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Article

Ursolic Acid Derivatives Down Regulate Inflammatory Mediators

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Ursolic acid (UA) is being investigated due to its anti-inflammatory potential, and structural modifications can enhance its biological activities. The aim of this study was to evaluate the immunomodulatory effect of the ursolic acid derivatives (UAD) in macrophages and in the carrageenan-induced paw edema model. RAW264.7 cells were cultured in the presence or absence of UA or UAD (**1-18**). Nitric oxide (NO), nuclear factor kappa B (NF-κB), tumor necrosis factor (TNF), and cellular viability were measured. 30 min before the carrageenan-induced paw edema, the UAD**1** and UAD**2** (200 mg kg^{-1}) were administered intraperitoneally. The results showed that UAD**2-4**, UAD**7**, UAD**9-11** had half maximal inhibitory concentration (IC_{50}) greater than 90 µM and were able to reduce NO, NF-κB and TNF production. Moreover, UAD**1** and UAD**2** reduced paw edema and IL-6 production. In conclusion, the results obtained demonstrated a variation in the response between the derivatives due to the chemical modifications, showing potential to reduce the inflammatory mediators, deserving further investigations.

Keywords: ursolic acid, macrophage, carrageenan, inflammation

Introduction

The inflammation processes are associated with the development of several diseases, acute or chronic, many of them of unclear origin, such as rheumatoid arthritis, multiple sclerosis and Crohn's disease.[1,2] The activation of macrophages is related to the production of inflammatory cytokine and transcription factor expression, such as tumor necrosis factor (TNF) and nuclear factor kappa B (NF-κB), respectively, playing a major role in the establishment of the inflammation.

The cytokines are involved in the activation of endothelial cells and leukocyte infiltration.[3] The TNF is a pro-inflammatory cytokine that has several functions, such as inducing the production of other cytokines and lipid mediators of inflammation, proliferation, cell differentiation and apoptosis.[4] Although the macrophage lineage cells are the main source of TNF, in inflammatory diseases, it can be produced by a wide variety of cells including neutrophils, fibroblasts and endothelial cells.[5]

The transcription factor NF-κB stands out, which plays a crucial role in the initiation and amplification of inflammation. NF-κB consists of a protein assembly located in the cytoplasm of cells and its activity is controlled by a family of inhibitor proteins, denominated IκB, which bind to the NF-κB dimmer, promoting its inhibition. This factor regulates the expression of several genes that code inducible nitric oxide synthase (iNOs), cyclooxygenase-2 (COX-2), TNF-α, interleukin-1-beta (IL-1β) and interleukin-6 (IL-6), which are involved in the pathogenesis of inflammatory diseases.[6-8]

Nitric oxide (NO) is a powerful inflammatory mediator, produced by macrophages and is involved in the regulation of several physiological processes. NO can be generated in excess during the host's response against damage caused by pathogens or other substances, contributing to the pathogenesis of various inflammatory disorders including tissue damage, septic shock, and rheumatoid arthritis.[9,10] Therefore, lipopolysaccharide (LPS)-induced

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NO production may reflect the degree of inflammation. The inhibition of NO production may demonstrate the ability of a substance to act as possible anti-inflammatory agent, although other mediators needed to be evaluated.[10,11]

Macrophages, when sensing pathogen-associated molecular patterns (PAMPs) by their pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), induce the synthesis of endogenous pyrogenic cytokines, among them interleukin 6 (IL-6). IL-6 constitutes an important inflammatory marker secreted by macrophages in response to pathogen invasion during acute inflammation.[12,13] Thus, IL-6 has been considered as an important mediator of fever and the acute phase response.[14,15]

The up-regulation of these inflammatory mediators, produced by the defense, is related to signs and symptoms, such as pain, fever, edema and, sometimes, irreversible tissue damage.[16,17] Thence, the down-modulation of the inflammatory mediators can be valuable to prevent and control the development of inflammatory diseases.[1,2,18] In this context, a disordered and excessive production of pro-inflammatory mediators may be related to the pathogenesis of several diseases and, therefore, these mediators are possible targets of therapeutic substances.[19]

The inhibition of inflammatory mediators and the immune response regulation, through immunosuppression activity, has been shown as a result of the treatment with ursolic acid (UA).[20-24] Among the different mechanisms of action of UA, it is possible to highlight its inhibitory action on the expression of NF-κB.[25,26]

Taking into consideration the promising activity of UA, the aim of this study was to synthesize ursolic acid derivatives (UAD) and to evaluate the improvement in its biological activity. The chemical modifications in the UA structure, create semisynthetic derivatives that demonstrated enhanced cytotoxic activity against tumor cell lines.[27] In a continuation of the work of Scherrer *et al.*,[27] this study evaluated the immunomodulatory effect of the UAD in macrophage response and in carrageenan-induced paw edema model.

Experimental

Reagents

Ursolic acid was purchased from Sigma-Aldrich (Saint Louis, MO, USA). The reagents and the solvents were used directly from the manufacturers. Tetramethylsilane (TMS) and CH_3I (Sigma-Aldrich), acetone, EtOAc, *t*-BuOOH, *n*-hexane, CH_2Cl_2, anhydrous diethyl ether, acetic anhydride and tween 20 (Merck, Darmstadt, Germany); K_2CO_3, $NaHCO_3$, $NaClO_2$, Na_2SO_4, $LiAlH_4$, BF_3-Et_2O, NaCl and pyridine (Sigma-Aldrich, Saint Louis, MO, USA) were employed to obtain ursolic acid derivatives. Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Grand Island, USA); RPMI-1640 (Lonza, Basel, Switzerland); bovine serum albumin (BSA), L-glutamine, streptomycin-penicillin, sulfanilamide, *N*-(1-naphthyl)-ethylenediamine hydrochloride, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum, H_3PO_4, carrageenan, phenylmethylsulfonyl fluoride (PMSF), benzethonium chloride, ethylenediaminetetraacetic acid (EDTA) and aprotinin (Sigma-Aldrich, Saint Louis, MO, USA); and IL-6, TNF and NF-κB (PS529)-PE 558423 (BD, Biosciences Pharmingen, San Diego, CA, USA) were used for the biological assays. All the other materials and solvents were of analytical reagent grade and used as received.

Spectral data

Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ on an AC200 (Bruker Corporation, Billerica, USA) at 200 MHz for ^1H and 50 MHz for ^{13}C, using TMS as internal reference for both nuclei. For each peak, chemical shift values are expressed in parts *per* million, followed by multiplicity, relative peak integration (when appropriate) and coupling constants (J) in hertz. High resolution mass spectra (HRMS) were obtained using a QSTAR XL spectrometer (Applied Biosystems, Foster City, USA). The spectra in the infrared (IR) were obtained in Spectrum One apparatus (PerkinElmer, Wellesley, USA) coupled to the diffuse reflectance accessory (ATR). The specific rotational power values $[\alpha]_D^{25}$ were measured on a 241 polarimeter (PerkinElmer, Wellesley, USA) at 20 ºC. Column chromatography was performed on Silica Gel 60 (230-400 mesh, Merck, Darmstadt, Germany), whereas thin-layer chromatography was carried out on Silica Gel 60 F254 plates (0.25 mm thick, Merck, Darmstadt, Germany). Solvents and reagents were used directly from the manufacturer or purified by standard procedures when required.

