Anti-inflammatory Effect of Birsonimadiol from Seeds of *Byrsonima crassifolia*

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Abstract The new compound identified as $16\alpha,23\alpha$ -dihydroxy- $3\beta,28\beta,30\alpha$ -triacetoxylean-12-ene, named birsonimadiol (BIR) was isolated from *Byrsonima crassifolia* seeds using activity-guided fractionation and structural elucidation was achieved based on extensive analysis of spectroscopic data. Effects of BIR on acute and chronic phases of inflammation were studied in edema induced using formaldehyde, 12-O-tetradecanoylphorbol-acetate (TPA)-induced ear edema (meloperoxidase activity), and histamine, carrageenan, cotton pellet granuloma, and adjuvant-induced arthritis. Anti-inflammatory activities of BIR were indicated based on reduction of edema levels induced in models of inflammation. Anti-inflammatory activities were also investigated in murine macrophage RAW264.7 cells. BIR suppressed production of nitric oxide (NO) and prostaglandin E_2 (PGE_2), decreased gene expression of cyclooxygenase-2 (COX-2), inhibited tumor necrosis factor (TNF)- α , and protein secretion of interleukin IL-6. Triterpene was an effective topical anti-inflammatory agent in experimental models of acute and chronic dermatitis and can be used in inflammatory disorders.

Keywords: anti-inflammatory, cytokines, triterpenoid, *Byrsonima crassifolia*

Introduction

Inflammation is associated with a number of chronic diseases, including rheumatoid arthritis, asthma, atherosclerosis, Alzheimer's disease, and others. One of the main factors involved in inflammation is nitric oxide synthase (iNOS), which is induced by lipopolysaccharide (LPS) and other inflammatory mediators, including cytokine interleukins, interferons, and tumor necrosis factor (TNF)- α (1). The tropical fruit tree *Byrsonima crassifolia* is consumed as a jelly, juice, candy, and liquor in México. Since pre-Hispanic times nanche has been used as a medicine with reports on ethnobotanical uses, including seeds for dysentery, infections, as an anti-inflammatory agent, treatment of diabetes, and for wound healing and burns (2).

Bioactivities of plant extracts and constituent esters (3), epicatechins (4), and glycolipids have been investigated (5). Extracts from leaves, fruits, and bark exhibited neuropharmacological effects (6), and antioxidant (7), antibacterial (8), and anti-trypanocidal activities (9). Hypoglycemic (10) and anti-inflammatory (11) activities of *B. crassifolia* and inhibitory activities of guaianolides from nanche seeds against protein glycation have been reported (12). Phytochemical studies have shown that oleanolic acid, acid derivatives, and triterpenoidal saponins exert anti-inflammatory activities (13). Therefore, in this study, anti-inflammatory effects of BIR against

agent-induced paw edema in mice and RAW264.7 cells were investigated.

Materials and Methods

General experimental procedures Infrared (IR) spectra were obtained using a Perkin-Elmer 1720 FTIR spectrometer (Waltham, MA, USA). A Bruker DRX-300 Nuclear Magnetic Resonance (NMR) spectrometer (Bruker, Billerica, MA, USA) operating at 599.19 MHz for 1H and 150.86 MHz for ^{13}C and using the UXNMR software package (Bruker) was used for NMR experiments. Chemical shifts were expressed as δ (ppm) using tetramethylsilane (TMS) as an internal standard. High resolution electron impact mass spectrometry (HREIMS) was performed on an HX 110 mass spectrometer (JEOL, Tokyo, Japan).

Plant material Nanche (*Byrsonima crassifolia* L. of Family Malpighiaceae) fruits were collected in Cuautla, Morelos State, Mexico in September of 2013 and were taxonomically authenticated at the Herbarium of Escuela Nacional Ciencias Biológicas- Instituto Politécnico Nacional (ENCB-IPN, D.F. Mexico, D.F.). A voucher specimen was stored for reference as No. 8976.

Experimental animals Male CD1 mice procured from the Bioterium of in September of 2013 weighing approximately 22-25 g., were used. Before and during experimentation, animals were fed a standard laboratory diet (Mouse Chow 5015; Purina, St. Louis, MO, USA) with free access to water. Mice were housed in a controlled environment at 25°C. Animals were acclimatized for a period of three days in the new environment before initiation of experimentation. Litter in cages was renewed 3x each week to ensure hygiene and maximum comfort for animals.

