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1. Title page

Cucurbitacin R Reduces the Inflammation and Bone Damage

Associated with Adjuvant Arthritis in Lewis Rats by suppression of

TNF-α in T Lymphocytes and Macrophages

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2. Running Title

a) Cucurbitacin R inhibits adjuvant arthritis

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d) Abbreviations: BOC, *N-tert*-butoxy-carbonyl-L-alanine *p*-nitrophenyl ester; EDTA, ethylendiaminetetraacetic acid; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; HBSS, Hanks' balanced salt solution; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenil-tetrazolium bromide; PBS, phosphate-buffered saline; PMNL, polymorphonuclear leukocytes; STAT,

signal transducer and activation of transcription; TNF-α, tumour necrosis factor-α; TPA, 12-*O*-

tetradecanoylphorbol 13-acetate.

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ABSTRACT

The aim of this study was to investigate the effects of cucurbitacin R on an experimental model of adjuvant-induced arthritis in rats. The treatment of arthritic rats with cucurbitacin R (1 mg /kg p.o. daily) modified the evolution of the clinical symptoms while the histopathology of paws demonstrated a reduction in the signs of arthritis. When compared with the control group, the radiography of the tibiotarsal joints of cucurbitacin R-treated rats showed a decrease in joint damage and soft tissue swelling of the footpad. The *in vivo* study of the expression of proinflammatory enzymes (nitric oxide synthase-2, cyclooxygenase-2) with the aid of the Western blot technique, and that of TNF-α and prostaglandin E_2 by means of ELISA demonstrated a clear decrease in both the enzymes and the mediators in paw homogenates. The analysis for prostaglandin E_2, nitric oxide and TNF-α production in RAW 264.7 macrophages, as well as that of TNF-α in human lymphocytes, indicated a reduction of all mediators. The expression of cyclooxygenase-2 was not modified in RAW 264.7 macrophages while the expression of nitric oxide synthase-2 was clearly diminished. Moreover, cucurbitacin R was found to inhibit STAT3 activation in the lymphocytes of both healthy and arthritic men. These experimental data on the chronic model, together with previously reported activity on acute and sub-chronic experimental models, justify the anti-inflammatory activity of cucurbitacin R and provide further evidence for the therapeutic potential of a group of natural products as anti-inflammatory agents.

Introduction

Arthritis is a chronic disease that affects several parts of the joints such as the cartilage, synovium, tendons, and muscles. Rheumatoid arthritis is a specific type of this disease characterized by chronic inflammation of the joints, with severe cartilage and bone damage. The precise causes are unknown, but genetics, infectious agents, environment, and hormonal effects have all been implicated. Treatments are focused on the reduction of pain, inflammation, and joint damage. The principal pharmacological agents are non-steroidal anti-inflammatory drugs, disease-modifying antirheumatic drugs, glucocorticoids, and specific inhibitors of the mediator response such as the tumor necrosis factor-α (TNF-α) antibody infliximab. Adjuvant arthritis is an experimental model unique to rats that is widely used for studying the physiology, biochemistry, and pharmacology of inflammation, and also as a model of cell-mediated autoimmnune disease, human arthritis, and chronic pain. Different parameters can be evaluated, such as weight evolution, paw swelling, histology, immunohistochemistry, and radiographic evaluation (Taurog et al., 1988). The origin and development are complex, with the implication of different inflammatory cells and cytokines. Thus, when the inflammatory cells migrate into the joint space between bones, there is inflammation of the membranes and damage to the bone and cartilage, with cytokines being released to transmit the signal. Neutrophils, macrophages, and lymphocytes all participate in the development and pathogenesis of arthritis (Solomon et al., 2005). Neutrophils generate reactive oxygen species, releasing hydrolytic enzymes, such as elastase (Bombini et al., 2004). Activated T lymphocytes increasing the levels of TNF-α (Skapenko et al., 2005), and activated macrophages generate oxygen and nitrogen reactive species, eicosanoids through cyclooxygenase-2, nitric oxide through the inducible form of nitric oxide synthase, and cytokines such as TNF-α (Park et al., 2004a;b). All these enzymes and mediators are involved in the development of rheumatoid arthritis, stimulating the leukocyte migration, inflammation in the joint, and tissue damage.

Superoxide anion, which is formed by inflammatory cells in chronic inflammation, is generally metabolized by the endogenous superoxide dismutase. However, when production of the former is extremely high, it overwhelms the action of the latter. In such situations, superoxide anion can mediate cell damage, increase the formation of chemotactic factors such as leukotriene B_4, and stimulate recruitment of neutrophils at sites of inflammation which could be aggravated (Salvemini et al., 2003). Because neutrophil elastase is involved in the destruction and inflammation of tissue in different diseases, including rheumatoid arthritis, the development of new inhibitors of this protease is an important research objective (Tremblay et al., 2003).

Nociception involves a complex interaction of peripheral and central nervous system structures extending from the skin, the viscera and the musculoskeletal tissues to the cerebral cortex. Various cells, mediators, and receptors are implicated in this process; therefore, the anti-inflammatory drugs currently used for treatment act by reducing the production and release of these mediators and by modifying the access to their targets. Ribeiro et al. (2000) demonstrated that the nociceptive response induced by acetic acid depends on the peritoneal resident macrophages and mast cells through the release of TNF-α, interleukin-1β, and interleukin-8.

Tayuya is a medicinal plant used in the folk medicines of Brazil, Peru and Colombia as an analgesic, anti-inflammatory and anti-rheumatic agent (Ríos et al., 1990). Cucurbitacin R isolated from tayuya roots, has shown anti-inflammatory activity in different experimental models of acute mouse ear oedema, acute mouse paw oedema and mouse ear inflammation (Recio et al., 2004). Moreover, Park et al. (2001;2004a) demonstrated that cucurbitacin R (called dihydrocucurbitacin D in these papers) inhibited nitric oxide generation from murine macrophages by blocking the activation of nuclear factor-κB, which is essential for the transcriptional activation of NOS induction. In 1999, Panossian et al. reported that cucurbitacin R-diglucoside, moderately stimulates the adrenal cortex to produce a slight increase in corticoid

secretion, thereby justifying its pharmacological properties. However, our previous results demonstrated that cucurbitacin R did not modify its anti-inflammatory properties when administered together with mifepristone, which implies that the glucocorticoid receptor is not involved in the mechanism of action of cucurbitacin R. However, when the compound was administered together with cycloheximide, the anti-inflammatory activity was clearly reduced (Recio et al., 2004). Recently, Escandell et al. (2006) demonstrated the anti-arthritic effect of a closely related compound, dihydrocucurbitacin B, which reduced the oedema, the tissue and bone damage.

In the present study, we report on the effects of cucurbitacin R on a chronic model of inflammation (adjuvant arthritis) in rats, the effects on cartilage and bone damage, and the effect on the principal mediators implicated in this experimental process.