Urs-12-ene-3β,28-diol (UAD**9**)

The ursolic acid (60 mg, 0.13 mmol) was reduced with $LiAlH_4$ (90 mg, 2.37 mmol) in diethyl ether, at room temperature for 20 h. At the end of this period, the excess $LiAlH_4$ was consumed with a drop of wet ether and then with a drop of water. The reaction mixture was extracted with saturated NaCl solution; the organic phase was dried over anhydrous Na_2SO_4 and the solvent eliminated in a rotary evaporator to afforded the white solid UAD**9** (50 mg, 86.5% yield). FTIR (ATR) ν_{max} / cm^{-1} 3642 (O-H), 2921 (C-H), 1045 (C-O);

$[\alpha]_D^{25}$ +71.10 (*c* 1.00, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.13 (t, 1H, *J* 3.6, H12), 3.54 (d, 1H, *J* 10.9 Hz, H28a), 3.20 (d, 1H, *J* 10.9 Hz, H28b), 3.18 (m, 1H, H3), 1.91 (dd, 1H, *J* 3.8, 8.8 Hz, H18), 1.10 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 0.79 (s, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 138.77 (C13), 125.06 (C12), 79.04 (C3), 69.93 (C28), 55.19 (C5), 54.05 (C18), 47.70 (C9), 42.08 (C14), 40.06 (C8), 39.39 (C20), 39.39 (C19), 38.81 (C1), 38.03 (C4), 36.90 (C10), 36.90 (C17), 35.24 (C22), 32.85 (C7), 30.68 (C21), 28.18 (C23), 27.27 (C15), 26.05 (C2), 26.05 (C27), 23.33 (C11), 23.33 (C16), 21.39 (C30), 18.37 (C6), 17.42 (C29), 16.83 (C26), 15.69 (C25), 15.69 (C24); HRMS *m/z*, calcd. for $C_{30}H_{50}O_2Na$: 465.7136, found: 465.3730.

Methyl 3-oxours-12-en-28-oate (UAD**10**)

Oxidation of the ursolic acid methyl ester UAD**1** (500 mg, 1.06 mmol) was carried out with pyridinium dichromate (PDC) (716 mg, 3.3 mmol) in anhydrous CH_2Cl_2 at room temperature under constant stirring for 3 h. The crude reaction product was chromatographed over silica gel (eluent *n*-hexane/EtOAc 8:2) to afford UAD**10** (371.9 mg, 74.7% yield). FTIR (ATR) ν_{max}/cm^{-1} 2978 (C-H), 1726 (C=O), 1706 (C=O), 1241 (C-O); $[\alpha]_D^{25}$ +89.20 (*c* 1.00, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.26 (t, 1H, *J* 3.4, H12), 3.60 (s, 3H, COOC$\underline{H}_3$), 2.23 (d, 1H, *J* 11.0 Hz, H18), 2.05 (s, 3H, COOC$\underline{H}_3$), 1.07 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.94 (d, 3H, *J* 6.1 Hz, CH_3), 0.85 (d, 3H, *J* 6.2 Hz, CH_3), 0.78 (s, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 217.75 (C3), 178.02 (C28), 138.29 (C13), 125.35 (C12), 55.26 (C5), 52.95 (C18), 51.49 (COO$\underline{C}H_3$), 48.14 (C17), 47.41 (C4), 46.78 (C9), 42.15 (C14), 39.47 (C8), 38.47 (C10), 39.32 (C1), 39.06 (C19), 38.88 (C20), 36.64 (C22), 34.21 (C2), 32.52 (C7), 30.68 (C21), 28.04 (C15), 26.57 (C23), 24.22 (C16), 23.48 (C27), 23.48 (C11), 21.53 (C24), 21.20 (C30), 19.62 (C6), 17.09 (C29), 16.90 (C26), 15.21 (C25); HRMS *m/z*, calcd. for $C_{31}H_{48}O_3Na$: 491.7082, found: 491.3449.

Methyl 3,11-dioxours-12-en-28-oate (UAD**11**)

The derivative UAD**10** (100 mg, 0.21 mmol) was submitted to oxidation with $NaClO_2$/*t*-BuOOH for 12 h. After 12 h of reaction, aqueous solution was added of 10% Na_2SO_3 and extracted with EtOAc. The organic phase was washed successively with saturated aqueous $NaHCO_3$ solution and water, dried on anhydrous Na_2SO_4 and the solvent was removed in a rotary evaporator to give the white solid UAD**11** (91 mg, 88.4% yield). FTIR (ATR) ν_{max}/cm^{-1} 2928 (C-H), 1726 (C=O), 1658 (C=O), 1199 (C-O); $[\alpha]_D^{25}$ +57.10 (*c* 1.00, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.61 (s, 1H, H12), 3.60 (s, 3H, COOC$\underline{H}_3$), 2.37 (s, 1H, H9), 1.27 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.24 (d, 3H, CH_3), 1.22 (d, 3H, *J* 6.2 Hz, CH_3), 1.05 (d, 3H, *J* 7.1 Hz, CH_3), 0.85 (d, 1H, *J* 6.1 Hz, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 217.35 (C3), 199.23 (C11), 177.25 (C28), 163.43 (C13), 130.61 (C12), 60.78 (C9), 55.41 (C18), 52.81 (C5), 51.96 (COO$\underline{C}H_3$), 47.70 (C17), 47.70 (C4), 44.54 (C14), 43.88 (C8), 39.76 (C1), 38.66 (C19), 38.66 (C20), 36.79 (C10), 35.94 (C22), 34.29 (C2), 32.41 (C7), 30.32 (C21), 28.48 (C15), 26.46 (C23), 23.88 (C16), 23.88 (C6), 21.46 (C27), 21.02 (C30), 21.02 (C24), 18.78 (C26), 15.58 (C25); HRMS *m/z*, calcd. for $C_{31}H_{46}O_4Na$: 505.6918, found: 507.3442.

28-Methoxy-11,28-dioxo-3,4-secours-12-ene-3,4-lactone (UAD**12**)

Derivative UAD**11** (50 mg, 0.10 mmol) was dissolved in CH_2Cl_2 (5 mL), following the addition of 77% *meta*-chloroperoxybenzoic acid (MCPBA) (55.3 mg, 0.25 mmol) and $NaHCO_3$ (147.2 mg, 1.75 mmol). After 24 h under magnetic stirring at room temperature, an additional quantity of 77% MCPBA (47.3 mg, 0.21 mmol) was added. The reaction mixture was maintained under the same conditions for an additional 16 h. EtOAc was added to the mixture, and it was sequentially washed with saturated aqueous $NaHCO_3$ and NaCl solutions, dried over anhydrous Na_2SO_4, and evaporated to dryness. The crude product (40.4 mg) was filtered over silica gel (eluent *n*-hexane/EtOAc 1:1) to afford UAD**12** (24.9 mg, 48.2% yield). FTIR (ATR) ν_{max}/ cm^{-1} 2923 (C-H), 1721 (C=O), 1654 (C=O), 1232 (C-O), 1030 (C-O); $[\alpha]_D^{25}$ +135.00 (*c* 0.5, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.65 (s, 1H, H12), 3.61 (s, 3H, COOC$\underline{H}_3$), 2.61 (m, 2H, H2a and H2b), 2.43 (d, 1H, *J* 11.1 Hz, H18), 1.47 (s, 3H, CH_3), 1.44 (s, 3H, CH_3), 1.37 (s, 3H, CH_3), 1.30 (s, 1H, CH_3), 0.98 (s, 1H, CH_3), 0.96 (s, 3H, CH_3), 0.88 (s, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 198.53 (C11), 177.21 (C3), 175.67 (C28), 163.21 (C13), 130.79 (C12), 85.66 (C4), 61.11 (C9), 54.45 (C5), 52.73 (C18), 51.96 (COOC$\underline{H}_3$), 47.74 (C17), 44.61 (C14), 43.99 (C8), 39.69 (C10), 38.88 (C1), 38.70 (C20), 38.70 (C19), 35.98 (C22), 32.34 (C7), 32.34 (C2), 30.35 (C21), 28.37 (C15), 26.05 (C24), 23.92 (C16), 23.92 (C6), 22.12 (C27), 20.98 (C30), 18.41 (25), 17.49 (C29), 17.12 (C26); HRMS *m/z*, calcd. for $C_{31}H_{46}O_5Na$: 521.6912, found: 521.3265.