Experiments reported in this study were performed following guidelines stated in Principles of Laboratory Animal Care (National Institute of Health publication, NIH) 85-23, revised 1985 (14) and the Mexican Official Normativity (NOM-062-ZOO-1999) (15). All experiments and protocols described in this study were approved by the Institutional Animal Ethical Committee of Escuela Nacional Ciencias Biológicas- Instituto Politécnico Nacional (ENCB-IPN, D.F. Mexico, D.F.) (Registered No. 639).

Extraction, bioassay-guided fractionation, isolation and, characterization of triterpenoid Seeds (10 kg) of the nanche fruit were dried in an air current oven Cascade Teck TFO-1 (Cascade TEK, Hillsboro, OR, USA) at 40°C then ground in a mill Universal Mill, type PXL & PPXL (Pallmann, New York, NY, USA) and subjected to extraction twice with hexane for 3 h. Extracts were combined and concentrated *in vacuo* in a Rotavapor buchi r-215 (BÜCHI Labortechnik AG, Flawil, Switzerland) to generate 572 g of residue. Hexane extracts were subjected to silica gel column chromatography and eluted using petroleum ether/acetone/hexane (2:1:0.5) and 6 fractions named F1-F-6 were obtained and subsequently tested for anti-inflammatory activities on edema induced using carrageenan. The paw edema volume was measured using a plethysmometer (7140; Ugo Basile S.R.L., Monvalle, Italy). Active fractions were pooled based on similarities of chemical content for thin plate chromatography (TLC) analysis. Eluted fraction F4 was subjected to additional silica gel column chromatography elution using methanol-chloroform (1:9) to produce the 7 fractions F4-1 to F4-7. The bioactive fraction (assay on edema induced carrageenan) F4-5 was subjected to silica gel column chromatography using petroleum ether/acetone/hexane (2:1:0.5) to yield the 8 subfractions F45-1 to F45-8. Subfraction F452 exhibited an anti-inflammatory activity (assay on edema induced using carrageenan) and was further purified using a preparative plate with ethyl ether/chloroform (1:5) to produce the 4 fractions F452-1 to F452-4, which were visualized using UV Shimadzu RF10A (Shimadzu, Kyoto, Japan) at 254 nm. Fraction F4522 was separated using a column packed with sephadex using a gradient of CHCl₃-MeOH from 10:1 to 5:1 to yield 144 mg of a compound with an anti-inflammatory effect assay in edema induced using formaldehyde, TPA-induced ear edema (myeloperoxidase activity), and histamine, carrageenan, cotton pellet granuloma, and adjuvant-induced arthritis and in murine macrophage RAW264.7 cells.

Carrageenin, histamine, and formaldehyde induced paw edema in mice and adjuvant-induced development of arthritis in mice BIR was orally administered to mice at 5, 10, and 20 mg/kg ($n=6$ for each dose). One hour after BIR administration, 0.1 mL of a 0.1% carrageenin suspension, 0.1 mL of 1 mg/mL histamine, 0.2 mL of 1% formaldehyde, and 0.02 mL of 5.0 mg/mL of steam-killed *Mycobacterium tuberculosis* (Difco, Detroit, MI, USA) was injected into the subplantar region of the right hind paw (15). The paw edema volume was measured using a plethysmometer (7140; Ugo Basile S.R.L.) before and 3 and 6 h after injection. Induced-arthritic animals were orally administered BIR or the non-steroidal anti-inflammatory drug indomethacin, or vehicle daily in the morning for 13 days.

12-O-tetradecanoylphorbol-acetate-induced ear edema Edema was induced in the right of auricle pavilion (ear) in the central part using topical application of 2.5 µg of 12-O-tetradecanoylphorbol-acetate (TPA) dissolved in 10 µL of acetone. Ear thickness was measured using an electronic caliper YT 202 (Yato Electronics Co., Ltd., Guangzhou, China) (16). Concentrations of BIR at 12.5, 0.25, or 0.50 mg, or dexamethasone at 0.05 mg were applied topically before TPA application. Myeloperoxidase (MPO) activity was determined in edematous ear tissue using a commercial test kit (Myeloperoxidase colorimetric activity assay Kit, K744-100; BioVision Inc., Los Angeles, CA, USA).

