

Dihydrocucurbitacin B inhibits delayed-type hypersensitivity reactions
by suppressing lymphocyte proliferation

José M. Escandell, M. Carmen Recio, Salvador Máñez, Rosa M. Giner, Miguel
Cerdá-Nicolás, Rosario Gil-Benso and José-Luis Ríos

*Departament de Farmacologia, Facultat de Farmàcia, Universitat de València, Burjassot,
Spain (J.M.E., M.C.R., S.M., R.M.G., J.-L.R.); Departament de Patologia, Facultat de
Medicina, Universitat de València, Valencia, Spain (M.C.-N., R.G.)*

Running Title:

a) Dihydrocucurbitacin B inhibits DTH reactions

b) Address correspondence to: Prof. José Luis Ríos, Departament de Farmacologia, Facultat de Farmàcia, Universitat de València, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain.

Phone / Fax: +34-963 544 973, E-mail: riosjl@uv.es

c)

Number of text pages: 28

Number of tables: 0

Number of figures: 9

Number of references: 28

Number of words in Abstract: 173

Number of words in Introduction: 691

Number of words in Discussion: 1085

d) Abbreviations

DTH, Delayed-type hypersensitivity; EDTA, ethylenediamine tetraacetic acid; ELISA, Enzyme Linked Immunoabsorbent Assay; EMSA, electrophoretic mobility shift assays; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NF-AT, nuclear factor of activated T cells; PBS, phosphate buffered

saline; PCR, polymerase chain reaction; SRBC, sheep red blood cells; STAT, signal transducer and activator of transcription; TNF- α , tumor necrosis factor- α .

e) Recommended section(s): Inflammation, Immunopharmacology, and Asthma

ABSTRACT

We have studied the effects of dihydrocucurbitacin B, a triterpene isolated from *Cayaponia tayuya* roots, on different models of delayed-type hypersensitivity (DTH) in mice, as well as on T lymphocyte proliferation and the mediators involved. In experiments with mice, dihydrocucurbitacin B inhibited the inflammatory reactions induced by oxazolone, dinitrofluorobenzene, and sheep red blood cells (SRBC), reducing both the edema and cell infiltration. Moreover, the analysis of inflamed tissues showed that dihydrocucurbitacin B reduced the presence of the most relevant cytokines implicated in these processes, including interleukin-1 β , interleukin-4, and tumor necrosis factor- α (TNF- α). Dihydrocucurbitacin B was also found to inhibit the proliferation of phytohemagglutinin-stimulated human T lymphocytes ($IC_{50} = 1.48 \mu M$), halting the cell cycle in the G₀ phase. In addition, the triterpene reduced the production of interleukin-2, interleukin-4, interleukin-10, and interferon- γ in human T lymphocytes and hampered the induction of the principal cyclins involved in the cell cycle, including A₁, B₁, D₂, and E₁. Finally, dihydrocucurbitacin B was found to exert a selective inhibition on the nuclear factor of activated T cells (NF-AT) in human lymphocytes without affecting the calcium influx. Taken together, these results suggest that dihydrocucurbitacin B curbs delayed-type hypersensitivity reactions by inhibiting NF-AT, which, in turn, suppresses the proliferation of the most relevant cells involved in DTH reactions, namely the T cells.

Introduction

Cucurbitacins, a group of phytochemicals with anti-inflammatory activities, have been shown to exert specific effects against various inflammatory processes, including contact dermatitis (Ríos et al., 2000; 2005). 23,24-Dihydrocucurbitacin B, a triterpenoid isolated from tayuya roots, has been shown to exert anti-inflammatory activity on different experimental models of inflammation (Recio et al., 2004). For example, it has a significant effect on adjuvant-induced arthritis in rats, reducing both inflammation and tissue damage and inhibiting the expression of several enzymes and mediators implicated in the inflammatory process, including elastase, cyclooxygenase, nitric-oxide synthase-2, and tumor necrosis factor- α (TNF- α) (Escandell et al., 2006). Moreover, dihydrocucurbitacin B has been found to possess potential anticancer properties and could have applications as an immunodepressant, acting by means of a mechanism in which the inhibition of both TNF- α and interleukin-1 β in lymphocytes is implicated. It is also possible that dihydrocucurbitacin B operates by activating both the Janus kinase-2 pathway along with that of the signal transducer and activator of transcription-3 (STAT3). This mechanism seems likely since a closely related compound, cucurbitacin B, has been shown to activate these pathways in diverse tumor cell lines (Sun et al., 2005).