Material and Methods

Reagents and Chemicals

Cucurbitacin R (Figure 1) was previously isolated from *Cayaponia tayuya* roots (Recio et al., 2004). Chemicals were obtained from Panreac (Barcelona, Spain) and Merck (Darmstadt, Germany). Biochemical reagents were from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA) and Santa Cruz (Santa Cruz, CA, USA). Biotin-conjugated goat anti-rabbit immunoglobulin G and avidin-biotin peroxidase complex were from (LSAB 2 System-HRP, Glostrup, Denmark).The kits for enzyme immunoassay of cytokines were from eBioscience (San Diego, CA, USA). Culture material and reagents were from Gibco (Langley, OK, USA), Sarstetd (Nümbrecht, Germany) and Nunc (Roskilde, Denmark). Complete mini EDTA free protease inhibitor cocktail was purchased from Roche (Mannheim, Germany).

Animals

Groups of female Lewis rats weighing 200-240 g (7 weeks old) and groups of Swiss female mice weighing 25-30 g (8 weeks old) from Harlan Interfauna Iberica (Barcelona, Spain) were used for arthritis and analgesia, respectively. All animals were fed a standard diet ad libitum. Housing conditions and all *in vivo* experiments were approved by the institutional Ethical Committee of the Faculty of Pharmacy according to the guidelines established by the European Union on Animal Care (CEE Council 86/609).

Adjuvant arthritis

Adjuvant arthritis was elicited in female Lewis rats by injecting *Mycobacterium butyricum* (0.1 ml, 10 mg/ ml) from Difco (Detroit, MI, USA) in mineral oil into the base of the tail. Paw

volumes were measured at the beginning of the experiment with a plethysmometer (Ugo Basile, Comerio, Italy). The initial oedema was evaluated by measuring the volume of both paws at day 16. Animals with paw volumes 1.1 ml larger than normal paws were then randomized into treatment groups. Cucurbitacin R (1 and 0.25 mg/kg), the reference drug ibuprofen (10 mg/kg, Sigma) and vehicle as control (olive oil, Carbonell, Córdoba, Spain) were administered orally once daily on days 17 to 23 and the paw oedemas were measured on the same days. Oedema was expressed as the increase in paw volume due to arthritis and the percentage of inhibition was expressed as the reduction in volume with respect to the control group. The rats were also weighed each day and weight variations were compared to those of the control group.

Clinical assessment and radiographic studies of arthritis

The rats were previously anaesthetized with halothane (Sigma) and placed on a radiographic box at a distance of 90 cm from the X-ray source. Radiographic analysis of normal and arthritic hind paws was performed by X-ray machine (Phillips X12, Germany) with a 40 kW exposition for 0.01 s. The following radiograph parameters were considered: inflammation, osteolisis, articular cartilage affectation and osteophyte formation, with the following criteria and scores: (0) no damage; (1) mild; (2) moderate, and (3) severe.

Histological evaluation and immunohistochemical localization of cyclooxygenase-2

Animals were previously anaesthetized and then sacrificed by decapitation and arthritic paws were amputated above the ankle and were placed in 4% formalin (Panreac). The paws were then trimmed, placed in descalcifying solution for 24 h, embedded in paraffin (Panreac), sectioned at 4 µm, stained with trichromic Masson (prepared with products from Sigma) and studied using light microscopy (Dialux 22 Leitz, Wetzlar, Germany).

Cyclooxygenase-2 was determined by immunohistochemistry. At day 24, the joint's organs were then trimmed, placed in decalcifying solution for 24 h and 8 µm sections were prepared

from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched

with 0.3% H_2O_2 (Panreac) in 60% methanol (Panreac) for 30 min. The sections were

permeabilized with 0.1% Triton X-100 (Sigma) in phosphate-buffered saline (PBS, Sigma) for

20 min. Non-specific adsorption was minimized by incubating the section in 2% normal goat

serum (Sigma) in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by

sequential incubation for 15 min with avidin and biotin (LSAB2). The sections were then

incubated overnight with primary anti- cyclooxygenase-2 (Santa Cruz) (1:500) with control

solutions. Control included buffer alone or non-specific purified rabbit immunoglobulin G

(Sigma). Specific labelling was detected with a biotin-conjugated goat anti-rabbit

immunoglobulin G (LSAB2) and avidin-biotin peroxidase complex (LSAB2).

Prostaglandin E_2, TNF-α and Western blot analysis in paw and stomach homogenates

For the measurement of prostaglandin E_2 in paw and stomach, the organs were homogenized

with a Polytron (Kinematica AG, Luzern, Switzerland) in 0.1 M phosphate buffer pH 7.4

containing 1 mM EDTA (Sigma), and 10 µM indomethacin (Sigma). Prostaglandin E_2 was

isolated using a C-18 SPE cartridge (Merck LiChrolut) and was determined by a specific

enzyme immunoassay kit from Cayman Chemical, employed according to the manufacturer's

instructions.

TNF-α ELISA and Western blot analysis of paws were performed with samples from control,

ibuprofen (Sigma) and cucurbitacin R. Skin was removed from paws, then they were

homogenized with a polytron (Kinematica AG) in 2.5 ml of 10 mM HEPES (Sigma), 2 mM

phenylmethilsulfonyl fluoride (Sigma), 100 mM EDTA (Sigma), 0.32 M sucrose (Sigma), 1

mM ditiothreitol (Sigma), 2 mg/ml aprotinin (Sigma) and complete mini EDTA free protease

inhibitor cocktail (Roche). Mixture was sonicated (3 cycles in 10 s) in a Branson sonifier 150

(Danbury, CT, USA) and centrifuged (Eppendorff centrifuge 5810R, Hamburg, Germany) at

10000 $\times g$ during 20 min at 4 ℃. TNF-α was measured by enzyme immunoassay (eBioscience), employed according to the manufacturer's instructions.

Protein in supernatant was measured by the Bradford method using bovine serum albumin (Sigma) as standard, and 30 µg of protein were loaded on 10% SDS-PAGE (products from Sigma) and transferred onto polyvinylidene difluoride membranes (GE Healthcare) for 90 min at 125 mA. Membranes were blocked in PBS-Tween 20 (Panreac PA) containing 5% w/v defatted milk (Santa Cruz). Membranes were incubated with anti- cyclooxygenase-2 polyclonal antiserum (1/1000) (Cayman), anti-nitric oxide synthase-2 antibody (1/1000) (Cayman) and β-actin (1/10000) (Sigma). Blots were washed and incubated with peroxidase-conjugate goat anti-rabbit immunoglobulin G (1/20000 dilution) (Sigma). The immunoreactive bands were visualized using an enhanced chemiluminiscence system (ECL, GE Healthcare).