Cotton pellet-induced granuloma Surgical implantation of 2 cotton pellets (10±1 mg) subcutaneously in the dorsal region of mice, one near each axilla followed the procedure of Swingle and Shideman (17). Concentrations of BIR to 5, 10, and 20 mg/kg or diclofenac sodium (10 mg/kg) was administered orally once daily for 7 days at 9.0 am. On 8th day, mice were sacrificed using an overdose of anesthetic ether, and the surgically implanted pellets covered by granulomatous tissue were dissected and dried to a constant weight (Fisher Scientific, Inc. Waltham, MA, USA.) at 50°C for 20 h.

Cell viability Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)-based colorimetric assay (18). Cells in 96 well plates at a density of 5×10^5 cells/well were exposed to BIR to concentrations of 80, 40, and 10 µL/well at 37°C and 5% CO₂ in air for 24 h. Then, 10 µL of MTT (5 mg/mL) was added to in phosphate buffer followed by further incubation (Thermo Scientific, Waltham, MA, USA) for 4 h at 37°C. After aspiration of the supernatant from wells, 100 µL of buffer was added for dissolution of formazan crystals. The absorbance of each well was then read at 570 nm using an ELISA plate reader. A decrease in absorbance values indicated a reduction in cell viability.

Cell culture and treatment The RAW264.7 murine macrophage cells were cultured at 37°C in Dulbecco's modified Eagle medium (DMEM) with 10% Fetal bovine serum (FBS) in 24-well-flat plates at a

density of 5×10^{-5} cells/well. The cells were treated with various concentrations 25, 50, and 100 $\mu\text{g/mL}$ of BIR or LPS 10 $\mu\text{g/mL}$ at 37°C for 48 h. LPS was used as a positive control for macrophage activation. Then the supernatants were collected for cytokines bioassays (19).

Interleukin 6 (IL-6) and COX-2 (cyclooxygenase) protein expressions, and nitric oxide (NO) levels, were measured in culture supernatants using a commercial ELISA assay kit (R&D Systems Inc., Minneapolis, MN, USA) for IL-6, and using Griess reagent (17). Levels of tumor necrosis factor- α (TNF- α) were quantified using a Quantikine ELISA kit (R&D Systems). IFN- γ levels were determined using a commercial ELISA Quantikine kit (R&D Systems). COX-2 levels were investigated using a COX-2 inhibitor screening assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). PGE_2 levels were quantified using a kit for prostaglandin E_2 (PGE_2) (MyBioSource, San Diego, CA, USA) following manufacturer instructions. Aminoguanidine (4 $\mu\text{g/mL}$) and celecoxib (20 $\mu\text{g/mL}$) were used as positive controls.

Statistical analysis Results are expressed as a mean $\pm$ standard error of the mean (SEM). Experimental group data were evaluated using a one-way analysis of variance (ANOVA) followed by Dunnett's t -test. Values of $p \leq 0.05$ were considered significant.

Results and Discussion

The compound isolated from a subfraction was identified as 16 α ,23 α -dihydroxy-3 β ,28 β ,30 α -triacetoxylean-12-ene, and named as birsonimadiol (BIR) based on analysis of spectral data, including IR, two dimensional NMR spectra, and comparison with previous reports (20–24). BIR exhibited strong absorption bands at 340.2, 176.3, and 165.1 cm^{-1} in the IR spectrum, indicating the presence of hydroxyl, carboxyl, and tri-substituted double bond functionalities, respectively. In HREIMS an ion was performed at $m/z=588.3692$ [$\text{M}]^+$ for assignment of the molecular formula $\text{C}_{34}\text{H}_{52}\text{O}_8$, supported by Distortionless enhancement by polarization transfer (DEPT) and ^1H NMR and ^{13}C NMR spectral data. ^{13}C NMR spectroscopic data indicated 9 degree of unsaturation. ^{13}C NMR, DEPT, and HSQC spectral data showed 8 methyls, 10 methylenes, 6 methines, and 10 quaternary carbons. Fragment-ion peaks at $m/z=571$ ($[\text{M}-\text{H}_2\text{O}+\text{H}]^+$), 553 ($[\text{M}-2\text{H}_2\text{O}+\text{H}]^+$), and a retro-Diels-Alder fragmentation-ion peak, (25) characteristic of an oleanane skeleton, at $m/z=264$ (29%) indicated an olean-12-ene derivative. Observation of Heteronuclear multiple bond correlation (HMBC) correlations from δ_{H} 5.50 (H-12) to C-9 (δ_{C} 47.69), C-14 (δ_{C} 42.34), and C-18 (δ_{C} 41.21) supported the double bond at C-12 (13). Signals at δ_{C} 127.82 (C-12), 137.51 (C-13), and a triplet at δ_{H} 5.50 characteristic of a vinylic proton were assigned to H-12 and the proton at δ_{H} 2.22 was assigned to H-18, showing correlation in the HMBC spectrum (Fig. 1) with carbon at δ_{C} 127.82 (C-12), δ_{C} 137.51 (C-13), δ_{C} 69.67 (C-17), and δ_{C} 41.32 (C-19), in good agreement with an olean-type skeleton (Fig. 1A).