3β-Acetoxyurs-12-en-28-oic acid (UAD**13**)

Derivative UAD**13** was prepared under standard acetylation conditions using acetic anhydride in pyridine. After 24 h of reaction, ice was added ground and stirred for 15 min. The reaction mixture was extracted with EtOAc and washed with 0.5 N HCl. The organic phase was

dried over anhydrous Na_2SO_4 and the solvent eliminated in rotary evaporator being obtained as white crystals (194.8 mg, 89.2% yield). FTIR (ATR) ν_{max}/ cm^{-1} 3661 (O-H), 2924 (C-H), 1733 (C=O), 1694 (C=O), 1242 (C-O), 1455 (C-O-H), 1369 (C-O); $[\alpha]_D^{25}$ +54.30 (*c* 1.00, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.29 (t, 1H, *J* 3.6 Hz, H12), 4.49 (m, 1H, H3), 2.17 (d, 1H, *J* 11.5 Hz, H18), 2.04 (s, 1H, $OCOCH_3$), 1.08 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.86 (d, 3H, *J* 6.1 Hz, CH_3), 0.86 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.80 (s, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.96 (C28), 171.12 ($OCOC\underline{H}_3$), 137.42 (C13), 126.24 (C12), 80.96 (C3), 55.35 (C5), 52.70 (C18), 49.35 (C17), 47.52 (C9), 42.22 (C14), 39.65 (C8), 39.14 (C19), 38.77 (C20), 38.40 (C1), 37.74 (C4), 36.90 (C10), 35.83 (C22), 33.04 (C7), 30.50 (C21), 28.11 (C23), 28.11 (C15), 24.25 (C16), 23.59 (C11), 23.59 (C2), 23.37 (C27), 21.39 ($COCOC\underline{H}_3$), 21.16 (C30), 18.22 (C6), 17.27 (C29), 17.01 (C26), 16.79 (C25), 15.62 (C24); HRMS *m/z*, calcd. for $C_{30}H_{50}O_2Na$: 465.7136, found: 465.3730.

(13*S*)-3β-Acetoxyurs-11-ene-28,13-lactone (UAD**14**)

The oxidation of derivative UAD**13** (54 mg, 0.10 mmol) with $NaClO_2$/*t*-BuOOH for 12 h afforded the white solid 3β-acetoxy-11-oxours-12-en-28-oic acid (50 mg, 90.1% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.56 (s, 1H, H12), 4.48 (dd, 1H, *J* 5.5, 10.9 Hz, H3), 3.57 (s, 3H, $COOC\underline{H}_3$), 2.29 (s, 1H, H9), 2.02 (s, 3H, $OCOC\underline{H}_3$), 1.22 (s, 3H, CH_3), 1.13 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 0.88 (s, 3H, CH_3). Then, the reduction of 3β-acetoxy-11-oxours-12-en-28-oic acid with $LiAlH_4$ in diethyl ether, at 0 ºC for 1 h, was followed by acetylation with acetic anhydride in pyridine and esterification with CH_3I/K_2CO_3. The final reaction product (43.3 mg) was chromatographed over silica gel (eluents *n*-hexane/EtOAc 9:1, 8:2 and 6:4) to afford UAD**14** (10 mg, 23% yield). FTIR (ATR) ν_{max}/cm^{-1} 2923 (C-H), 1755 (C=O), 1728 (C=O), 1238 (C-O); $[\alpha]_D^{25}$ +41.00 (*c* 0.5, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.95 (dd, 1H, *J* 1.5, 10.4 Hz, H12), 5.54 (dd, 1H, *J* 3.0, 10.4 Hz, H11), 4.50 (m, 1H, H3), 2.05 (s, 3H, $COOC\underline{H}_3$), 1.16 (s, 3H, CH_3), 1.15 (s, 3H, CH_3), 1.05 (s, 3H, CH_3), 1.00 (d, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.86 (s, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 180.00 (C28), 171.11 ($O\underline{C}OCH_3$), 133.36 (C12), 128.95 (C11), 89.70 (C13), 80.66 (C3), 60.59 (C18), 54.86 (C5), 52.96 (C9), 45.13 (C17), 41.97 (C14), 41.75 (C8), 38.70 (C19), 38.15 (C20), 38.00 (C1), 37.89 (C4), 36.31 (C10), 31.35 (C7), 31.20 (C22), 30.87 (C21), 27.78 (C23), 25.58 (C15), 23.37 (C2), 22.86 (C16), 21.39 ($COO\underline{C}H_3$), 19.22 (C30), 18.96 (C26), 18.04 (C25), 17.93 (C29), 17.60 (C6), 16.09 (27), 16.09 (C24); HRMS *m/z*, calcd. for $C_{32}H_{48}O_4Na$: 519.7186, found: 519.3459.

3-Hydroxy-oxime-urs-12-en-28-oato (UAD**15**)

Derivative UAD**1** (500 mg, 1.06 mmol) was dissolved in CH_2Cl_2 (20 mL), following the addition of PDC (716 mg, 3.3 mmol). The reaction mixture was maintained for 3 h under magnetic stirring at room temperature. The crude product was filtered over silica gel (eluent *n*-hexane/EtOAc 1:1) to afford methyl 3-oxours-12-en-28-oate (371.9 mg). Then, methyl 3-oxours-12-en-28-oate (100 mg, 0.21 mmol) was dissolved in EtOH (10 mL) following the addition of anhydrous pyridine (0.1 mL) and $(NH_3OH)Cl$ (25 mg, 0.36 mmol). The reaction mixture was maintained for 3 h under magnetic stirring at reflux and 95-100 ºC temperature. The EtOH was evaporated and EtOAc was added to the mixture, than it was sequentially washed with saturated aqueous NaCl solution, dried over anhydrous Na_2SO_4, evaporated to dryness to afford UAD**15** (98 mg). FTIR (ATR) ν_{max}/ cm^{-1} 3660 (O-H), 2945 (C-H), 1721 (C=O), 931 (N-O); $[\alpha]_D^{25}$ +22.00 (*c* 1.00, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.24 (sl, 1H, H12), 3.59 (s, 3H, $COOCH_3$), 3.06 (m, 1H, H2b), 2.22 (m, 1H, H2a), 2.22 (d, 1H, *J* 11 Hz, H18), 1.14 (s, 3H, $C27H_3$), 1.04 (s, 3H, $C25H_3$), 1.02 (d, 3H, $C23H_3$), 0.93 (s, 3H, $C30H_3$), 0.84 (d, 3H, *J* 6.2 Hz, $C29H_3$), 0.76 (s, 3H, $C26H_3$), 0.76 (s, 3H, $C24H_3$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 178.10 (C28), 166.85 (C3), 138.26 (C13), 125.51 (C12), 55.82 (C18), 52.96 (C5), 51.52 ($COOCH_3$), 44.14 (C17), 47.11 (C9), 42.11 (C14), 40.35 (C4), 39.58 (C8), 39.06 (C19), 38.88 (C20), 38.66 (C1), 37.04 (C10), 36.64 (C22), 32.74 (C7), 30.68 (C2), 30.68 (C21), 28.00 (C15), 27.34 (C23), 24.25 (C16), 24.25 (C11), 23.33 (C27), 21.20 (C24), 21.20 (C30), 19.07 (C6), 17.01 (C26), 17.01 (C29); HRMS *m/z*, calcd. for $C_{32}H_{48}O_4Na$: 506.3610, found: 507.3305.

3β-Hydroxy-11-oxours-12-en-28-oate (UAD**16**)