Analgesia

Cucurbitacin R (10 mg/kg) and ibuprofen (Sigma) (30 mg/kg) were administered orally 1 h, before the i.p. administration of 1% acetic acid (Panreac) solution (0.1 ml). Control animals received vehicle in the same experimental conditions. Immediately after acetic acid injection, each animal was isolated in an individual cage to be observed during 20 min. The number of writhing and stretching was recorded. Analgesic activity was expressed as the percentage of writhing reduction referred to the control group.

Determination of cell viability

The cytotoxicity of compounds on cells was performed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenil-tetrazolium bromide (MTT) assay. Human neutrophils, human lymphocytes, and murine RAW 264.7 macrophages were exposed to cucurbitacin R at concentration of 100 µM in a microplate at the assayed time in the different experiments, and then 100 µl per well of a

0.5 mg/ml solution of MTT (Sigma) were added and incubated at 37 ℃ until blue deposits were visible. The coloured metabolite was dissolved in dimethyl sulfoxide (Panreac). Absorbance was measured at 490 nm using a Labsystems Multiskan EX plate reader (Midland, Canada). Results were expressed in absolute absorbance readings; a decrease indicated a reduction in cell viability.

Preparation of human neutrophils for elastase release/activity and radical superoxide assays

Neutrophils were obtained from human blood buffy coats. The leukocytes present in the residual blood were isolated by sedimentation with 2% (w/v) dextran (Sigma) in 0.9% NaCl (Panreac) at room temperature. The supernatant was centrifuged at $300 \times g$ for 10 min at 4 ℃. Rest of erythrocytes was lysed by hypotonic treatment. The pellet was resuspended in PBS and cells were purified by the Ficoll-Paque gradient density method (GE Healthcare). Neutrophils (95% purity) were resuspended in PBS containing 1.26 mM Ca^{2+} and 0.9 mM Mg^{2+} (Sigma).

Elastase release assay: human neutrophils (2.5×10^6) were preincubated for 5 min with the test compound (100 µM) or vehicle. Then they were stimulated by the addition of cytochalasin B (10 µM, Sigma) and fMLP (10 nM, Sigma) for 10 min at 37 ℃; 15 min later the samples are placed on ice and centrifuged (0 ℃, 10 min, $3220 \times g$). Supernatant aliquots (200 µl) were mixed with 5 µl of 0.3% BOC (Sigma) in the 96-well microtiter plate, then incubated at 37 ℃ for 30 min. Elastase activity was detected in using BOC as substrate and *p*-nitrophenol release was measured at 414 nm. α_1-Antitrypsin (Sigma) was used as reference (90 µg/ml).

Elastase activity assay: human neutrophils (20×10^6) were suspended in 8 ml of Ca^{2+}/Mg^{2+} Hanks' balanced salt solution (HBSS, Sigma) in a falcon tube. Then 80 µl of TPA (65 µg/ml, Sigma) were added and the cells incubated at 37 ℃ for 30 min. The samples are centrifuged (0

ºC, 10 min, 3220 ×g). Supernatant aliquots corresponding to 1.25×10^6 neutrophils were incubated for 15 min with 10 µl of the test compound to a final concentration of 100 µM, before proceeding as in the release assay. The rest of the protocol was as described above.

Radical superoxide assay: Neutrophils (2.5×10^6), obtained as described above, were suspended in 500 µl HBSS containing Ca^{2+} and Mg^{2+} (Sigma) and test compounds at different concentrations, the mixture was incubated for 5 min at 37 ºC. Superoxide release was induced by addition of 5 µl TPA (final concentration 1 µM, Sigma) and after 10 min. incubation at 37 ºC, the mixture was centrifuged at 3220 ×g. Reaction was detected by nitroblue tetrazolium (100 µM, Sigma) reduction. The precipitate was dissolved in 500 µl dimethyl sulfoxide-HCl (95:5) (Sigma) in an ultrasonic bath (Branson sonifier 150) and measured using a Labsystem Multiscan EX plate reader at 560 nm.

Determination of TNF-α, nitric oxide and prostaglandin E$_2$ production, and nitric oxide synthase-2 and cyclooxygenase-2 activity, in RAW 264.7 macrophages

Macrophages RAW 264.7 were cultured in DMEM medium (Gibco) containing 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 10% foetal bovine serum (Gibco). Cells were removed from the tissue culture flask using a cell scraper and resuspended until a final relation of 1×10^6 cells/ml.

Macrophages RAW 264.7 (1×10^6 cells/ml) were co-incubated in 96-well culture plate (200 µl) with 1 µg/ml of lipopolysaccharide (1 µg/ml, Sigma) at 37 ºC for 24 h in the presence of test compound (100-25 µM) or vehicle. Nitrites were determined in culture supernatant by Griess reagent (Sigma). The prostaglandin E$_2$ production was determined in culture supernatant by a specific enzyme immunoassay kit from Cayman Chemical, employed according to the

manufacturer's instructions, and TNF-α by a specific enzyme immunoassay kit from

eBioscience.

Macrophages RAW 264.7 (1×10^6 cells/ml) were stimulated with lipopolysaccharide (1 µg/ml,

Sigma) in the absence of test compound. After 24 h, cells were washed and fresh medium

supplemented with L-arginine (0.5 mM, Sigma) and arachidonic acid (5 µM, Sigma) and

incubated for 2 h in presence of test compounds (100-25 µM). Supernatants were colleted for

the measurement of nitrite and prostaglandin E$_2$ accumulation for the last 2 h. In this

experiment, the metabolites assessed as an index of nitric oxide synthase-2 and

cyclooxygenase-2 activity.

Determination of TNF-α production in T lymphocytes

Peripheral lymphocytes were obtained from the blood of both healthy and arthritic men; the

latter had been clinically diagnosed with rheumatoid arthritis according to the criteria of the

American Rheumatism Association (Arnett et al., 1988). Cells were isolated by the Ficoll-

Paque gradient density method (GE Healthcare). T lymphocytes were isolated by depletion of

adherent cells on plastic dishes (95% purity). Under sterile conditions, cells were resuspended

to a concentration of 1×10^6 cells/ml in RPMI-1640 medium (Gibco), supplemented with 10%

heat-inactivated foetal calf serum (Gibco), 100 U/ml penicillin (Gibco) and 100 µg/ml

streptomycin (Gibco). T lymphocytes were cultured with phytohemagglutinin (25 µg/ml,

Sigma) alone or with cucurbitacin R at different concentrations (40-3 µM) for 24 hours. The

cells supernatants were colleted and assayed for TNF-α by enzyme immunoassay

(eBioscience), employed according to the manufacturer's instructions.

Western blot analysis

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Cellular lysates from RAW 264.7 macrophages (10^6 cells per well) incubated for 18 h with lipopolysaccharide (1 μg/ml, Sigma) and dexamethasone (10μM, Sigma) or test compound at different concentrations (100-25 μM) were obtained with lysis buffer (1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl and 25 mM Tris, pH 7.4, all from Sigma, and a complete mini EDTA-free protease inhibitor cocktail from Roche). After centrifugation (10000 ×g, Eppendorf centrifuge 5810R, Hamburg, Germany), proteins were determined in the supernatants by means of the Bradford method. 50 μg of protein were developed as described below in the section on the Western blot analysis for arthritic paws.