Fig. 1. (A) Structure of 16 α ,23 α -dihydroxy-3 β ,28 β ,30 α -triacetoxylean-12-ene, (Birsonimadiol, BIR), (B) Correlation HC HMBC spectrum

BIR displayed characteristic resonances for 5 tertiary methyls at δ_{H} 0.77, δ_{H} 0.84, δ_{H} 1.03, δ_{H} 1.07, and δ_{H} 1.45 (3H each, Me-24, Me-25, Me-26, Me-27, and Me-29, respectively), an oxygenated methine at δ_{H} 3.89 (1H, dd, $J=4.5, 10.9$ Hz), and an oxygenated methylene δ_{H} 3.55 (1H, d, $J=11$ Hz). The hydroxyl function at C-16 was assigned an α -configuration on the basis of HMBC correlations of the proton at δ_{H} 3.62 (H-16), and signals at δ_{C} 42.34 (C-14) (Fig. 1B). Relative configurations of H-3 and H-16 were supported by a nuclear Overhauser effect spectroscopy (NOESY) is useful for determining which signals from protons that are close to each other in space even if they are not bonded experiment, where in enhancements were observed between δ_{H} 4.41 (H-3) and δ_{H} 1.51 (H-5), δ_{H} 3.62 (H-16) and δ_{H} 2.22 (H-18), and δ_{H} 1.29 (H-27).

Signals for primary alcoholic functions at δ_{H} 3.55 (1H, d, $J=11$ Hz, H-23 α) and δ_{H} 3.61 (1H, d, $J=11$ Hz, H-23 β) were observed. Proton signals of H-23 showed cross peaks with 5 α and 6 α proton signals in the NOESY spectrum and, thus, the hydroxymethyl must have been in the α -orientation. Acetoxymethyl signals at δ_{H} 2.10, δ_{H} 2.03, and δ_{H} 2.21 in the ^1H NMR spectrum, and signals at δ_{C} 178.59, δ_{C} 173.56, and δ_{C} 174.12 in the ^{13}C NMR spectrum were due to 3 acetoxymethyl groups. Signals at δ_{H} 4.41 must have corresponded to H-3 on the

Table 1. Anti-inflammatory effect of the Birsonimadiol on edema induced with formaldehyde, carrageenan, histamine mice paw, cotton pellet granuloma and adjuvant-induced arthritis

Grupos mg/kg	% inhibition of edema				
	Formaldehido 6 h	Carragenina 6 h	Histamine 3 h	Cotton pellet 7 d	Arthritis 13 d
Control	0	0	0	0	0
Birsonimadiol (5)	44±8.42	50± 4.67	68±10.34	45±3.87	41± 2.90
Birsonimadiol (10)	63±6.78 ^{a1)}	63±5.23 ^a	87±4.98 ^a	56±5.39 ^a	58± 5.38 ^a
Birsonimadiol (20)	74±9.38 ^a	69±9.12 ^a	93±9.69 ^a	64±4.73 ^a	72± 6.29 ^a
Indomethacin (10)	73±7.21 ^a	67±7.29 ^a	-	62±6.11 ^a	75± 7.14 ^a
Diclofenac (10)	-	-	91±6.87 ^a	-	-

¹⁾Data are mean±SD values ($n=3$). Results indicated percent reduction in paw volumen compared with the control group treat only inflammatory agents. Dunnett test ^a $p<0.05$

carbon bearing the acetoxy group, verified via HMBC analysis. Correlations observed δ_H 4.41 (H-3), δ_C 27.49 (C-2), 43.11(C-4), 63.52 (C-23), and 14.32 (C-24) indicated that H-3 must have been in the axial position, and the acetoxy group must have been in the β -position. Furthermore, the presence of 2 other acetoxy groups was confirmed on the basis of ions $m/z=322$ [$C_{18}H_{26}O_5$], 264 [$C_{16}H_{24}O_3$], and 206 [$C_{14}H_{22}O$] produced by loss of 2 acetate groups in rings D and E.