Derivative methyl 3-oxours-12-en-28-oate (UAD**10**, 100 mg, 0.21 mmol) was dissolved in EtOAc (5 mL), following the addition of 0.2 mL of *t*-BuOOH 6 M at *n*-decane (1.20 mmol) and $NaClO_2$ (66 mg; 0.73 mmol). The reaction mixture was maintained for 12 h under magnetic stirring at reflux and 100-110 ºC temperature. After this time, Na_2SO_3 aqueous solution 10% was added and extracted with EtOAc. The organic phase was washed successively with saturated aqueous $NaHCO_3$ solution and water; dried over anhydrous Na_2SO_4 and the solvent was removed on a rotary evaporator to afford 3,11-dioxours-12-en-28-oate (91 mg). Then, the derivative 3,11-dioxours-12-en-28-oate (UAD**11**, 50 mg, 0.10 mmol) was dissolved in 5 mL of the mixture of MeOH/THF (1:1) following the addition of $CeCl_3.7H_2O$ (164 mg; 0.44 mmol) and 22 mg of $NaBH_4$ (0.57 mmol). The reaction mixture was maintained for 6 h under magnetic stirring at room temperature. The

crude product was filtered over silica gel (eluent *n*-hexane/EtOAc 1:1) to afford methyl 3-oxours-12-en-28-oate (371.9 mg). The solvent was evaporated, added Et_2O and it was washed with saturated aqueous NaCl solution, dried over anhydrous Na_2SO_4, evaporated to dryness to afford UAD**16** (32.25 mg). FTIR (ATR) ν_{max} / cm^{-1} 3675 (O-H), 2970 (C-H), 1725 (C=O), 1659 (C=O); $[\alpha]_D^{25}$ +108.57 (*c* 0.7, $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 5.60 (s, 1H, H12), 3.61 (s, 3H, $COOCH_3$), 3.22 (m, 1H, H3), 2.80 (m, 1H, H2a), 2.75 (m, 1H, H2b), 2.42 (d, 1H, *J* 11.2 Hz, H18), 2.30 (s, 1H, H9), 1.12 (s, 3H, $C27H_3$), 0.99 (s, 3H, $C25H_3$), 0.96 (d, 3H, *J* 5.0 Hz, $C30H_3$), 0.90 (s, 3H, $C23H_3$), 0.86 (d, 1H, *J* 6.1 Hz, $C29H_3$), 0.79 (s, 3H, $C26H_3$), 0.79 (s, 3H, $C24H_3$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 200.01 (C11), 177.29 (C28), 162.96 (C13), 130.73 (C12), 78.83 (C3), 61.52 (C9), 55.01 (C18), 52.76 (C5), 51.92 ($COOCH_3$), 47.70 (C17), 44.72 (C14), 43.77 (C8), 39.17 (C1), 38.66 (C19), 38.66 (C20), 37.19 (C10), 37.19 (C4), 36.01 (C22), 33.07 (C7), 30.35 (C21), 28.44 (C15), 28.15 (C23), 27.34 (C2), 23.99 (C16), 21.05 (C30), 21.05 (C27), 18.89 (C26), 17.49 (C6), 17.16 (C24), 16.28 (C29), 15.65 (C25); HRMS *m/z*, calcd. from $C_{31}H_{44}O_4$ [M + Na]: 503.3137, found: 503.3442.

3-Oxo,11-hydroxyurs-12-en-28-oate (UAD**17**)

To a solution of the derivative 3-oxours-12-en-28-oate (50 mg, 0.11 mmol) in 4.0 mL of CH_2Cl_2 was added 5,10,15,20-tetrakis (pentafluorophenyl) porphyrin (3.5 mg, 3 mol%) and MCPBA 55% (44.32 mg, 0.26 mmol). The reaction mixture was maintained under stirring and inert atmosphere at −78 °C for 30 h. At the end of this period, saturated aqueous solutions of $NaHCO_3$ and $Na_2S_2O_3$ were added, followed by extraction with EtOAc. The organic phase was washed with saturated aqueous NaCl solution, dried over anhydrous Na_2SO_4 and the solvent removed on a rotary evaporator, yielding 44.9 mg of a residue. This was chromatographed on a silica gel column (packed column dimensions: 10 × 220 mm) using *n*-hexane:EtOAc mixtures (9:1; 8:2; 1:1) as eluents. The fractions were pooled according to the profiles at three groups: starting material (the derivative 3-oxours-12-en-28-oate, 17.4 mg) was recovered from the first combined fractions group, while the second combined fractions group provided 13.3 mg (30%) of the derivative 3,11-dioxours-12-en-28-oate, and the third combined fractions group provided 25.1 mg of the UAD**17** (56%) mixture of isomers. FTIR (ATR) ν_{max} / cm^{-1} 3646 (O-H), 2921 (C-H), 1726 (C=O), 1706 (C=O), 1241 (C-O); $[\alpha]_D^{25}$ +65.10 (*c* 1.00, $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 5.30 (d, 1H, *J* 3.6 Hz, H12), 4.29 (dd, *J* 3.6 and 8.8 Hz, 1H, H11), 3.61 (s, 3H, $COOCH_3$), 2.27 (d, 1H, *J* 10.4 Hz, H18), 1.10 (s, 3H, $C27H_3$), 1.06 (s, 3H, $C25H_3$), 0.96 (d, 3H, *J* 5.3 Hz, $C30H_3$), 0.94 (s, 3H, $C23H_3$), 0.91 (s, 3H, $C26H_3$), 0.88 (d, 3H, *J* 6.4 Hz, $C29H_3$), 0.84 (s, 3H, $C24H_3$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 217.91 (C3), 178.03 (C28), 139.88 (C13), 129.55 (C12), 68.27 (C11), 55.62 (C9), 52.18 (C5), 51.78 (C18), 51.71 ($COOCH_3$), 47.74 (C17), 47.37 (C4), 42.89 (C8), 42.89 (C14), 40.68 (C1), 38.73 (C19), 38.73 (C20), 38.26 (C10), 36.42 (C22), 34.54 (C7), 34.43 (C2), 30.57 (C21), 28.07 (C15), 26.94 (C23), 24.14 (C16), 23.44 (C27), 21.31 (C30), 19.69 (C6), 18.78 (C29), 18.22 (C26), 17.16 (C24), 17.01 (C25); HRMS *m/z*, calcd. for $C_{31}H_{44}O_4$ [M + Na]: 503.3137, found: 503.3453.

3β,28-Dihydroxyurs-9,12-diene (UAD**18**)

In a suspension of $LiAlH_4$ (62 mg, 1.63 mmol) and 2.0 mL of dry ethyl ether was added the derivative 3,11-dioxours-12-en-28-oate (62 mg, 0.13 mmol), solubilized in 4.0 mL of dry ethyl ether. The reaction mixture was kept under stirring at room temperature for 4 h. At the end of this period, the excess $LiAlH_4$ was removed by dripping with wet ether followed by water trickling. 2 mol L^{-1} HCl was added, extracted with EtOAc, and the organic phase was washed with saturated aqueous NaCl solution. The organic phase was dried over anhydrous Na_2SO_4, and after removal of the solvent on a rotary evaporator, 44 mg of a residue was obtained. This was subjected to fractionation on a silica gel column, using the *n*-hexane:EtOAc (8:2 and 1:1) and EtOAc as eluents. The fractions were pooled according to the profiles and the derivative UAD**18** (5.2 mg, 9%) was obtained from the first fractions group. FTIR (ATR) ν_{max} / cm^{-1} 3662 (O-H), 2941 (C-H), 1690 (C=C), 1240 (C-O); $[\alpha]_D^{25}$ +7.80 (*c* 3.5, $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 5.58 (d, 1H, *J* 6.0 Hz, H11), 5.51 (d, 1H, *J* 6.0 Hz, H12), 3.60 (d1, 3H, *J* 11 Hz, H28a), 3.24 (d, 1H, *J* 11 Hz, H28b), 3.22 (m, 1H, H3), 1.21 (s, 3H, $C27H_3$), 1.16 (s, 3H, $C25H_3$), 1.03 (s, 3H, $C23H_3$), 0.94 (d, 3H, *J* 5 Hz, $C30H_3$), 0.95 (s, 3H, $C26H_3$), 0.83 (d, 3H, *J* 5 Hz, $C29H_3$), 0.82 (s, 3H, $C24H_3$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 155.09 (C9), 140.19 (C13), 123.45 (C12), 115.29 (C11), 78.76 (C3), 70.21 (C28), 52.37 (C18), 51.12 (C5), 43.11 (C8), 40.72 (C14), 39.36 (C19), 38.73 (C20), 38.07 (C4), 38.07 (C17), 37.30 (C22), 35.17 (C1), 31.93 (C7), 30.61 (C21), 29.77 (C15), 28.26 (C23), 27.93 (C2), 25.47 (C16), 25.47 (C27), 21.53 (C30), 18.33 (C6), 17.66 (C26), 17.31 (C29), 15.69 (C25), 15.69 (C24); HRMS *m/z*, calcd. for $C_{30}H_{44}O_2$ [M + Na]: 459.3239, found: 459.3553.