For the STAT3 assay, human lymphocytes from healthy and arthritic patients were treated for 4 h with vehicle or cucurbitacin R (50 μM) and then stimulated with either interleukin-6 (20 ng/ml, Prepotech, London, UK) for 30 min or phytohemagglutinin (25 μg/ml, Sigma) for 2 h. The cells were then lysed with RIPA buffer (1% Triton X-100, 1% deoxycholic acid, 150 mM NaCl, 50 mM Tris pH 7.4 and 0.1% sodium dodecyl sulphate), 2 mM sodium orthovanadate and 25 mM NaF, all from Sigma, and a complete mini EDTA-free protease inhibitor cocktail from Roche. After centrifugation (10000 ×g), proteins were determined in the supernatants by means of the Bradford method. 100 μg of protein were run on 7.5% SDS-PAGE gel (Santa Cruz). The protein was transferred to nitrocellulose and then blotted as described above for phospho-specific STAT3 (Santa Cruz).

Statistics

Data were expressed as mean ± S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett´s t-test for multiple comparisons. When comparing with control group, values of P less than 0.05 were considered significant. Inhibition percentages (%I) were calculated from the differences between drug treated group and control animal treated only with the inflammatory agent. Inhibitory concentration-50 (IC_{50}) and

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effective dose-50 (ED_{50}) were calculated from the dose/response linear regression plots.

Western blot quantification has carried out with Scion image software version 1.0.0.1

(Frederick, MA, USA).

Results

Evolution of induced arthritis in rats

The arthritis was clearly developed in the immunized rats two weeks after challenge.

Periarticular erythema, oedema, and a reduction of paw function were observed as clear signs

of the evolution of the inflammation, and a one hundred percent incidence of adjuvant arthritis

in the rats was observed by day 16. All rats experienced a dramatic increase in paw volume

from 1.1 to 2.5 ml during the 17 days after challenge, after which time the paw volume of the

control group stabilized until the end of the experiment. Cucurbitacin R (orally, at 1 mg/kg, 7

days) significantly reduced hind paw swelling by 48% while at 0.25 mg/kg a reduction of 10%

was observed (not significant, data not shown). The reference drug ibuprofen (10 mg/kg)

reduced the swelling by 58% under the same experimental conditions (Figure 2A). The blank

group showed no increase in hind paw volume during the experiment. There was a decrease in

weight in all arthritic animals, but while the weight in all groups stabilized during the

experiment, the weight loss was lower in the group treated with cucurbitacin R (Figure 2B).

Still, as mentioned above, cucurbitacin R did not significantly modify the oedema at 0.25

mg/kg respect to the control group.

Radiography of the tibiotarsal joint of rats demonstrated a severe inflammation in the control

group with severe periarticular inflammation, osteolisis of tarse bone, calcification of Achilles

talon, affectation of distal tibia and severe alteration of proximal metatarsiane bones, bone

resorption and joint erosion (Figure 3A), whereas the radiography of the tibiotarsal joint of the

cucurbitacin R-treated rats indicated a decrease in joint damage as well as in soft tissue swelling of the footpad, and only light osteolisis of tarse-metatarse zone with light calcification (Figure 3C). Ibuprofen-treated rats show similar radiographic characteristics than the problem group (Figure 3B).

Histopathological study production of mediators *in vivo*

The histopathological study of paws obtained from the control group showed characteristic signs of severe skin inflammation (Figure 4A) and arthritis, with massive mixed infiltration (neutrophils, macrophages, and lymphocytes), lesions with marked oedema and severe absesses, severe erosion of bone and cartilage (Figure 4B), and bone destruction (Figure 4C), articular inflammation, pannus, and large cysts with abundant granulation tissue. More than 50% of the bone surfaces were affected; bone regeneration by osteoide was more than 75% (Figure 4C) and the thickness of periosteal regeneration was above 500 μm. The histological analysis of joint sections obtained from cucurbitacin R-treated rats showed that the degree of arthritis was clearly attenuated (Figure 5C) as compared to the control group (Figure 5B). In animals treated with ibuprofen, the histological signs of arthritis decreased (Figure 5D) while the blank group showed no characteristic arthritic lesions (Figure 5A).

Immunohistochemical analysis of joint sections obtained from adjuvant arthritic rats revealed a positive staining for cyclooxygenase-2 (Figure 6A). In contrast, this was reduced in the joints of cucurbitacin R- (Figure 6B) and ibuprofen-treated rats (Figure 6C). Moreover, cucurbitacin R inhibited the *in vivo* production of prostaglandin E_2 by 94% and reduced both the nitric oxide synthase-2 and cyclooxygenase-2 expression in paw homogenates as demonstrated with Western blot analysis (Figure 7A). Prostaglandin E_2 production in the stomach homogenates was also reduced (Figure 7B), although there was no visible damage in the tissue (data not

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shown). Cucurbitacin R inhibited TNF-α production in arthritic paws by 76% and ibuprofen reduced it by 68% (Figure 7C).

Analgesia, elastase release and elastase activity, and effect on superoxide radical

Cucurbitacin R administered orally at 10 mg /kg showed significant analgesic activity, with a 47% reduction in writhing as compared to the control group (*$p<0.05$). The group treated with ibuprofen (30 mg/kg) showed a 50% inhibition (*$p<0.05$). In addition, cucurbitacin R had no effect on superoxide radical (data not shown). Cucurbitacin R at 100 μM inhibited elastase release by 50% (*$P<0.05$), but had no effect at lower concentrations, nor did it modify the enzyme activity.

Cells viability

As assessed by the mitochondrial reduction of MTT after 24 h, cucurbitacin R did not affect the cellular viability of human neutrophils, human lymphocytes, or murine RAW 264.7 cells. This finding indicates that the compound is not toxic at doses of 100 μM or lower.

Nitrite, prostaglandin E$_2$ and TNF-α production in RAW 264.7 macrophages and lymphocytes

During the induction phase, cucurbitacin R inhibited nitrite production by 42% at a final concentration of 100 μM (24 h), by 22% at 30 μM, and by 14% at 10 μM. The positive control, L-NAME (Sigma), a specific inhibitor of nitric oxide synthase-2, inhibited the nitrite production by 98% at 1 mM whereas dexamethasone (Sigma) inhibited this production by 37% at 10 μM (Figure 8A). In addition, cucurbitacin R inhibited prostaglandin E$_2$ production by 53%, whereas dexamethasone at 10 μM reduced the prostaglandin E$_2$ production by only 43% (Figure 8B).

