic resonance (NMR) spectroscopy was performed in D_2O using a Bruker high-resolution DPX300 NMR spectrometer at 300 MHz. MS was conducted using a ThermoElectron LTQ ion trap mass spectrometer, operating in the negative ion mode. The HPLC method was developed and validated according to Filip et al.[15] and performed with a Varian 9000 instrument using a diode array detector. A C_{18} column (Gemini 5 μm, 150 × 4.6 mm) was used. Solvent A: H_2O/AcOH (98:2); solvent B: MeOH/AcOH (98:2). Gradient: 15% B to 40% B, 30 min; 40% B to 75% B, 10 min; 75% B to 85% B, 5 min. Flow rate: 1.2 mL/min. Detection: 325 nm. A Rheodyne injector fitted with a 20 μL loop was used.

Plant Material. *Urtica circularis* was collected in Estancia "La Merced", Corrientes, Argentina, in November 2007 and identified by Dr. Martha Gattuso. A voucher specimen (no. 054) is deposited at Facultad de Ciencias Químicas, U.N.R., Argentina.

Extraction and Isolation. The dried aerial parts of *U. circularis* were ground into a fine powder and extracted by maceration with 80% ethanol. Then, the extract was concentrated and lyophilized. A major component of the extract was purified by preparative column chromatography in Sephadex LH20 using Cl_2CH_2 (100%) as solvent and then increasing the proportion of MeOH until MeOH (100%). Afterward, preparative paper chromatography was performed on Whatman No. 3 using first *n*-BuOH−HOAc−H_2O, 4:1:1 (twice), and then water as solvents. The purity of the isolated compound was checked by HPLC analysis and was 97% on the basis of peak area integration. Vicenin-2 (5,7,4′-trihydroxyflavone 6,8-di-*C*-glucoside, **1**, 0.08% w/w) was identified by spectroscopic data measurement (UV−vis, ^{1}H NMR, ESIMS) and by comparison with literature values.[16−18]

Phytochemical Analysis. The *U. circularis* extract total phenol content was determined by the Folin−Ciocalteu colorimetric method described by Singleton et al.[27] The absorbance was measured at 760 nm and compared with a gallic acid calibration curve. The result was expressed as gallic acid equivalents per gram of extract.

In Vivo Anti-inflammatory Activity. *Drugs.* Indomethacin, morphine sulfate, naloxone, lambda carrageenan, and TPA were purchased from Sigma Chemical Co. (St Louis, MO). Acetic acid and formalin were acquired from Merck (Darmstadt, Germany).

Animals. Female Swiss mice weighing 25−30 g and Sprague−Dawley female rats weighing 180−200 g were used following the guidelines and experimental use of animals as described by the National Institutes of Health.[28] The animals had free access to a standard commercial diet and water ad libitum and were kept in rooms maintained at 22 ± 1 °C with a 12 h light/dark cycle.

Ear Edema in Mouse Assay. Ear edema was induced according to the method of De Young et al.[29] The right ear of each mouse received TPA

(0.125 μg/μL acetone solution) as a topical application (10 μL for each side of the ear). The ethanol extract of *U. circularis* (dissolved in acetone) was applied topically immediately after TPA at doses of 0.8, 2, and 5 mg/ear. The left ear, used as a control, received the vehicle. Indomethacin (1 mg/ear/20 μL) was used as a reference compound. Four hours after TPA administration, the animals were sacrificed and disks of 6 mm diameter were removed from each ear and their weights determined. Swelling was measured as the difference in weight between the punches from right and left ears, and the percent inhibition of edema was calculated in comparison with control animals.

Carrageenan-Induced Edema in Rats Assay. Paw swelling was induced by subplantar injection of 0.1 mL 1% sterile lambda carrageenan in saline solution into the right hind paw.[30] The *U. circularis* ethanol extract was administered ip 30 min before carrageenan injection at doses of 100 and 300 mg/kg. Indomethacin (10 mg/kg) was used as reference compound. The control group received only the vehicle (1 mL/kg ip). The inflammation was quantified by measuring the volume displaced by the paw in a plethysmometer (Ugo Basile) at times 0 and 1, 3, 4, and 5 h after carrageenan injection. The difference between the left and the right paw volumes (indicating inflammation) was determined.

In Vitro Anti-inflammatory Activity. *Sample.* Compound **1**, obtained from the ethanol extract of *U. circularis*, was used for experimental in vitro assays, solubilized in ethanol. The concentration of ethanol in the cell culture was 0.5%.

Cell Culture. Peritoneal macrophages were obtained from female mice handled following the guidelines and experimental use of animals described in a National Institutes of Health publication.[28] Peritoneal cells (PC) were harvested by sterile lavage with 20 mL of sterile PBS, from animals, and adjusted to 1×10^6 cell/mL. Macrophages were purified from PC by adherence onto 96-well flat-bottomed tissue culture plates in RPMI 1640 (Sigma, San Diego, CA) supplemented with penicillin/streptomycin and glutamine and 10% heat-inactivated fetal calf serum (Gibco, Rockville, MD). No adherent cells were removed after 2 h at 37 °C and complete medium was added. The adherent macrophage monolayer showed 90% purity according to morphologic analysis on nonspecific esterase staining. The viability of the macrophages was determined immediately by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO). Briefly, 1×10^6 cells/mL, isolated from animals, were incubated in 100 μL of RPMI 1640 containing 10 μL of 5 mg/mL MTT.[31] Macrophages (1×10^6 cell/mL) were incubated without any drug (basal values) and with LPS in the absence or presence of different concentrations of **1** (0.01 to 1000 μg/mL). The cells were incubated with **1** for 24 h, and LPS (Sigma, St. Louis, MO), used to stimulate macrophages, was added at a concentration of 1 μg/mL for 16 h.

TNF-α Determination. The production of TNF-α was determined for basal macrophages and macrophages treated with LPS and treated with LPS plus different concentrations of vicenin-2 (**1**, 0.01 to 1000 μg/mL). TNF-α was determined in the supernatants of macrophage cultures using a commercial mouse TNF-α ELISA kit (Chemicon International, Inc., Temecula, CA). Cells without any treatment were used as control basal.

NF-κB Assay. Compound **1** (0.01 to 1000 μg/mL) was studied for its effects on NF-κB translocation in LPS-stimulated macrophages. NF-κB translocation was determined using a Cayman Chemical NF-κB (p65) kit (cat. no. 10007889), a nonradioactive, sensitive method for detecting specific transcription factor DNA binding activity in nuclear extracts. The nuclear absorbance measured represented NF-κB translocation.

Total Nitrite Determination. Total nitrite production by macrophages was determined after incubation with LPS, using Griess reagent.[23] Cells (basal, LPS treated, and LPS plus **1**-treated) were collected, centrifuged at 800*g* for 10 min, then incubated with Griess reagent for 20 min in the dark, and measured spectrometrically at 540 nm.

Statistical Analysis. Data in the in vivo assays were analyzed for statistical significance of differences between treated and control group by analysis of variance followed by Dunnett's or Bonferroni tests. Values were considered to be significantly different at $p < 0.05$. Data of in vitro assays were analyzed by analysis of variance plus Dunnett's or Student's tests. Significant differences were determined when $p \leq 0.05$.

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