Cell culture

RAW264.7 macrophage cell lines were placed in 96-well plates, containing RPMI-1640 supplemented (2.0 mM L-glutamine, 100.0 µg mL^{-1} of streptomycin and penicillin, 5% fetal bovine serum), at 1 × 10^6 cells mL^{-1}

or 2 × 10^5 cells mL^{-1} by 3 or 48 h, respectively. Cells were incubated at 37 °C in a 5% CO_2 atmosphere in the presence or absence of the UA or ursolic acid derivatives (UAD**1**, UAD**2**, UAD**3**, UAD**4**, UAD**5**, UAD**6**, UAD**7**, UAD**8**, UAD**9**, UAD**10**, UAD**11**, UAD**12**, UAD**13**, UAD**14**, UAD**15**, UAD**16**, UAD**17** or UAD**18**) at 15, 30, 60 or 90 µM. The compounds have been solubilized in DMSO, never exceeding 0.1% (v/v), and diluted in RPMI-1640 before the use. The DMSO concentration was determined to allow the solubilization of the UAD and UA but without affecting the RAW264.7 viability.[18]

MTT assay

Cellular viability was measured using the MTT assay. After 48 h of culture, the supernatants were removed and the cells were incubated with 100 µL of supplemented RPMI-1640 and 10 µL of MTT (5.0 mg mL^{-1}), during 4 h at 37 ºC in a 5% CO_2 atmosphere. After purple formazan crystal formation, the supernatants were gently removed and crystal products were solubilized with DMSO. Complete solubilization was obtained by shaking the plates. The optical density (OD) values were determined in the Multiskan microplate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific, Waltham, MA, USA) at 560 nm wavelength. The cellular viability was presented as the half maximal inhibitory concentration (IC_{50}) calculated using Prism software (GraphPad Prism 5.00, San Diego, CA, USA).[28]

Nitric oxide concentration in RAW264.7

The NO concentration was measured by the Griess method, in the supernatant of the 48 h of culture. To perform the test, 100 µL of the supernatant from each well was transferred to 96-well plates and an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine hydrochloride, 5% H_3PO_4) was added. The NO concentration was determined by comparison with a standard sodium nitrite solution and the values were determined by the Multiskan microplate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific, Waltham, MA, USA) at 540 nm wavelength.

NF-κB concentration in RAW264.7

The cells that were cultured for 3 h were detached after this period and stained for the analysis of the p65 expression (indirectly NF-κB),[27] following the instructions of the manufacturer. The cells were acquired in the FACSVerse (BD, Biosciences Pharmingen, San Diego, CA, USA) and analysed in the FCS Express software (De Novo software, Pasadena, CA, USA).[29]

Induction of acute inflammation by carrageenan-induced paw edema

BALB/c (female) mice 6-8 weeks old (n = 6 *per* group) were obtained from the animal care facilities of the Federal University of Minas Gerais, Belo Horizonte, MG, Brazil, and maintained in micro isolator cages. All procedures were in accordance with the principles of the Brazilian Code for the Use of Laboratory Animals and were approved by the committee on the use of laboratory animals from UFVJM (Protocol No. 03/2018r). The mice were monitored for clinical signs of toxicity after the treatments. Initially, the mice were weighed (26.57 ± 0.31 g) and their right and left paws were measured (0 h) with a pachymeter (Mitutoyo, Kawasaki, Japan). The treatments, phosphate buffered saline (PBS), dexamethasone (0.5 mg kg^{-1}), UA (200 mg kg^{-1}), UAD **1** (200 mg kg^{-1}) and UAD**2** (200 mg kg^{-1}), were administered intraperitoneally (100 µL) 30 min before the induction of the edema. Dexamethasone was used as a positive control treatment due to its anti-inflammatory activity. Carrageenan (2.5%) was dissolved in PBS, and 20 µL injected into the left footpad, and 20 µL of PBS into the right footpad of all groups. The left and the right paws were measured after 1, 2, 3 and 4 h after the injection of carrageenan and the differences were calculated. The magnitude of the carrageenan-induced paw edema was determined as follows: [paw edema / mm] = [footpad thickness of carrageenan / mm] – [footpad thickness of PBS / mm].[30]

Cytokine production

Cytokine production was assayed by enzyme-linked immunosorbent assay (ELISA) using commercially available antibodies according to the instructions of the manufacturer (BD Biosciences Pharmingen, San Diego, CA, USA). The plates were read at the Multiskan microplate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific, Waltham, MA, USA) at 450 nm wavelength.[18] Supernatants of the 48 h macrophage culture were used to evaluate the TNF. The TNF production (%) was calculated using the formula (X1/X2) × 100, considering X1 the TNF production (pg mL^{-1}) of stimulated and treated cells and X2 the mean TNF production (pg mL^{-1}) of stimulated and untreated cells. The IL-6 productions were measured in the mice paw and lymph nodes, after euthanasia. Paw tissue and lymph nodes were removed and homogenized (100 mg mL^{-1}) in the extraction solution containing 0.4 M NaCl, 0.05%

Tween 20, 0.5% BSA, 0.1 M PMSF, 0.1 M benzethonium chloride, 10 mM EDTA and 20 kIU mL^{-1} aprotinin. The homogenate was centrifuged at 2000 ×g for 15 min at 4 °C and supernatants were collected to determine the concentration of IL-6.

Statistical analysis

The results represent at least three independent experiments and are presented as the mean ± standard error of the mean (SEM). All data were analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni posttests (GraphPad Prism 5.00, San Diego, CA, USA),[28] and the differences were considered significant at $p < 0.05$.

Results and Discussion

The ten UA semisynthetic derivative compounds were synthesized (Figures 1 and 2) with previously described structural modifications. The synthesis of derivatives UAD**1** to UAD**8** was previously published by Scherrer *et al.*[27] The structures were elucidated by NMR spectra recorded in $CDCl_3$ on a Bruker AC200 at 200 MHz for ^{1}H and 50 MHz for ^{13}C. The obtained spectra of the compounds correspond to the data in the literature.[31,32] Spectral data of the methyl 3β-hydroxyurs-12-en-28-oate (UAD**1**), methyl 3β-acetoxy-11-oxours-12-en-28-oate (UAD**2**), methyl 3β-acetoxy-11-oxours-12-en-28-oate (UAD**3**) and methyl 3β-acetoxyurs-9(11), 12-dien-28-oate (UAD**4**), urs-12-ene-3β,11,28-triol (UAD**5**), (17*S*)-3β-hydroxy-22 (17 → 18)-abeours-11-en-28-al (UAD**6**), (urs-11, 13(18)-diene-3β, 28-diyl diacetate) (UAD**7**), (13*S*)-13, 28-epoxyurs-11-en- 3β-ol (UAD**8**) were previously published.[27]

Biological activity

The cytotoxicity index (IC_{50}) and NO production by RAW264.7 macrophages, treated with UA or the UADs, were shown in the Table 1. The IC_{50} was obtained by the MTT test of the unstimulated cells treated with UA or UADs and the respective controls corresponding to untreated cells. Besides the viability, the Table 1 shows the results of NO production by RAW264.7 macrophages treated with UA or the UADs, stimulated with LPS and interferon (IFN)-γ after 48 h of culture.

The compounds UA, UAD**1**, UAD**2**, UAD**3**, UAD**6**, UAD**8** and UAD**9** were able to reduce the NO production, compared to the untreated stimulated RAW264.7 cells at all tested concentrations. The compounds UAD**4**, UAD**5**, UAD**7**, UAD**10**, UAD**17** and UAD**18** reduced the NO production at 30, 60 and 90 µM. Only the UAD**12** and UAD**15** were not able to reduce NO production (Table 1). The IC_{50} was greater than 90 µM when the cells were treated with UAD**2**, UAD**3**, UAD**4**, UAD**7**, UAD**9**, UAD**10**, UAD**11**, UAD**14** and UAD**15**. The UAD**1** was the closest to the IC_{50} of the UA (Table 1).