Delayed-type hypersensitivity (DTH) is a reaction triggered by antigen-specific T cells that can be induced by different allergens. The most common of these are oxazolone and dinitrofluorobenzene, which both induce contact dermatitis, and sheep red blood cells (SRBC), which lead to tuberculinic reactions. Contact hypersensitivity, a type of DTH, occurs in two phases: induction and elicitation. In the first phase, the hapten reacts with protein to form a complex that is recognized by Langerhans cells, which then migrate to the lymph nodes, where CD4⁺ lymphocytes recognize the antigen. Further contact with the hapten gives rise to the recruitment of antigen-specific T cells, resulting in erythema, edema, and, consequently, severe

dermal inflammation (Scott et al., 2002). These symptoms are caused by the release of mediators by Tc1 cells (interferon- γ), Th1 cells (interleukin-2, interferon- γ , and TNF- α), Th2 cells (interleukin-4 and interleukin-10), and Langerhans cells (interleukin-12 and interleukin-18) (Garaczi et al., 2004), together with the release of nitric-oxide by keratinocytes and Langerhans cells (Ross and Reske-Kunz, 2001). There is also a concomitant release of interleukin-1 β (Zunic et al., 1998), leukotrienes, and prostaglandins by macrophages (Meurer et al., 1988).

The application of oxazolone to mice produces both swelling and increased tissue levels of the aforementioned mediators. For example, after sensitization with oxazolone, CD8⁺ Tc1 cells increase interferon- γ production, which plays a relevant role in the inflammatory cutaneous reaction (Xu et al., 1996). The biochemical processes in DTH reactions induced by dinitrofluorobenzene are similar, with some variations observed depending on the time between application and measurement. What is clear is that both CD4⁺ Th1 cells and CD8⁺ Tc1 lymphocytes are required as effector cells for oxazolone- and dinitrofluorobenzene-induced contact hypersensitivity to develop fully (Wang et al., 2000). DTH induced by SRBC, on the other hand, is an immunological reaction mediated by specifically committed T cells. It peaks consistently at 18 h, elicits the maximum response at day 4 after immunization, and then decreases progressively until the fourth month (Hurtrel et al., 1984).

The pharmacological response to different agents depends on the mechanism implicated in the anti-inflammatory process after a DTH reaction. Thus, contact hypersensitivity reactions may be reduced either by specific inhibitors of cytokine expression, including interleukin-1 β and TNF- α (Zunic et al., 1998), or by pro-inflammatory enzymes such as inhibitors of inducible nitric-oxide synthase (Ross and Reske-Kunz, 2001) and corticosteroids (Belsito, 2000). Interestingly, while specific inhibitors of cyclooxygenase or 5-lipoxygenase were found to

reduce the presence of individual mediators, they had no effect on the swelling in oxazolone-induced DTH in mice. In fact, only corticosteroids reduced both the edema and the presence of inflammatory mediators in this case (Meurer et al., 1988).

In this study, we describe the pharmacological effects of dihydrocucurbitacin B on three models of DTH induced by different agents in order to gain insight into the anti-inflammatory activity of this compound on different DTH processes. In addition, we outline the effects of dihydrocucurbitacin B on different pro-inflammatory mediators and on the lymphocyte cell cycle in order to better understand its possible mechanism of action.

Material and Methods

Reagents and Chemicals

Dihydrocucurbitacin B (Figure 1) was previously obtained from tayuya roots (Recio et al., 2004). Chemical and biochemical reagents were purchased from Merck (Darmstadt, Germany), Panreac (Barcelona, Spain), and Sigma-Aldrich (St. Louis, MO, USA). Antibodies were obtained from Santa Cruz biotechnologies (Santa Cruz, CA, USA). Enzyme linked immunoabsorbent assay (ELISA) kits for cytokine detection were purchased from eBioscience (San Diego, CA, USA) and biotin-conjugated goat anti-rabbit immunoglobulin G and avidin-biotin peroxidase complex LSAB 2 System-HRP were obtained from Dako (Glostrup, Denmark).

Animals

Groups of six, female, 8-week-old Swiss mice weighing 25-30 g (Harlan Interfauna Iberica, Barcelona, Spain) were used. 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 (University of Valencia, Spain) in accordance with the guidelines established by the European Union on Animal Care (CEE Council 86/609).