When the enzymes were induced prior to the treatment (post-induction phase assay), cucurbitacin R and dexamethasone had no effect on nitrite production, which implies that neither of them have a direct effect on the enzyme. However, L-NAME inhibited nitrite production by 42% due to a direct inhibition of the enzyme (Figure 8A). In the cyclooxygenase-2 post-induction phase assay, cucurbitacin R at 100 µM inhibited the enzyme activity by 60%, whereas indomethacin at 10 µM reduced it by 81% (Figure 8B). In this case, both compounds directly modify the activity of the enzyme cyclooxygenase-2.

These findings were corroborated after an assay involving the co-incubation of cucurbitacin R at different concentrations on lipopolysaccahride-stimulated RAW 264.7 macrophages to assess the possible effects of cucurbitacin R on the expression of nitric oxide synthase-2 or cyclooxygenase-2 (Figure 8C). The results clearly indicate that in the presence of cucurbitacin R at 100 and 50 µM, nitric oxide synthase-2 expression was inhibited by 57% and 40%, respectively. Cyclooxygenase-2 induction, however, was not affected. As was expected, the reference compound dexamethasone inhibited the expression of both enzymes.

Cucurbitacin R inhibited TNF-α production in a concentration-dependent manner with an IC_{50} value of 65 µM (52 − 78, $r^2 = 0.9866$) in RAW 264.7 macrophages (Figure 9A), 29 µM (26.0 − 36.5, $r^2 = 0.9705$) in healthy human lymphocytes (Figure 9B) and 7 µM (4.0 − 8.8, $r^2 = 0.9612$) in lymphocytes from arthritic patients (Figure 9C). The differences in potency between human and mouse cells may be attributable to the fact that the tests were performed on different cells from different species and also because the stimuli used were not the same. In the human lymphocytes, is due to the difference in TNF-α production and the sensibility of cells from arthritic and healthy patients.

STAT-3 activation in human lymphocytes from healthy men and arthritic patients

Finally, cucurbitacin R inhibited the activation of STAT-3 by interleukin-6. It should be noted that there were clear differences between human lymphocytes obtained from healthy men and those from arthritic patients. For example, while in the latter group the STAT3 was activated, this was not the case in the lymphocytes from healthy donors. In both instances, however, cucurbitacin R was found to inhibit the activation of STAT3 induced by interleukin-6 (Figure 10).

Discussion

TNF-α and interleukin-1β play a relevant role in the pathogenesis of adjuvant arthritis, but other mediators such as interleukin-6, interleukin-15, interleukin-18, and leukotriene B$_4$ are also implicated in the process, with some of them playing an important role in neutrophil recruitment during immune-inflammation. TNF-α, which is involved in inflammation, differentiation and proliferation of T and B cells, and bone resorption, is the primary agent in the inflammatory process (Rioja et al., 2004) while interleukin-1β is responsible for the destruction of cartilage and bone (Cuzzocrea et al., 2000). Blocking TNF-α suppresses the inflammation and ameliorates cartilage destruction (Cuzzocrea et al., 2000), as this cytokine not only plays a relevant role in leukocyte recruitment to the articulations (Issecuts et al., 1994), but also regulates nitric oxide synthase-2 and cyclooxygenase-2 expression in the synovial tissue and cartilage of arthritic rats (Amin et al., 1999). This is significant as the metabolites of these enzymes, nitric oxide and prostaglandin E$_2$, have an essential function in the development of the inflammatory process (Fahmi, 2004).

Besides TNF-α, interleukin-6 also plays an important role in arthritis. In fact, a recent series of *in vivo* studies using models of experimental arthritis have established the role of interleukin-6 in joint destruction, leukocyte recruitment, apoptosis, and T-cell activation (Scheller et al., 2006). For its part, interleukin-15 is implicated in the recruitment and activation of T

lymphocytes and neutrophils; its neutralization has been found to produce an amelioration of inflammation and articular destruction in murine collagen-induced arthritis (Baslund et al., 2005). Interleukin-18 has the ability to induce the production of tumor necrosis factor-α and interleukin-1β in mononuclear cells, and to initiate a cytokine cascade with concomitant expression of pro-inflammatory markers such as chemokines, nitric oxide, adhesion molecules, and matrix metalloproteinase-9 (Muhl and Pfeilschifter, 2004). The inflammatory synovial thus predominantly contains neutrophils while, in addition to cytokines, leukotrienes are among the inflammatory mediators expressed in the inflamed joint (Chen et al., 2006). In patients with rheumatoid arthritis, elevated levels of leukotriene B_4 correlate with disease severity while leukocytes in the synovial fluid highly express BLT1 (Kim et al., 2006). Taking all this into consideration and in light of our results, the reduction of inflammation as well as that of cartilage destruction brought about by cucurbitacin R is probably due to decreases in TNF-α production, the induction of both nitric oxide synthase-2 and cyclooxygenase-2 observed in the damaged tissue, and a reduction in cell infiltration, but not to a decrease in leukotriene B_4 production, as was demonstrated in a previous paper (Recio et al., 2004).

Cucurbitacins are usually reported as cytotoxic compounds, but there are clear differences between the toxicity and activity of cucurbitacins depending on the pattern of substitution. There is described only a few studies on the *in vivo* toxicity cucurbitacins (Ríos et al., 2005). One conclusion was that their range of toxicity was between 2 and 12.5 mg/kg. However, in previous tests involving the *in vivo* toxicity of a mixture of cucurbitacins, including cucurbitacin R, we obtained an LD_{50} of 375 mg/kg p.o. and 67 mg/kg i.p. (Ríos et al., 1990). Musza et al. (1994) demonstrated that the presence of an acetyl group at C-25 of the chain clearly increases the toxicity while Oh et al. (2002) showed that the presence of a double bond at C-23 also produces an increase in the cytotoxicity of these compounds. The fact that these structural features are implicated in the pharmacological activity was further bolstered by work

carried out by Jayaprakasam et al. (2003), who demonstrated the selective inhibition of cucurbitacins with the acetyl group at C-25 against cyclooxygenase-2 versus cyclooxygenase-1 while showing that the same pattern of inhibition did not hold in the case of deacetyl derivatives such as cucurbitacin R. However, in our work this cucurbitacin (without an acetyl group) inhibited the cyclooxygenase-2 induction in paws and the prostaglandin E_2 production in both paws and stomach, which implies an inhibition of the activity of both cyclooxygenase-1 and cyclooxygenase-2. The results obtained for ibuprofen-treated rats were similar in stomach, but activity in paws was lower at higher dose, which could imply similar anti-inflammatory effect with lower gastric side effect at the same dose. In addition, in tests on the effects on isolated cells, cucurbitacin R at 100 µM reduced the prostaglandin E_2 production when administered before and after the induction of the enzyme, a fact which, together with the Western blot analysis in RAW 264.7 cells of cyclooxygenase-2, demonstrates the inhibition of the activity of the enzyme.