The carbonyl signal at δ_C 178.59 was correlated with signals at H-16 and H-18 indicating an acetoxy group at C-28. Identification of 28-oate functionality was achieved on the basis of previous reports (26-28). The β -axial configuration of H-18 was supported by multiplicity and the coupling constant of δ_H 3.22 (H-18, dd, $J=14.1$, 4.0 Hz) (19). Observation of important NOE correlations of δ_H 3.22 (1H, H-18) with δ_H 5.50 (1H, H-12) indicated an α -orientation of the acetoxy group at C-30 (20,21). The HMBC spectrum showed correlations of both H-19 and H-21 to C-30, confirming the acetoxy group at C-30. Therefore, the structure was identified as 16 α ,23 α -dihydroxy-3 β ,28 β ,30 α -triacetoxy-olean-12-ene, designated herein as Birsonimadiol (Fig. 1A).

BIR was evaluated for anti-inflammatory effects in acute and chronic models. Oral administered of BIR at the dosages of 5, 10, and 20 mg/kg showed a dose-dependent inhibitory effect against carrageenan, a cotton pellet granuloma model, histamine, and formaldehyde-induced mouse paw edema. BIR caused significant ($p<0.05$) inhibition of acute inflammatory process in carrageenan induced paw edema after 6 h of BIR administration at a dosage of 20 mg/kg (69%), compared with controls (Table 1).

The carrageenan model is associated with activation of the cyclooxygenase pathway (13). Edema development in a carrageenan-induced paw edema model in mice is characterized as a biphasic curve. The first phase occurs within 1 h of injection due to histamine and serotonin components and the trauma of injection (29). Prostaglandins participate in development of the second phase, which occurs approximately 3 h post-injection. Prostaglandins play a key role in the generation of the inflammatory response. Their biosynthesis is significantly increased in inflamed tissue and they contribute to the development of the cardinal signs of acute

inflammation. BIR reduced levels of inflammation during both phases. Thus, BIR probably acted via inhibition of release or synthesis of acute phase mediators, such as serotonin and histamine, and other pro-inflammatory mediators involved in the early phase of inflammation. The effect of BIR in the second phase of inflammation was probably due to inhibition of the cyclo-oxygenase enzyme, leading to inhibition of prostaglandin synthesis.

BIR showed the highest anti-inflammatory activity at the end of carrageenan testing (6 h), indicating some association with prostaglandin in inflammatory function, and causing a response similar to the indomethacin-induced response. The effect of BIR probably involved other inflammatory mediators, such as histamine, 5-hydroxytryptamine, and bradykinin.

Histamine acts with prostaglandins for induction of both phases of edema, and increasing vascular permeability (30). An increase in the paw volume of control group mice, was observed at 2 h after histamine injection. BIR caused significant ($p<0.05$) inhibition in the percentage of volume increase of histamine-induced paw edema in mice, in comparison with the standard drug diclofenac sodium at a dosage of 10 mg/kg (91%), with maximal inhibition at a BIR dosage of 20 mg/kg (93%). In the histamine-induced rat paw inflammation model, both BIR and diclofenac sodium significantly ($p<0.05$) decreased the inflammation volume of paw edema at 1, 2, and 3 h after histamine injection, compared with controls.

The effect of BIR on chronic inflammation was studied in cotton pellet induced-fibrovascular tissue growth in mice. BIR inhibited formation of granulomatous tissue (Table 1). At a dosage of 20 mg/kg, BIR produced a 64% decrease in granuloma tissue weight. Indomethacin produced a 62% decrease in granuloma tissue weight at a dosage of 10 mg/kg. Reduction in the granuloma tissue weight due to BIR indicated suppression of the proliferative phases of inflammation.