The percentage of NF-κB expression by RAW264.7 was shown in Table 2. The UA, UAD**1**, UAD**2**, UAD**3**, UAD**4**, UAD**5**, UAD**6**, UAD**7**, UAD**8**, UAD**11**, UAD**12**,

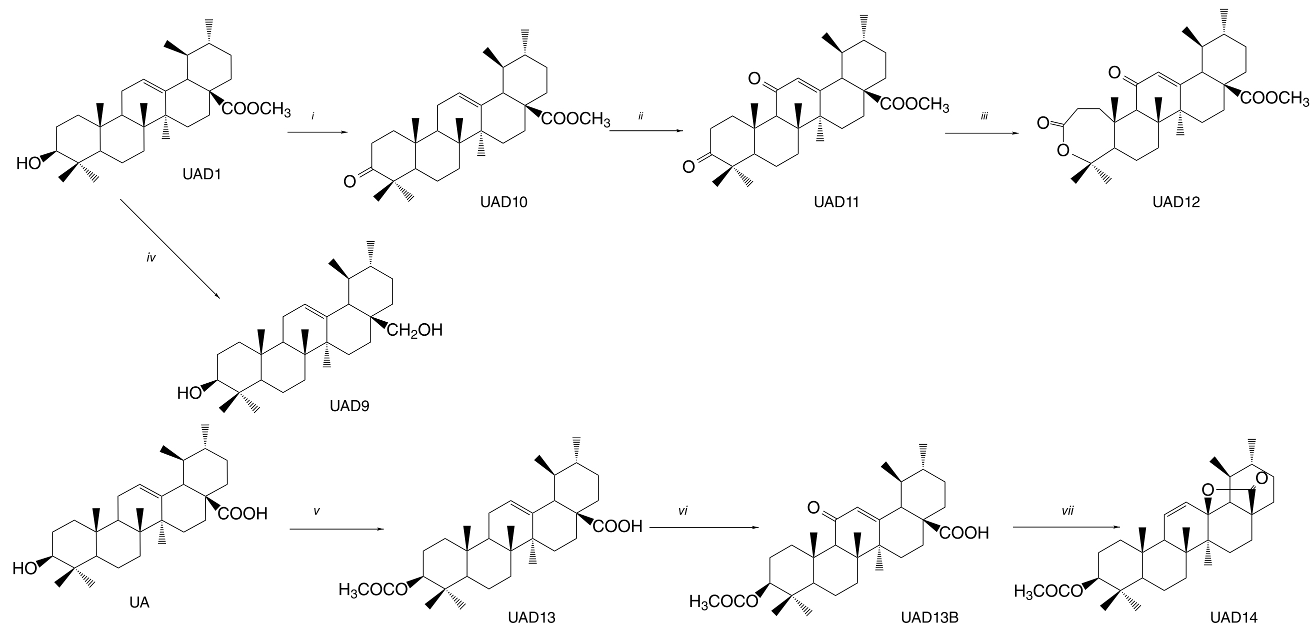

Figure 1. Synthesis of the ursolic acid derivatives. Reaction conditions *i*: pyridinium dichromate (PDC), room temperature; *ii*: $NaClO_2$/*t*-BuOOH; *iii*: MCPBA/$NaHCO_3$, room temperature; *iv*: $LiAlH_4$, room temperature; *v*: acetic anhydride/pyridine, room temperature; *vi*: $NaClO_2$/*t*-BuOOH, 100 °C; *vii*: $LiAlH_4$, 0 °C; acetic anhydride/pyridine; CH_3I/K_2CO_3, room temperature.

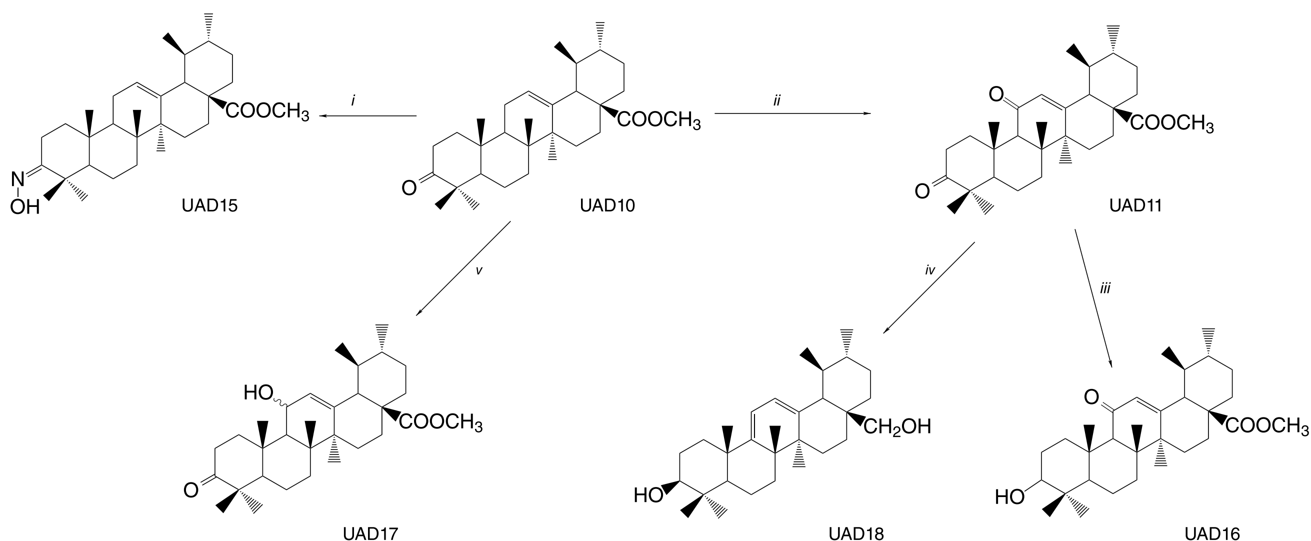

Figure 2. Synthesis of the ursolic acid derivatives. Reaction conditions *i*: pyridine/$(NH_3OH)Cl$, 95-100 °C; *ii*: EtOAc/*t*-BuOOH, *n*-decane/$NaClO_2$/Na_2SO_3, 100-110 °C (reflux); *iii*: MeOH/THF, $CeCl_3.7H_2O$/$NaBH_4$, room temperature; *iv*: $LiAlH_4$/diethyl ether, room temperature; *v*: 5,10,15,20-tetrakis(pentaflu orophenyl) porphyrin/MCPBA, −78 °C.

Table 1. NO production by RAW264.7 macrophages treated with UA or UAD, after 48 h of culture and cytotoxicity index (IC_{50})

Compound[a]	NO production / µM				
	15 µM[b]	30 µM[b]	60 µM[b]	90 µM[b]	IC_{50} / µM
UA	9.65 ± 0.51[c]	3.05 ± 1.16[c]	1.99 ± 0.15[c]	2.12 ± 0.83[c]	17.06
UAD**1**	3.98 ± 0.44[c]	4.69 ± 0.07[c]	2.63 ± 0.37[c]	1.66 ± 0.07[c]	9.64
UAD**2**	8.05 ± 0.31[c]	8.55 ± 0.47[c]	6.69 ± 1.10[c]	4.75 ± 0.61[c]	> 90
UAD**3**	9.99 ± 1.85[c]	9.13 ± 1.03[c]	7.96 ± 1.33[c]	6.10 ± 1.76[c]	> 90
UAD**4**	14.32 ± 0.63	10.47 ± 0.93[c]	8.05 ± 1.35[c]	8.94 ± 0.27[c]	> 90
UAD**5**	11.41 ± 1.33	8.39 ± 1.10[c]	2.98 ± 0.38[c]	4.56 ± 0.27[c]	39.89
UAD**6**	10.72 ± 1.63[c]	10.75 ± 0.19[c]	4.08 ± 0.72[c]	0.93 ± 0.21[c]	74.62
UAD**7**	11.51 ± 2.40	8.18 ± 2.22[c]	7.09 ± 0.99[c]	5.83 ± 1.53[c]	> 90
UAD**8**	10.79 ± 1.03[c]	9.85 ± 0.60[c]	3.63 ± 0.53[c]	3.46 ± 0.70[c]	47.25
UAD**9**	10.98 ± 1.75[c]	10.87 ± 0.67[c]	5.95 ± 0.30[c]	5.51 ± 0.95[c]	> 90
UAD**10**	12.19 ± 1.11	9.86 ± 0.87[c]	5.96 ± 0.15[c]	6.05 ± 2.12[c]	> 90
UAD**11**	10.11 ± 2.37	10.28 ± 2.82	9.15 ± 0.08[c]	9.89 ± 1.26[c]	> 90
UAD**12**	21.06 ± 1.02	13.23 ± 1.55	12.77 ± 1.21	12.55 ± 0.54	88.76
UAD**13**	20.44 ± 3.29	15.32 ± 4.58	13.32 ± 1.35	8.57 ± 0.75[c]	34.60
UAD**14**	16.33 ± 1.41	13.77 ± 1.89	10.98 ± 1.61	7.25 ± 1.92[c]	> 90
UAD**15**	14.76 ± 0.34	17.34 ± 2.24	10.82 ± 1.70	10.59 ± 2.22	> 90
UAD**16**	13.59 ± 0.57	10.28 ± 4.06	9.02 ± 0.07[c]	7.62 ± 1.83[c]	24.60
UAD**17**	12.46 ± 2.61	10.45 ± 0.63[c]	8.91 ± 1.26[c]	6.01 ± 0.42[c]	80.05
UAD**18**	12.46 ± 1.38	10.52 ± 1.35	8.58 ± 1.30[c]	6.98 ± 0.56[c]	63.40