Our aim in studying the effects of cucurbitacin R on a model of experimental analgesia was thus to see the *in vivo* response to an algogenic stimulus. While previous experiments using doses of 5 mg/kg of cucurbitacin R produced no effect on the writhing induced by acetic acid in mice, in the present investigation we obtained a significant reduction of writhing when a higher dose of 10 mg/kg was used. This result corroborates findings previously reported by Peters et al. (1997), who tested a mixture of cucurbitacins against acetic acid-induced algesic response in mice. These same authors (Peters et al., 2003) demonstrated that inhibition of nitric oxide formation and cyclooxygenase activity is implicated in the analgesic activity of the cucurbitacins studied. Moreover, they demonstrated the peripheral effects of the treatment since cucurbitacins abolished the abdominal contortions in the writhing test, but had no effect in the hot plate and rota-rod test. Ribeiro et al. (2000) demonstrated that the nociceptive response induced by acetic acid depends on the peritoneal resident macrophages and mast cells through

the release of TNF-α, interleukin-1β, and interleukin-8. As for TNF-α, it has been found to play a pivotal role in the genesis of the nociceptive writhing response in mice. This effect should thus be diminished if TNF-α production is inhibited, a theory which may explain the *in vivo* effect of cucurbitacin R on the writhing test in mice.

The role of nitric oxide in the pathogenic damage of induced arthritis is subject to some controversy. It has been shown, for example, that the induction of nitric oxide synthase-2 and the consequent production of nitric oxide is one of the causes of pathogenesis of chronic inflammation in arthritis (McCartney-Francis et al., 2001), with non-specific inhibitors of NOS reducing experimental arthritis in animals (McCartney-Francis et al., 1993; Connor et al., 1995). However, selective inhibitors of inducible nitric oxide synthase have been found to exacerbate the chronic inflammatory response in experimental arthritis (Abramson et al., 2001; McCartney-Francis et al., 2001). Thus, since TNF-α regulates nitric oxide synthase-2 through nitric oxide production (Amin et al., 1999; Fahmi, 2004), and since cucurbitacin R was shown to reduce both the TNF-α production and nitric oxide synthase induction in cell culture without modifying the enzyme activity, this compound may actually be effective in reducing tissue damage, as the suppression of nitric oxide following treatment with TNF-α antagonists results in a reduction of inflammation and of the associated synovial pathology (Wahl et al., 2003).

Cucurbitacin R may be responsible for modifying nuclear factor-κB activation, since it not only inhibited the nitric oxide generation from murine macrophages activated with lipopolysaccharide and interferon-γ, but also blocked nuclear factor-κB activation, which is essential for the transcriptional activation of nitric oxide synthase induction (Park et al., 2001). Still, in our most recent experiment, cucurbitacin R did not seem to affect the nuclear factor-κB activation (data not shown). The difference in results could be due to the effect on the activation of the JAK2 and STAT3 pathway, which implicates the induction of apoptosis and

inhibition of cell growth via the modification of extracellular transduction from cytokines and growth factors, especially since STAT3 has been shown to inhibit immune responses by blocking the expression of pro-inflammatory factors (Wang et al., 2004). In fact, Sun et al. (2005) studied some related cucurbitacins and demonstrated that cucurbitacin Q inhibits the activation of STAT3, cucurbitacin A inhibits Janus kinase-2, and cucurbitacins B, E, and I inhibit the activation of both. This produces a consequent decrease in the pro-inflammatory factors, including TNF-α and interleukin-1β, which are produced by these cells in tissue as demonstrated by the fact that a negative effect on STAT3 reduces the TNF-α production in experimental arthritis while increasing the apoptosis of macrophages, synovial fibroblasts, and lymphocytes (Liu and Pope, 2003). Our experimental data confirm the inhibition of STAT3 activation in lymphocytes from both healthy and arthritic men. Moreover, Graness et al. (2006) recently studied cucurbitacin I and demonstrated that it produces alterations in the actin cytoskeleton and focal adhesions, which may modulate cell morphology, migration, adherence, and gene expression.

In conclusion, cucurbitacin R demonstrated an anti-inflammatory effect on an experimental model of induced arthritis, reducing both tissue and bone damage. The mechanism of action involves the reduction of TNF-α production, as well as the production of both nitric oxide and prostaglandin E_2. In addition, the induction of nitric oxide synthase is reduced, but there is no effect on cyclooxygenase. In these features, the inhibition of STAT3 activation is implicated while that of nuclear factor-κB is not.

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Footnotes.

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Legends for figures

Figure 1. The chemical structure of cucurbitacin R.

Figure 2**.** Rat footpad and body weight evolution in adjuvant arthritis. **A**) Effect of cucurbitacin R (1 mg /kg, p.o.), and ibuprofen (10 mg /kg, p.o.) on footpad swelling in adjuvant arthritis. Footpad volume was measured each day. Each point represents mean ± S.E.M. (vertical lines) in footpad volume. * $P < 0.05$; ** $P < 0.01$ compared with control, n = 8 rats/group. **B**) Effect of cucurbitacin R (1 mg /kg, p.o.), and ibuprofen (10 mg /kg, p.o.) on body weight. Rats were weighted daily. Each point represents mean ± S.E.M. in body weight. Two independent experiments were performed with similar results.

Figure 3. Radiography of the tibiotarsal joint of rats with adjuvant-induced arthritis 24 days after immunization. **A**) Control group: severe periarticular inflammation, tarse osteolisis, calcification of Achiles tendon insertion, moderate affectation of distal tibia and severe affectation of proximal metatarse bones; **B)** Ibuprofen group: mild periarticular inflammation, mild osteolisis and inflammation of plantar zone and tibio-tarse articulation; moderate calcification. **C)** Cucurbitacin R: mild periarticular inflammation, mild osteolisis of tarse-metatarse zone and mild calcification. X-Ray score for each parameter: (0) no damage; (1) mild; (2) moderate, and (3) severe. The figures are representative of paws of each experiment.

Figure 4. Histopathological analysis of haematoxylin-eosin-stained sections of paws obtained from arthritic rats without treatment (control group, 10×). **A**) Severe skin inflammation (a), with erosion of bone (b); **B**) bone destruction, presence of osteoclasts (arrows); **C**) bone destruction, inflammation and osteoclasts (a), bone formation with osteoblasts (b) and osteocytes (c). The figures are representative of at least three different images obtained from three different samples.

Figure 5. Haematoxylin-eosin-stained section (10×) of paws from; **A**) Blank: non-arthritic rats without treatment; **B**) Control (arthritic rats without treatment): presence of necrosis and severe inflammation (abscesses) of synovial tissue, articulation and bone; **C**) Arthritic rats treated with cucurbitacin R (1 mg/kg/day orally during 7 days): inflammation was reduced respect to the control group; and **D**) Arthritic rats treated with ibuprofen (10 mg/kg/day orally during 7 days): signs of inflammation were reduced respect to the control group. The figures are representative of at least three different images obtained from three different samples.