Delayed-type hypersensitivity (DTH) induced by oxazolone, dinitrofluorobenzene, and sheep red blood cells (SRBC)

Female mice were sensitized through topical application of 20 μ l of 0.2% (v/v) dinitrofluorobenzene (Sigma-Aldrich) on days 1 and 2. Challenge was then performed on day 6 by applying dinitrofluorobenzene (20 μ l, 0.2% v/v) on the inner and outer ear surfaces. Dihydrocucurbitacin B (0.1, 0.3, and 0.5 mg/ear) in acetone was topically applied (20 μ l) to the ear 2, 24, 48, and 72 h after challenge. Ear thickness measurements of the treated and control groups were taken with a micrometer (Mitutoyo Series 293, Kawasaki, Japan) and the edema was calculated for each ear as the difference in thickness before treatment and 24, 48, and 72 h after challenge.

In the oxazolone-induced delayed hypersensitivity assay (Bas et al., 2007) mice were treated with dihydrocucurbitacin B at 0.1, 0.3, and 0.5 mg/ear while in the SRBC assay (Bas et al., 2007) dihydrocucurbitacin B was administered i.p. (10 mg/kg). Inhibition percentages are expressed with respect to the control group, which was treated only with the inducing agent (oxazolone, dinitrofluorobenzene, or SRBC). Doses were established according to previous data obtained for this compound by Recio et al. (2004) and Escandell et al. (2006).

Histology and immunohistochemistry

Ear samples fixed in 4% neutral-buffered formalin were cut longitudinally as described previously by Escandell et al. (2007). The inflammatory cells – lymphocytes, macrophages, and neutrophils – were counted in the papillary and in the reticular dermis/subcutis layers within an

area of 1000 μm^2 . For all immunohistochemistry assays (Escandell et al., 2007), antibodies (Santa Cruz biotechnologies) of CD4⁺ (SC1140) and CD8⁺ (SC7188) were used.

Cytokine measurement from *ex vivo* samples

Tests to determine the presence of cytokines in ears and paws from the control and dihydrocucurbitacin B-treated groups were performed. The skin was removed from the ears and paws, which were then homogenized with a polytron (Kinematica AG, Luzern, Switzerland) in 2.5 ml of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma-Aldrich), 2 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich), 100 mM ethylenediamine tetraacetic acid (EDTA, Sigma-Aldrich), 0.32 M sucrose (Sigma-Aldrich), 1 mM dithiothreitol (Sigma-Aldrich), 2 mg/ml aprotinin (Sigma-Aldrich), and a complete mini EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The mixture was sonicated (3 cycles in 10 s) in a Branson 150 sonifier (Danbury, CT, USA) and centrifuged (Eppendorff centrifuge 5810R, Hamburg, Germany) at 10000 $\times g$ for 20 min at 4° C. TNF- α , interleukin-1 β , and interleukin-4 were measured with the aid of an enzyme immunoassay (eBioscience, San Diego, CA, USA) employed according to the manufacturer's instructions.

T lymphocyte proliferation assay

Peripheral lymphocytes were obtained from human blood buffy-coats. Cells were isolated as described by Bas et al. (2007). A volume of 200 μl of cell suspension was applied onto each well of a 96-well flat-bottomed plate (Nunc A/S, Roskilde, Denmark) with or without 25 $\mu\text{g}/\text{ml}$ phytohemagglutinin (Sigma-Aldrich). Either dihydrocucurbitacin B at concentrations ranging from 1.0 – 2.5 μM or 5 μM dexamethasone were added to the cells. The plates were incubated in 5% CO₂-air-humidified atmosphere at 37° C for 4 days, after which T cell proliferation was determined with the aid of a modified colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium (MTT) assay. Controls consisted of lymphocytes with phytohemagglutinin (100% activity) or medium (0% activity). There were also control samples with nonstimulated lymphocytes. The kinetic studies were performed after the addition of dihydrocucurbitacin B (final concentration: 3 μ M) at 0, 3, 6, 10, 18, 24, 48, 72, and 96 h after stimulation. Lymphocyte proliferation measurements were performed as described above.

Cell cycle analysis

T lymphocytes were adjusted to a final relation of (1×10^6) cells/ml before use and then 1 ml of cell suspension was placed into each well of a 24-well flat-bottomed plate (Nunc), with or without 25 μ g/ml phytohemagglutinin. Then, either dihydrocucurbitacin B (2.5 μ M), dexamethasone (5 μ M), or aphidicolin (0.75 μ M) (Sigma-Aldrich) were added to the cells. The plates were incubated under a 5% CO₂-air-humidified atmosphere at 37° C for different time periods, after which the cells were harvested through centrifugation, washed in phosphate buffered saline (PBS, pH 7.2), and fixed in 70% ethanol (Sigma-Aldrich) for 30 min at -20° C. The cells were then washed and stained once with PBS. DNA was stained with propidium iodide (4 μ g/ml, Sigma-Aldrich) containing 100 μ g/ml of ribonuclease A (Sigma-Aldrich). Flow cytometry analysis was carried out on a Beckman Coulter Epics XL-MCL device (Fullerton, CA, USA).