Effects of BIR in formaldehyde-induced paw edema in mice are shown in Table 1. Formaldehyde produces inflammation via proliferation and migration of fibroblasts that are used in formation of connective tissue (31). Therefore, formaldehyde-induced paw edema is used as a model for assessment of anti-proliferative effects. BIR produced significant ($p<0.05$) suppression of formaldehyde-

induced paw edema in mice at a dosage of 20 mg/kg (74% inhibition), compared with controls. Thus, BIR exhibited a strong inhibitory effect against proliferation of fibroblasts, and probably also exerted a connective tissue modulation effect.

Triterpenoid exhibited an anti-arthritis activity in the *Mycobacterium tuberculosis*-induced-adjuvant arthritis test in mice (Table 1) with a maximum effect of 72% protection relative to control at a dosage of 20 mg/kg. Suppression of this response by BIR indicated an immunosuppressive activity (32). Adjuvant arthritis, an experimental model of autoimmune disease in rodents, is induced via immunization with *Mycobacterium tuberculosis* (MT).

The effect of BIR on acute inflammation was tested in a TPA-induced edema model. BIR at a dosage of 0.50 mg applied topically to the ear displayed significant ($p<0.05$) inhibition of 86% against ear swelling in mice, compared with controls (Table 2). TPA is a phorbol ester that induces skin inflammation and a hyperproliferative response with infiltration of neutrophils, and also stimulates phospholipase A2 (PLA2) and, as a consequence, release of prostaglandins and arachidonic acid occurs (33). The anti-inflammatory activity of BIR could be due to an inhibition effect against prostaglandin synthesis.

MPO is an enzyme that is present in the azurophilic granules of neutrophils that can be colorimetrically detected (33). MPO resulted in marked inhibition of MPO activity ($p<0.05$) (Table 2) and, consequently, of edema formation and migration of polymorphonuclear leukocytes induced using TPA.

Cytotoxicity of different amounts of BIR in RAW264.7 cells was determined in order to define appropriate concentrations for measurement of inhibitory activities against inflammation. Cultures were incubated overnight with 1-150 $\mu\text{g/mL}$ BIR and viability was determined. BIR at concentrations as high as 150 $\mu\text{g/mL}$ did not markedly affect the viability of RAW264.7 cells ($p<0.05$). Examination of BIR effects on LPS-induced production of NO, COX-2, IL-6, IFN- γ , and TNF- α in cells were limited to nontoxic quantities of BIR.

Effects of BIR in RAW264.7 cells were determined. Incubation with BIR for 24 h in the presence of LPS significantly ($p<0.05$) inhibited COX-2 protein and nitrite (NO) expressions in mouse macrophage RAW 264.7 cells in a dose-dependent manner, compared with controls (Table 3). There was a significant ($p<0.05$) decrease in COX-2

Table 2. Effect of topically applied of Birsonimadiol on ear edema induced by TPA and myeloperoxidase activity

Dosis mg/ear	Δ Ear thickness (mm) (% inhibition)	MPO absorbance at 470 nm
Control	0.092 $\pm$ 0.0023	0.19 $\pm$ 0.0017
Birsonimadiol (0.125)	0.064 $\pm$ 0.0052 ^{a1)} (69)	0.10 $\pm$ 0.0023 ^b (48)
Birsonimadiol (0.25)	0.074 $\pm$ 0.0019 ^a (80)	0.07 $\pm$ 0.0018 ^b (63)
Birsonimadiol (0.50)	0.079 $\pm$ 0.0026 ^a (86)	0.05 $\pm$ 0.00121 ^b (74)
Dexametasone 0.05 mg/ear	0.075 $\pm$ 0.0098 ^a (81)	0.04 $\pm$ 0.0054 ^b (79)

¹⁾Data as means $\pm$ SD of six mice. ^a $p<0.005$, ^b $p<0.001$ compared to the vehicle.

and NO production in mice treated with 100 $\mu\text{g/mL}$ BIR, compared with LPS Group mice (Table 3). Thus, COX-2 lead to neutrophil migration and reduced edema formation. COX is a critical inflammatory enzyme that converts arachidonic acid to prostaglandins (PGs) (28). The inducible isoform COX-2 is expressed during inflammatory disease in many cells, including macrophages and fibroblasts, and mediates release of great quantities of pro-inflammatory prostaglandins at the site of inflammation (34). NO plays a role as a vasodilator, an immune regulator, and a neurotransmitter in tissues, and is involved in the pathophysiology of inflammation.