[a]Compounds: ursolic acid (UA) and ursolic acid derivative (UAD); [b]treatment concentration; [c]$p < 0.05$ in relation to the nitric oxide (NO) production of the untreated stimulated RAW264.7 cells (14.77 ± 2.52). IC_{50}: half maximal inhibitory concentration.

UAD**13**, UAD**14**, UAD**15** and UAD**18** inhibited the NF-κB percentage expression at all concentrations tested. The UA, UAD**1**, UAD**2**, UAD**3** showed a dose-dependent inhibition (Table 2).

The percentage of TNF produced by RAW264.7 was inhibited by the UAD**7**, UAD**12**, UAD**13**, UAD**14**, UAD**15** and UAD**18** in all concentrations tested. The UAD**1**, UAD**10**, UAD**11** and UAD**16** reduced the TNF production in concentrations above 15 µM. All the compounds reduced the production of TNF at 60 and 90 µM (Table 3).

A study[33] has already shown that NF-κB is a well-known transcription factor in the inflammatory process,

Table 2. Percentage of the NF-κB expression by RAW264.7 macrophages treated with UA and UAD, after 3 h of culture

Compound[a]	NF-κB expression / %			
	15 μM[b]	30 μM[b]	60 μM[b]	90 μM[b]
UA	13.59 ± 0.29[c]	7.91 ± 0.11[c]	8.15 ± 0.31[c]	6.95 ± 0.20[c]
UAD**1**	12.77 ± 0.32[c]	11.98 ± 0.51[c]	9.65 ± 0.08[c]	3.03 ± 0.11[c]
UAD**2**	15.11 ± 0.28[c]	14.78 ± 0.19[c]	11.27 ± 0.32[c]	8.45 ± 0.08[c]
UAD**3**	15.06 ± 0.15[c]	14.45 ± 0.09[c]	10.43 ± 0.22[c]	8.76 ± 0.20[c]
UAD**4**	9.77 ± 0.37[c]	11.11 ± 0.20[c]	9.17 ± 0.26[c]	10.47 ± 0.16[c]
UAD**5**	15.42 ± 0.20[c]	12.22 ± 0.23[c]	12.75 ± 0.18[c]	13.96 ± 0.24[c]
UAD**6**	12.08 ± 0.12[c]	12.08 ± 0.06[c]	13.97 ± 0.16[c]	13.93 ± 0.27[c]
UAD**7**	12.09 ± 0.11[c]	11.65 ± 0.16[c]	11.10 ± 0.18[c]	11.84 ± 0.14[c]
UAD**8**	13.64 ± 0.19[c]	15.12 ± 0.16[c]	14.96 ± 0.12[c]	14.54 ± 0.15[c]
UAD**9**	16.15 ± 0.48	16.27 ± 0.19	11.75 ± 0.12[c]	15.52 ± 0.24[c]
UAD**10**	14.66 ± 0.23[c]	17.73 ± 0.29	16.67 ± 0.34[c]	18.40 ± 0.35[c]
UAD**11**	12.71 ± 0.27[c]	14.81 ± 0.29[c]	15.26 ± 0.31[c]	14.04 ± 0.17[c]
UAD**12**	9.04 ± 0.55[c]	13.66 ± 0.54[c]	11.29 ± 0.86[c]	11.98 ± 0.11[c]
UAD**13**	9.64 ± 0.23[c]	11.62 ± 0.24[c]	11.21 ± 0.23[c]	8.96 ± 0.09[c]
UAD**14**	11.07 ± 0.19[c]	9.968 ± 0.06[c]	11.79 ± 0.26[c]	9.37 ± 0.28[c]
UAD**15**	10.08 ± 0.17[c]	9.708 ± 0.19[c]	10.36 ± 0.18[c]	9.34 ± 0.19[c]
UAD**16**	22.69 ± 0.46	16.29 ± 0.21	15.66 ± 0.08	3.38 ± 0.11[c]
UAD**17**	17.20 ± 0.50	15.14 ± 0.23[c]	15.12 ± 0.29[c]	13.39 ± 0.10[c]
UAD**18**	11.27 ± 0.03[c]	11.72 ± 0.19[c]	12.12 ± 0.14[c]	11.49 ± 0.18[c]

[a]Compounds: ursolic acid (UA) and ursolic acid derivative (UAD); [b]treatment concentration; [c]$p < 0.05$ NF-κB expression by RAW264.7 macrophages treated with UA or UADs *versus* RAW264.7 not treated (NF-κB expression control: 17.24 ± 0.41). NF-κB: nuclear factor kappa B.

Table 3. Percentage of the TNF production by RAW264.7 macrophages treated with UA and UADs

Compound[a]	TNF production / %			
	15 μM[b]	30 μM[b]	60 μM[b]	90 μM[b]
UA	> 100	90.01 ± 8.08[c]	26.13 ± 5.98[c]	23.98 ± 2.09[c]
UAD**1**	> 100	74.55 ± 9.52[c]	21.07 ± 0.33[c]	17.98 ± 1.79[c]
UAD**2**	> 100	89.05 ± 6.69	31.73 ± 2.18[c]	30.41 ± 1.36[c]
UAD**3**	> 100	> 100	33.48 ± 2.08[c]	33.01 ± 2.72[c]
UAD**4**	> 100	91.13 ± 15.32	41.80 ± 2.78[c]	32.64 ± 4.11[c]
UAD**5**	> 100	> 100	48.02 ± 1.28[c]	32.38 ± 1.60[c]
UAD**6**	> 100	> 100	36.66 ± 2.82[c]	28.24 ± 1.03[c]
UAD**7**	67.02 ± 1.72[c]	37.97 ± 3.85[c]	15.78 ± 0.81[c]	14.82 ± 2.14[c]
UAD**8**	> 100	79.98 ± 12.35	19.55 ± 0.63[c]	14.43 ± 0.71[c]
UAD**9**	> 100	> 100	19.83 ± 0.63[c]	14.56 ± 1.83[c]
UAD**10**	84.01 ± 6.38	42.33 ± 0.96[c]	17.58 ± 1.13[c]	16.26 ± 0.69[c]
UAD**11**	77.09 ± 8.78	55.80 ± 3.63[c]	21.72 ± 1.52[c]	16.87 ± 0.56[c]
UAD**12**	49.87 ± 1.07[c]	19.48 ± 1.19[c]	6.50 ± 0.25[c]	4.71 ± 0.43[c]
UAD**13**	51.30 ± 2.07[c]	24.66 ± 1.48[c]	9.69 ± 0.55[c]	12.39 ± 1.03[c]
UAD**14**	53.41 ± 2.69[c]	16.34 ± 0.73[c]	10.92 ± 1.41[c]	10.81 ± 0.59[c]
UAD**15**	61.05 ± 3.85[c]	27.68 ± 2.01[c]	11.50 ± 1.14[c]	10.46 ± 0.60[c]
UAD**16**	93.81 ± 4.97	50.35 ± 1.39[c]	10.81 ± 0.92[c]	4.67 ± 0.46[c]
UAD**17**	> 100	> 100	74.88 ± 0.55[c]	40.87 ± 3.34[c]
UAD**18**	69.51 ± 1.20[c]	71.82 ± 4.95[c]	61.87 ± 4.40[c]	25.20 ± 2.09[c]

[a]Compounds: ursolic acid (UA) and ursolic acid derivative (UAD); [b]treatment concentration; [c]$p < 0.05$ percentage of the tumor necrosis factor (TNF) production by RAW264.7 macrophages treated with UA and UADs *versus* RAW 264.7 not treated (100%).

responsible for inducing the transcription of several pro- and anti-inflammatory mediator genes, including TNF. In the present work, the reduction in the expression of NF-κB was accompanied by the reduction in the production of TNF, probably due to the incorporated transformations to obtain the UADs, which improved the compounds capacities to act on the NF-κB, partly reported by other authors.[20,34] Also, these modifications generated UADs with lower cytotoxicity than UA, except for the UAD**1**.