Figure 6. Immunohistological analysis of cyclooxygenase-2 expression in rat footpad tissues obtained from: **A)** Arthritic rats without treatment (control group); **B)** Arthritic rats treated with cucurbitacin R (1 mg/kg/day orally during 7 days); and **C)** Arthritic rats treated with ibuprofen (10 mg/kg/day orally during 7 days). Arrows indicate the presence of cells expressing enzyme. n = 3. The figures are representative of at least three different images obtained from three different samples.

Figure 7. Cucurbitacin R (1 mg/kg) suppressed cyclooxygenase-2 and nitric oxide synthase expression in arthritic rat paws, prostaglandin E_2 production in stomachs and arthritic rat paws and TNF-α production in arthritic rat paws. **A**) Effects of cucurbitacin R and ibuprofen on cyclooxygenase-2 and nitric oxide synthase expression in arthritic rat paws and inhibition percentage of ibuprofen and cucurbitacin R-treated rats versus control group. Data were normalized with β-actin. **B**) Prostaglandin E_2 productions in arthritic rat paw and stomach homogenates from ibuprofen-, and cucurbitacin R-treated groups. **C**) Effect of ibuprofen and cucurbitacin R on TNF-α production in arthritic rat paws. *$P<0.05$ and **$P<0.01$ (Dunnett's *t*-test). Data are the mean of three independent experiments. The figures are representative of three different experiments.

Figure 8. **A)** Effects of cucurbitacin R (25, 50 and 100 µM), dexamethasone (10 µM) and L-NAME (1 mM) on nitric oxide production in RAW 264.7 murine macrophages during induction and post-induction phases. **B)** Effects of cucurbitacin R (25, 50 and 100 µM), dexamethasone (10 µM) and indomethacin (10 µM) on prostaglandin E_2 production in RAW 264.7 murine macrophages during induction and post-induction phases. **C)** Effects of dexamethasone and cucurbitacin R on cyclooxygenase-2 and nitric oxide synthase-2 expression in RAW 264.7 cells; macrophages were treated with 100, 50 and 25 µM of cucurbitacin R for 1 h, and then activated with lipopolysaccharide. The results are expressed as mean ± S.E.M.; *$P<$ 0.05, and **$P<$ 0.01 compared with control (Dunnett's t-test). Three independent experiments were performed with similar results.

Figure 9. Effects of dexamethasone and cucurbitacin R on TNF-α. **A)** Macrophages RAW 264.7; cells were treated with 100 to 6.25 µM of cucurbitacin R for 1 h, and then activated with lipopolysaccharide (1 µg/ml). **B)** Human lymphocytes from healthy men; cells were treated with 100 to 3.75 µM of cucurbitacin R for 1 h, and then activated with phytohemagglutinin (25 µg/ml). **C)** Human lymphocytes from arthritic patients; cells were treated with 100 to 3.75 µM of cucurbitacin R for 1 h, and then activated with phytohemagglutinin (25 µg/ml). The results are expressed as mean ± S.E.M.; *$P<0.05$ and **$P<0.01$ compared with control (Dunnett's t-test). Three independent experiments were performed with similar results. At 100 µM cucurbitacin R did not show toxicity against RAW 264.7 cells nor human lymphocytes.

Figure 10. Activation of STAT3 in interleukin-6 stimulated human T lymphocytes. Cells were analyzed by Western blotting with specific anti-phospho-STAT3. Lymphocytes were stimulated with interleukin-6 (20 ng/ml) for 30 min. **A)** T cells from arthritic patients, n = 3; **B)** T cells from healthy donors, n = 5. Three or five independent experiments were performed with similar results.

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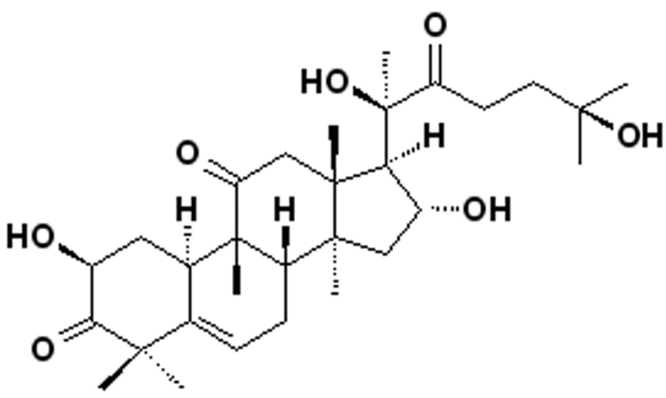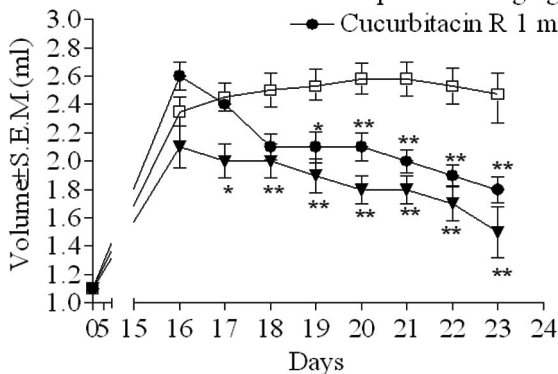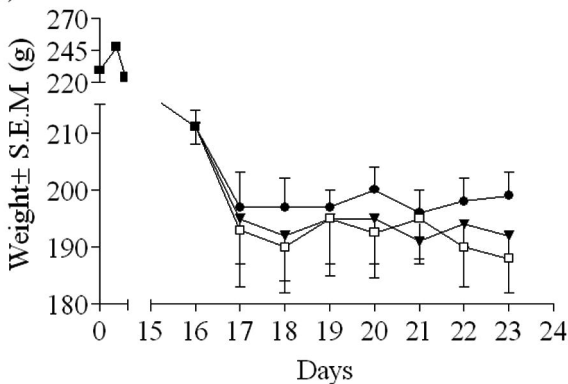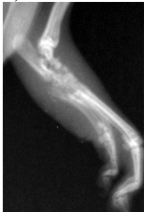
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Osteolisis	(3)	(1)	(2)
Articular cartilage affection	(3)	(1)	(2)
Osteophyte formation	(3)	(1)	(1)