Determination of cell viability and cytotoxicity

T lymphocytes were cultured to a final relation of 1×10^6 cells/ml in a supplemented medium and stimulated with phytohemagglutinin (25 μ g/ml). After four days, 100 μ l of a solution of 50 μ g/ml of propidium iodide was added and cell viability was measured 30 min later with a flow cytometry Beckman Coulter Epics XL-MCL device.

Determination of cytokine production in T lymphocytes

T lymphocytes (2×10^5 cells/well) were cultured with phytohemagglutinin alone or in combination with various concentrations of dihydrocucurbitacin B for 4 days. The cell supernatants were then collected and assayed for the presence of interleukin-2, interleukin-4, interleukin-10, and interferon- γ by means of an enzyme linked immunoabsorbent assay (ELISA, detection limit of immunoassay: 4 pg/ml) (eBioscience, San Diego, CA, USA).

Extraction of total cellular RNA

Cells (5×10^6) were treated with or without phytohemagglutinin and cultured with dihydrocucurbitacin B for 18 h. T cells were collected and total RNA was extracted with the aid of RNeasy mini spin (50) columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions and then redissolved in diethyl pyrocarbonate- (Sigma-Aldrich) treated H₂O. The concentration of the extracted RNA was calculated by measuring the optical density at 260 nm. The ratio of the optical density at 260 nm to that at 280 nm was always higher than 1.8.

RT-Polymerase Chain Reaction (PCR)

Aliquots of 1 μ g of RNA were transformed to first strand complementary deoxyribonucleic acid (cDNA) with Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega Biosciences, San Luis, CA, USA). Then, 2.5 μ l of the resulting cDNA were mixed with 0.75 μ M primers (Invitrogen, Langley, OK, USA) of interferon- γ (sense: 5'-GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC-3'; antisense: 5'-CTCCTTTTTTCGCTTCCCTGTTTTAGCT GCT GG-3'), interleukin-2 (sense: 5'-AACAGTGCACCTACTTCAAG-3'; antisense: 5'-GTTGAGATGATGCTTTGACA-3'), interleukin-4 (sense: 5'-ATGGGTCTCACCTCCCAACTGCT-3'; antisense: 5'-GAACACTTTGAATATTTCTCTCTCAT-3'), interleukin-10 (sense: 5'-ATCCCCCAAGCTGAGAACCAAGACCCA-3'; antisense: 5'-

TCTCAAGGGGCTGGGTCAGCTATCCCA-3'), and β -actin (sense: 5'-GCAGAGCAAGAGAGGCATCC-3'; antisense: 5'-CTGTGGTGGTGAAGCTGTAG-3').

The thermocycler conditions were 94° C for 1 min, with an annealing temperature of 60° C for 1 min and an elongation temperature of 72° C for 1 min for the first 30 cycles, followed by an elongation temperature of 72° C for 10 min. After the reaction, the amplified product was removed from the tubes and run on 2% agarose gel (Sigma-Aldrich).

Western blot analysis

Cellular lysates from lymphocytes incubated for 24 h with phytohemagglutinin were obtained as described previously by Escandell et al. (2007). For cyclins A (Santa Cruz, H-230), B (Santa Cruz, H-433), D₂ (Santa Cruz, H-289), and E (Santa Cruz, C-19), membranes were incubated with their respective polyclonal antibodies (1:200 dilution). For β -actin (Sigma-Aldrich), membranes were incubated with monoclonal antibody (1:10000 dilution). Western blot quantification was carried out with the aid of Scion image software version 1.0.0.1 (Frederick, MA, USA).

Electrophoretic mobility shift assays (EMSA)

Human lymphocytes were incubated with 2.5 μ M dihydrocucurbitacin B for 1 h. The cells were then stimulated with phytohemagglutinin for an additional 1 h, after which the nuclear protein was extracted. The protein content of the supernatant was determined with the aid of Bradford reagent and then 10 μ g of nuclear protein were loaded into a 6% non-denatured polyacrilamide gel. EMSAs were carried out with a DIG-gel shift kit (Roche Diagnostics, Mannheim, Germany).