PGE₂ induced using LPS is overproduced during inflammation and overexpression is related to inflammation and pain. To confirm the effect of BIR on LPS-induced PGE₂ production, cells were incubated with BIR for 1 h, then activated using 10 $\mu\text{g/mL}$ LPS for 24 h. Production of PGE₂ was significantly ($p<0.05$) inhibited by BIR in a concentration-dependent manner, compared with controls (Table 3). Thus, PGE₂, COX-2, and NO were key regulatory molecules in the inflammatory process.

Triterpene and dexamethasone showed significant ($p<0.05$) suppressive effects against IL-6, IFN- γ , and TNF- α in macrophage cells treated with BIR at concentrations of 25 and 100 $\mu\text{g/mL}$, compared with controls (Table 3). TNF- α , an early stage inflammation mediator, induces synthesis of IL-6 or serotonin, resulting in activation of T cells

Table 3. Inhibitory effect of BIR on NO, TNF- α , IFN- γ , IL-6, PGE₂, and COX-2 levels

Grupos ($\mu\text{g/mL}$)	NO (μM)	TNF- α (pg/mL)	IL-6 (%)	IFN- γ (pg/mL)	COX-2 (%)	PGE ₂
Control	1.4 $\pm$ 0.05	16.3 $\pm$ 3.17	100	472.5 $\pm$ 8.92	3	-
LPS	13.5 $\pm$ 1.76	-	-	-	100	32.12 $\pm$ 4.43
Birsonimadiol (25)	9.2 $\pm$ 2.04 ^{ab1)}	10.6 $\pm$ 3.17 ^b	69	17.19 $\pm$ 5.13 ^b	75	26.45 $\pm$ 3.80 ^a
Birsonimadiol (50)	7.1 $\pm$ 1.33 ^{ab}	7.3 $\pm$ 1.78 ^b	74	13.23 $\pm$ 4.22 ^b	68	14.29 $\pm$ 5.03 ^a
Birsonimadiol (100)	5.9 $\pm$ 1.75 ^{ab}	5.5 $\pm$ 1.96 ^b	86	9.81 $\pm$ 1.28 ^b	62	8.77 $\pm$ 2.38 ^a
Aminoguanidine (4)	2.9 $\pm$ 0.94 ^{ab}	-	-	-	-	-
Dexamethasone (10)	-	4.3 $\pm$ 1.07 ^b	90	7.43 $\pm$ 2.16 ^b	42	-
Celecoxib (20)	-	-	-	-	57	-
NS 398 (10 μM)	-	-	-	-	-	9.48 $\pm$ 2.67 ^a

¹⁾The value shown are the means $\pm$ SD of three independent experiments; ^a $p<0.05$, vs LPS-treated group; ^b $p<0.001$ vs. control group; Significant differences between groups were determined one way ANOVA followed by Dunnett's method.

and inflammation-related cells. IFN- γ , a pleiotropic cytokine involved in regulation of nearly all phases of immune and inflammatory responses, coordinates a diverse array of cellular programs via transcriptional regulation of immunologically relevant genes. Cytokine analysis showed that BIR had an inhibitory effect against endotoxin-induced inflammation and indicated that BIR exerts an immunoregulatory function in some forms of inflammatory disease. Study of the influence of BIR isolated from *B. crassifolia* on acute phase inflammatory markers, such as tumor necrosis factor and interleukin-6, is important. Thus, compounds that suppress TNF- α , IFN- γ , and IL-6 expression are also expected to show anti-inflammatory effects.

Birsonimadiol isolated from seeds of *Byrsonima crassifolia* exerted anti-inflammatory effects in both acute and chronic inflammatory models when administered orally or topically to mice. BIR inhibited *in vitro* production/release of TNF- α , IL-6, IFN- γ , PGE₂, COX-2, and NO in lipopolysaccharide-stimulated macrophages. Thus, the anti-inflammatory activity of BIR was probably related to suppressive effects against pro-inflammatory cytokines and cellular immune responses. BIR is a potential therapeutic drug for treatment of inflammation.

Disclosure The authors declare no conflict of interest.

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