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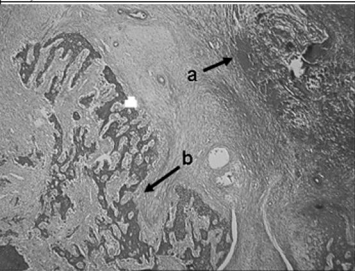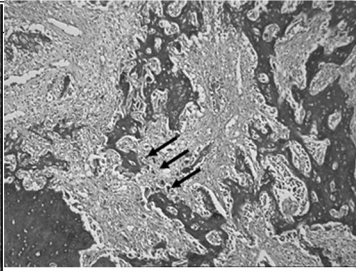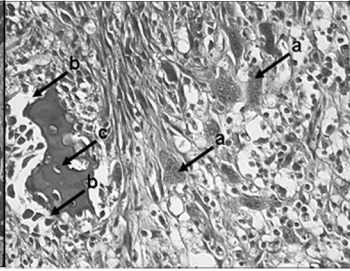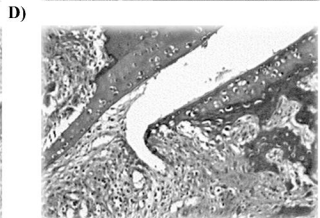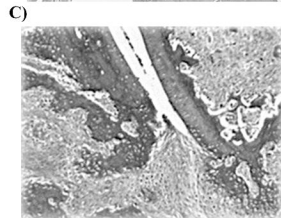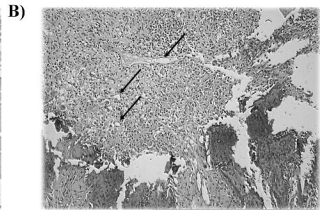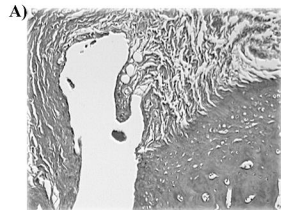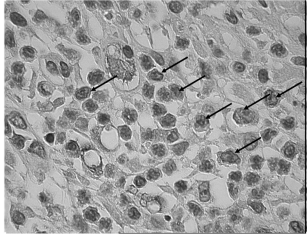
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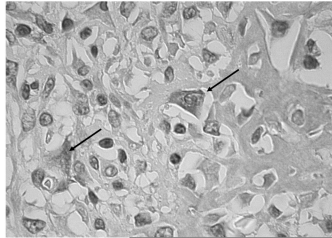
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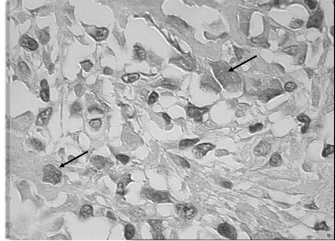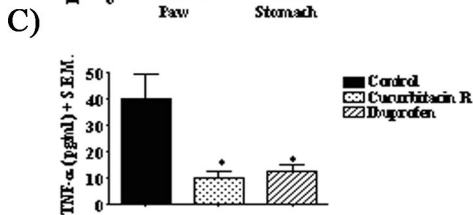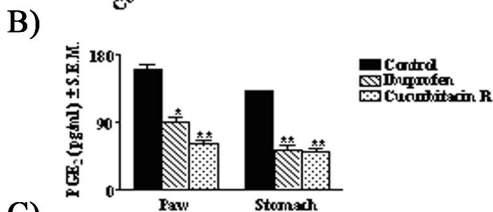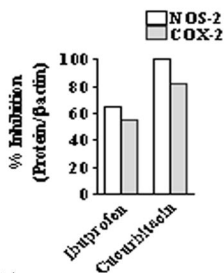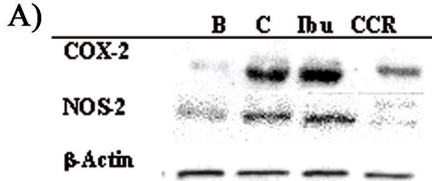
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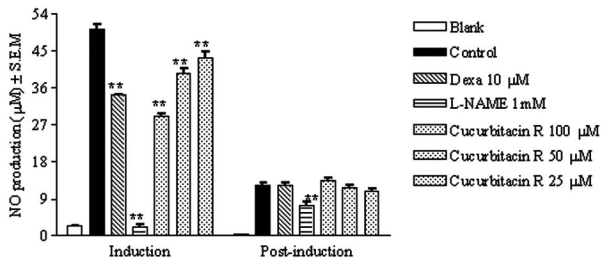
B)
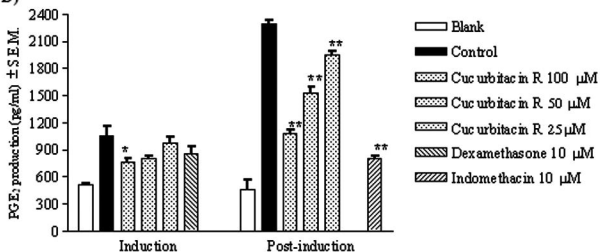
C)

		B	C	D	CCR		
Concentration (µM)	-	-	10	100	50	25
COX-2							
NOS-2							
β-Actin							
LPS	-	+	+	+	+	+

A)

$IC_{50} = 65 \ \mu M$

Legend:
- ☐ Blank
- ■ Control
- ▨ Dexamethasone 10 μM
- ▦ Cucurbitacin R

y-axis: [TNF-α] ± S.E.M. (ng/ml)
x-axis (μM): 100, 50, 25, 12.5, 6.25

B)

$IC_{50} = 29 \ \mu M$

y-axis: TNF-α ± SEM (pg/ml)
x-axis (μM): 100, 50, 37, 25, 19, 12.5, 6, 3

C)

$IC_{50} = 7 \ \mu M$

y-axis: TNF-α ± SEM (pg/ml)
x-axis (μM): 100, 50, 25, 18, 9, 4, 3, 1.5
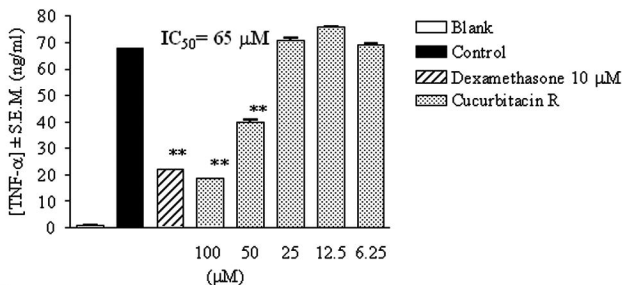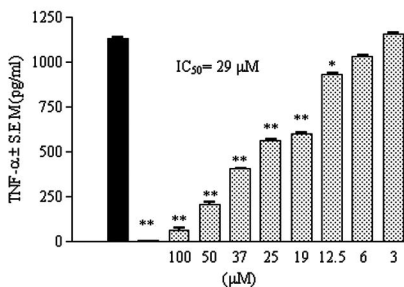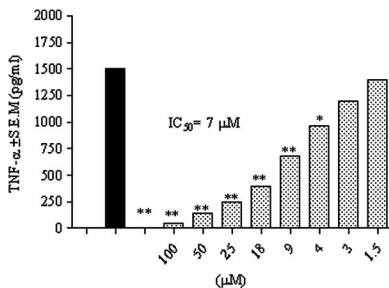
A)	Blank	IL-6	CCR+IL-6
STAT3-P Arthritic lymphocytes			
β-actin			

B)	Blank	IL-6	CCR+IL-6
STAT3-P Normal individues			
β-actin			