Determination of calcium cell influx

Lymphocytes (0.5×10^6) were placed into supplemented RPMI with 1.5 mM Ca^{2+} and then 4 μM of FLUO-3 (Invitrogen) were added for each incubation. The cells were then treated with dihydrocucurbitacin B (2.5 μM) and the suspension was mixed and incubated for 1 h at 37° C in 5% CO_2 in air. The FLUO-3 loaded cells were then measured for 30 s before administering lectins. Changes in relative fluorescence intensity were recorded at 10 s intervals for 300 s with the aid of a Beckman Coulter Epics flow cytometer. All flow cytometric experiments were repeated at least three times.

Statistics

Data were expressed as mean $\pm$ S.E.M. First, a one-way analysis of variance (ANOVA) was performed, followed by Dunnett's *t*-test for multiple comparisons. When making comparisons with the control group, values of *P* less than 0.05 were considered significant. Inhibition percentages (%I) were calculated from the differences between the drug-treated group and control animals treated only with the inflammatory agent. Inhibitory concentration-50 (IC_{50}) and effective dose-50 (ED_{50}) values were calculated from dose/response linear regression plots.

Results

Effects on DTH induced by oxazolone, dinitrofluorobenzene, and SRBC

The administration of dihydrocucurbitacin B at different doses (0.1, 0.3, and 0.5 mg/ear) reduced the oxazolone-induced DTH reaction, but had no clear dose-response effect. Thus, at 24 h, edema inhibition was 39% (0.3 mg/ear), with no increase in the effect observed at higher doses. At 72 h, the effect was actually higher at a dose of 0.3 mg/ear than at a dose 0.5 mg/ear (Fig. 2A). For dinitrofluorobenzene-induced DTH, the compound inhibited the reaction at doses of 0.3 mg/ear (29%, 30%, and 47%, at 24, 48, and 72 h, respectively) and 0.5 mg/ear (26%, 54%, and 60%, at 24, 48, and 72 h, respectively), but had no significant effect at 0.1

mg/ear (Fig. 2B). Interestingly, it reduced the DTH reaction by 56% when administered together with the allergenic product (data not shown). In the case of the DTH reaction induced by SRBC in mouse paws, dihydrocucurbitacin B at a dose of 10 mg/kg (i.p.) clearly and significantly reduced the reaction by 54% at 18 h and by 57% at 24 h, but the effect disappeared at 48 h (Fig. 2C). Finally, the levels of TNF- α , interleukin-1 β , and interleukin-4 were all reduced by dihydrocucurbitacin B in animals treated with oxazolone (Fig. 2D) or dinitrofluorobenzene (Fig. 2E). In both cases there was a correlation between the reduction of cytokines and the anti-edema effect, although the effect of dihydrocucurbitacin B was higher in the dinitrofluorobenzene model (Fig. 2E).

The histological study of the ears from the control group treated only with oxazolone showed a characteristic inflammatory lesion that affected the dermis with both edema and cell infiltration in which the ratio of neutrophils:lymphocytes:macrophages was 3:2:1 (Fig. 3A1). The ear treated with dihydrocucurbitacin B (Fig. 3B1) presented histological changes with respect to the control group. The differences included the infiltration of fewer neutrophils, a milder inflammatory lesion, a reduction of edema and inflammatory cell infiltration, and a reduction of epithelium thickness. Other parameters of the inflammatory process such as papillomatosis and spongiosis were also attenuated in both dihydrocucurbitacin B-treated groups. There was no clear difference in the CD4⁺ and CD8⁺ populations between the treated and control groups (data not shown).

The ears from mice treated only with dinitrofluorobenzene (control group) showed a characteristic inflammatory lesion similar to that described above (Fig. 3A2). In contrast, the ears treated with dihydrocucurbitacin B (Fig. 3B2) presented a milder inflammatory lesion along with reduced edema and inflammatory cell infiltration. Papillomatosis and spongiosis were also attenuated in the dihydrocucurbitacin B-treated group. While dihydrocucurbitacin B

reduced T cell infiltration in inflamed tissue, once again there was no clear effect on the CD4⁺ and CD8⁺ populations in the treated group (data not shown).

The histological examination of the paws of animals treated only with SRBC (control group) showed a characteristic inflammatory lesion in the connective periarticular tissues, as well as intense, acute inflammation with an abundance of polymorphonuclear leukocytes (data not shown). The paws treated with dihydrocucurbitacin B presented a mild inflammation with reduced infiltrate of a mixture of polymorphonuclear leukocytes and lymphocytes (data not shown).