The structural modification of the UAD**2**, consists of the esterification of both C-3 and C-28 carbons,[35] which may be the factor that improved its capacity to reduce NO, being the compound that best reduced NO at 90 μM, without altering cell viability. Also, it was able to inhibit the expression of NF-κB in a dose response manner and to reduce the production of TNF at concentrations of 60 and 90 μM.

The UAD**1** and UAD**2** were chosen to be used as a treatment on carrageenan-induced paw edema, for its similar results to UA in inhibiting inflammatory mediators, such as NF-κB and NO, at the lowest concentrations tested. However, it is possible to recognize the potential in others UAD that deserves to be tested in the inflammatory models.

Both, UAD**1** and UAD**2**, showed an effective reduction of paw edema of mice, as well as dexamethasone, compared to carrageenan group, who received only PBS treatment. The reduction in paw edema is already observed in the 2nd h and persists until the 4th h (Figure 3).

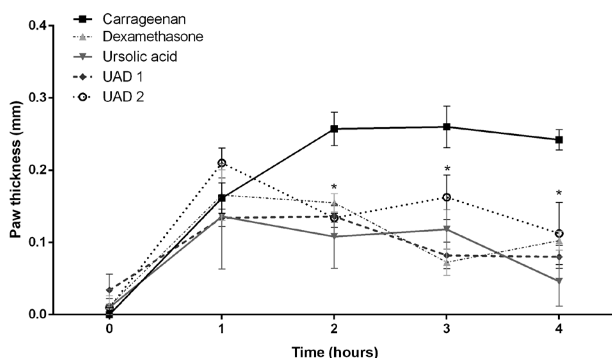

Figure 3. Carrageenan-induced paw edema. Female BALB/c mice (n = 6 *per* group) were treated intraperitoneally (i.p.) (100 μL) with PBS (carrageenan group), UAD**1** (200 mg kg^{-1}), UAD**2** (200 mg kg^{-1}), ursolic acid (200 mg kg^{-1}) or dexamethasone (0.5 mg kg^{-1}), 30 min before the induction of the model. Paw edema [Paw edema / mm] = [footpad thickness of carrageenan / mm] – [footpad thickness of PBS / mm] were monitored until 4 h. *$p < 0.05$ UAD**1**, UAD**2**, UA and dexamethasone *versus* carrageenan group.

Regarding the analysis of IL-6 in the supernatant of the paw and lymph node macerates, a reduction of IL-6 was observed in the groups treated with UA and the derivatives UAD**1** and UAD**2** compared to the carrageenan group (Figures 4 and 5). Different studies[36-38] have shown that UA and derivatives are able to reduce the inflammatory marker IL-6. Rapid IL-6 production is also known to contribute to host defense during infection and tissue injury, but excessive IL-6 synthesis and dysregulation of IL-6 receptor signaling are involved in inflammatory and autoimmune disorders. Thus, various therapeutic agents have been evaluated to inhibit the cytokine itself or targets associated with its signaling pathway.[39,40]

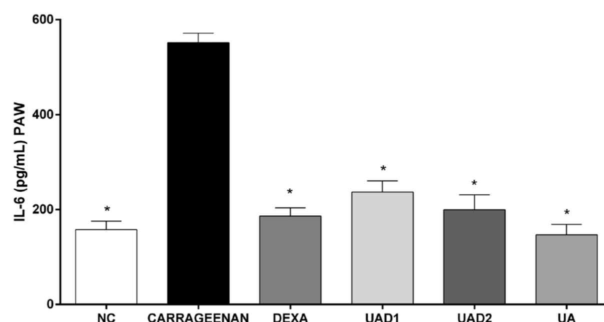

Figure 4. IL-6 in paw macerates. Female BALB/c mice (n = 6 *per* group) were treated i.p. (100 μL) with PBS (carrageenan group), UAD**1** (200 mg kg^{-1}), UAD**2** (200 mg kg^{-1}), ursolic acid (UA 200 mg kg^{-1}) or dexamethasone (DEXA 0.5 mg kg^{-1}) and not treated group negative control (NC). After euthanasia, the paws were macerated and the supernatant was collected to dose IL-6 by ELISA. *$p < 0.05$ UAD**1**, UAD**2**, UA, dexamethasone and NC *versus* carrageenan group.

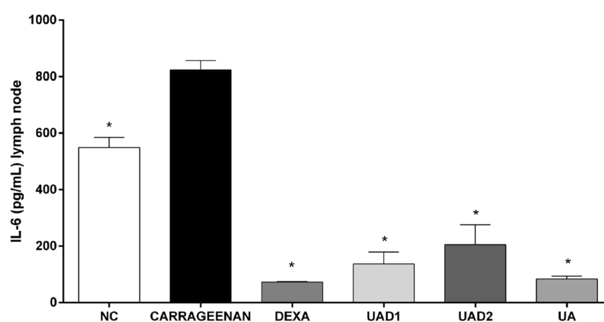

Figure 5. IL-6 in lymph node. Female BALB/c mice (n = 6 *per* group) were treated i.p. (100 μL) with PBS (carrageenan group), UAD**1** (200 mg kg^{-1}), UAD**2** (200 mg kg^{-1}), ursolic acid (UA 200 mg kg^{-1}) or dexamethasone (DEXA 0.5 mg kg^{-1}) and not treated group negative control (NC). After euthanasia, the lymph nodes were macerated and the supernatant was collected to dose IL-6 by ELISA. *$p < 0.05$ UAD**1**, UAD**2**, UA, dexamethasone and NC *versus* carrageenan group.

Conclusions

The results obtained demonstrated a variation in the response between the derivatives, due to their chemical modifications, but with potential to reduce the inflammatory mediators evaluated. Regarding paw edema, the UAD**1** and UAD**2** proved to be as efficient as dexamethasone, an anti-inflammatory used in clinical practice. Furthermore, the investigation of the other derivatives, in *in vivo* models of inflammation, deserves to be expanded.

Supplementary Information

Supplementary information about the compounds spectrum data (Figures S1-S35) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Elaine C. Scherrer was responsible for running the laboratory work and the analysis of data and data curation and writing original draft; Ydia M. Valadares for conceptualization, funding acquisition, in chemical synthesis, chemical analysis and writing original draft; Bárbara G. R. Fernandes for running the laboratory work and supporting the analysis of data and data curation; Karla A. Ramos for supporting the laboratory work and data analysis; Paloma E. Carvalho for supporting the laboratory work and data analysis; Fernando S. Silva for the conceptualization, biological analysis, funding acquisition and writing-review and editing the manuscript; Maiara R. Salvador for supporting the laboratory work and data analysis; Jeferson G. da Silva for chemical analysis, funding acquisition and writing original draft; Alessandra P. Carli for biological analysis and critical review of the manuscript; Caio C. S. Alves for conceptualization, funding acquisition, resources and writing original draft, writing - review and editing; Ângelo M. L. Denadai for funding acquisition, resources and the revision of the manuscript in this resubmission; Sandra B. R. Castro lead the conceptualization, project administration, funding acquisition, visualization and writing the original draft.

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