Analysis of cell toxicity and effect of dihydrocucurbitacin B on T-lymphocyte proliferation

Dihydrocucurbitacin B exhibited no cell toxicity at doses of 10 μ M or lower. In the cell proliferation assay, T lymphocytes were first incubated with phytohemagglutinin for 4 days, which stimulated cell proliferation by 300%. Addition of dihydrocucurbitacin B to these activated T lymphocytes totally inhibited cell proliferation. Thus, the compound was found to suppress phytohemagglutinin-treated cell proliferation by 100% at 2.5 μ M, with an IC₅₀ value of 1.49 μ M ($r^2 = 0.9998$, range assayed 2.5 – 1.0 μ M, 5 dilutions, n = 3).

Effect of dihydrocucurbitacin B on the cell cycle

T lymphocytes stimulated with phytohemagglutinin typically enter into the G₁ phase within 2-4 h, the S phase at 18-24 h, and finally reach the G₂/M phase after 36-48 h. Because dihydrocucurbitacin B was found to inhibit cell proliferation totally (see above), we set out to determine in which phase the T cell cycle was arrested. We found that resting T lymphocytes stayed mainly in the G₀/G₁ phase 96 h after incubation with or without dihydrocucurbitacin B. Cells incubated without the compound and stimulated with phytohemagglutinin changed from

the G_0/G_1 phase to the S and G_2/M phases (Fig. 4A) while stimulated cells that had been previously treated with dihydrocucurbitacin B (2.5 μM), generally remained in the G_0/G_1 phase with only a very reduced passage to the S phase. Dexamethasone (5 μM) also modified the cycle of phytohemagglutinin-stimulated cells, halting it in the G_0 phase of lymphocyte proliferation. In a parallel assay, aphidicolin (0.75 μM) stopped the cycle in the S phase. In addition, a blank group was assayed to determine the normal evolution of the cell cycle (Fig. 4A). After running all the assays, we calculated the percentage of T cells in the G_0/G_1 , S, and G_2/M phases in each case. The results are shown in Figure 4B. In an additional study, the effect of dihydrocucurbitacin B on the cell cycle and proliferation of T lymphocytes after phytohemagglutinin stimulation was examined. The results indicate that addition of dihydrocucurbitacin B (2.5 μM) 0 and 3 h after phytohemagglutinin stimulation inhibited the passage of the cells from G_0/G_1 to S phase by 96% and 97%, respectively. In contrast, addition of dihydrocucurbitacin B (2.5 μM) between 6 and 72 h produced a gradual increase in the percentage of cells in the S and G_2/M phases (Fig. 5A). Again, the percentage of T cells in the various phases was calculated in each case (Figure 5B).

Effects of dihydrocucurbitacin B on cytokine production in human T lymphocytes

We then went on to study the production of interferon- γ , interleukin-2, interleukin-4, and interleukin-10 by T lymphocytes treated with dihydrocucurbitacin B at different concentrations (2.5 – 1.0 μM) in order to gain insight into the relationship between these cytokines, cell proliferation, and cell cycle progression, as well as to determine the implications of their inhibition on the inflammatory process. The effects of dihydrocucurbitacin B on cytokine production (pg/ml) were compared with those from the blank group (non-treated cells), the control group (phytohemagglutinin-stimulated cells), and the positive control group (phytohemagglutinin-stimulated cells treated with 5 μM dexamethasone). The compound was

found to inhibit significantly the production of interferon- γ , interleukin-2, and interleukin-10 in stimulated cells (Fig. 6B,6C,6D). There was also a slight reduction of interleukin-4 production, but the effect was not significant (Fig. 6A).

Because the production of cytokines in activated T lymphocytes decreased in the presence of dihydrocucurbitacin B, we also carried out an RT-PCR analysis to determine the effects of this compound on the cytokine mRNA expression in activated T cells. After extracting total cellular RNA from activated T lymphocytes in either the presence or absence of dihydrocucurbitacin B (2.5 μ M), we found that mRNA levels of interferon- γ , interleukin-2, interleukin-4, and interleukin-10 from stimulated T lymphocytes were all attenuated by dihydrocucurbitacin B (Fig. 7A, line 4) with respect to the control (Fig. 7A, line 2), which measured β -actin mRNA expression (Fig. 7A). Moreover, a densitometry analysis demonstrated that the ratios of cytokine mRNA decreased with respect to β -actin mRNA in phytohemagglutinin-stimulated T lymphocytes (Fig. 7B).

Effects of dihydrocucurbitacin B on cyclin expression and transcription factor in human lymphocytes and on calcium cell influx

As indicated above, the phytohemagglutinin-stimulated cells remained in the G_0/G_1 phase, with only very reduced passage to the S phase when cocultured with dihydrocucurbitacin B. Because cyclins are involved in the progression of cells throughout the cell cycle, we tested the effects of dihydrocucurbitacin B on the most relevant cyclins in this process. At a concentration of 2.5 μ M, dihydrocucurbitacin B was found to inhibit the expression of cyclins A₁, B₁, D₂, and E₁ (Fig. 8A) as was demonstrated by densitometry analysis (Fig.8B).

We went on to test the effect of dihydrocucurbitacin B on the nuclear factor of activated T cells (NF-AT) and the influx of calcium. NF-AT is controlled by calcineurin while a Ca²⁺-dependent phosphatase controls and activates the genes that encode cytokines and their receptors in

response to antigenic stimulation of immune cells. Binding sites for NF-AT proteins are present in the promoter/enhancer regions of several inducible genes, including the cytokines interleukin-2, interleukin-4, and interferon- γ , along with other genes related to the cell cycle. While dihydrocucurbitacin B (2.5 μ M) inhibited the activation of NF-AT in phytohemagglutinin-stimulated T lymphocytes (Fig. 9, line 4) with respect to the control group (Fig. 9, line 3), it did not modify the calcium influx into these same cells (data not shown).

Discussion

Application of a hapten such as oxazolone or dinitrofluorobenzene after a previous sensitization produces severe edema in the reticular dermis, mild edema in the papillary dermis, and severe mixed inflammatory cellular infiltration throughout the dermis and subcutis (Chapman et al., 1986). Different mediators are implicated in these processes, including nitric-oxide, arachidonate metabolites, and proinflammatory cytokines such as interleukin-1 β , interleukin-2, interleukin-4, TNF- α , and interferon- γ , which all play a relevant role in the development of contact hypersensitivity in mice after stimulation with allergens (Thomson et al., 1993; Xu et al., 1996; Ross et al., 1998; Bruch-Gerharz et al., 1998).

Our results demonstrate that when administered topically, dihydrocucurbitacin B not only suppresses the inflammatory response, but also reduces both the edema and the cellular infiltrate while simultaneously reducing the expression of interleukin-1 β , interleukin-4, and TNF- α in inflamed tissue. Interleukin-1 β is required for hapten-specific T cell priming in the sensitization phase after challenge as well as for the induction and production of TNF- α , which is necessary for the induction of local inflammation during the elicitation phase (Nakae et al., 2003). Furthermore, the production of TNF- α and interleukin-1 β regulates the expression of nitric-oxide synthase and cyclooxygenase-2 (Bruch-Gerharz et al., 1998; Amin et al., 1999). Interleukin-4, in contrast, is a key cytokine in the development of T cells, especially Th₂ cells

(Nagai et al., 2000). These data justify in part the *in vivo* effects of dihydrocucurbitacin B on oxazolone- and dinitrofluorobenzene-induced DTH. Moreover, dihydrocucurbitacin B has been found to reduce the production of interferon- γ , interleukin-2, and interleukin-10 in human T lymphocytes *in vivo*. We had previously demonstrated that dihydrocucurbitacin B reduces TNF- α production in both lymphocytes and macrophages and also reduces nitric-oxide production in inflamed tissue as well as in RAW 264.7 macrophages (Escandell et al., 2006). However, in the latter case, dihydrocucurbitacin B exerted this activity only when added before the induction of nitric-oxide synthase, showing no activity when added after the enzyme had already been induced (Escandell et al., 2006). This is a clear indication that the compound does not act through direct inhibition of this particular synthase.

In the case of cyclooxygenase-2, a direct inhibition of the enzyme was hypothesized because the effect occurred both before and after the induction of the enzyme in RAW 264.7 macrophages (Escandell et al., 2006). This data is in agreement with a previous report by Jayaprakasam et al. (2003), which demonstrated that cucurbitacins with an acetyl group at C-28 of the chain, as is the case with dihydrocucurbitacin B, selectively inhibit cyclooxygenase-2. In a similar vein, we had previously demonstrated that leukotriene B₄ production was not affected by rat neutrophils (Recio et al., 2004). In contact hypersensitivity induced by oxazolone or dinitrofluorobenzene in mice, levels of both prostaglandins and leukotrienes increase.

Curiously, inhibitors of cyclooxygenase and lipoxygenase, which are responsible for the production of prostaglandins and leukotrienes, respectively, had no direct effect on the inflammation itself. Rather, these inhibitors acted through reduction of arachidonate metabolite levels. Indeed, only corticoids, which exert their influence at a higher level than the COX and LOX pathways, are capable of reducing swelling, cell infiltration, and levels of arachidonate metabolites all together (Zunic et al., 1998; Meurer et al., 1988). However, in one of our previous studies (Recio et al., 2004) we observed no different effect for dihydrocucurbitacin B

when the glucocorticoid receptor was blocked by mifepristone, a finding which indicates that the compound does not act by means of a corticoid-like mechanism. In fact, the most likely mechanism involved is protein synthesis because when applied together, cycloheximide reduces the activity of dihydrocucurbitacin B. We have now found additional support for invoking this mechanism, since a direct inhibition of NF-AT was observed when we analyzed the proliferation of T lymphocytes. We thus have a clearer idea of the implications of NF-AT on the immune response after treatment with dihydrocucurbitacin B and the consequent disruption of the cell cycle. This, in turn, is corroborated by several different lines of evidence, which suggest that NF-AT factors are major players in the control of the cell cycle of T cells (Serfling et al., 2000). In fact, the principal event in immune responses is the activation and clonal expansion of T lymphocytes, which, in the process of differentiation, spontaneously arrest in the G_0 phase and remain quiescent for long periods until exposed to specific antigens or mitogens, such as phytohemagglutinin, which stimulate the T lymphocyte receptors and initiate a cascade of events including cell proliferation and differentiation (Kuo et al., 2000). After recognizing the stimulant agent, NF-AT is dephosphorylated through calcium cell influx, producing the activation of the calmodulin – calcineurin system. Subsequently, NF-AT migrates to the nucleus and, together with activating protein-1, activates the synthesis of interleukin-2 and other mediators implicated in immune and inflammatory responses (Crabtree, 1999; Serfling et al., 2000). This stimulation leads to lymphocyte proliferation, which is controlled at specific stages of the cell cycle by different cyclin-dependent kinases. These are activated by specific cyclins during a particular phase of the cell cycle. The cyclins themselves are a family of proteins implicated in the cell cycle that are synthesized and destroyed during this process. When lymphocytes are stimulated, the proliferation machinery starts and the cyclin-dependent kinases are activated by cyclin D and E during the G_1 phase, cyclin A in the S phase, and cyclin B for the transition from the G_2 to the M phase. When

lymphocytes are treated with dihydrocucurbitacin B, the expression of cyclins is clearly inhibited and the cell cycle is arrested in the G₀ phase. The idea that this occurs through the direct inhibition of NF-AT dephosphorylation is supported by the fact that the calcium influx remains unchanged. Although Escandell et al. (2006) had previously reported that dihydrocucurbitacin B at the same concentrations had no effect on either NF-AT or activating protein-1 through any of the stimuli used in Jurkat cells, we have now demonstrated that this compound clearly inhibits the NF-AT transcription factor in human T lymphocytes. This finding helps explain the results obtained with this compound, such as the inhibition of interleukins and interferon- γ production observed during our study.

In conclusion, dihydrocucurbitacin B has been found to exert an anti-inflammatory effect in different experimental models of DTH by modifying both the proliferation of T lymphocytes. The mechanism of action of dihydrocucurbitacin B includes a reduction in the expression of cyclins and cytokines through the inhibition of NF-AT. This leads to a consequent reduction of the mRNA expression of the cytokines implicated in DTH reactions, such as interleukin-2 and interferon- γ .

References

- Amin AR, Attur M, and Abramson SB (1999) Nitric oxide synthase and cyclooxygenases: distribution, regulation and intervention in arthritis. *Curr Opin Rheumatol* 11:202–209.
- Bas E, Recio MC, Máñez S, Giner RM, Escandell JM, López-Ginés C, Ríos JL (2007) New insight into the inhibition of the inflammatory response to experimental delayed-type hypersensitivity reactions in mice by scropolioside A. *Eur J Pharmacol* 555: 199-210.
- Belsito DV (2000) The diagnostic evaluation, treatment, and prevention of allergic contact dermatitis in the new millennium. *J Allergy Clin Immunol* 105: 409–420.
- Bruch-Gerharz D, Ruzicka T, and Kolb-Bachofen V (1998) Nitric oxide and its implications in skin homeostasis and disease. A review. *Arch Dermatol Res* 290: 643–651.
- Chapman JR, Ruben Z, and Butchko GM (1986) Histology of and quantitative assays for oxazolone-induced allergic contact dermatitis in mice. *Am J Dermatopathol* 8:130-138.
- Crabtree GR (1999) Generic signals and specific outcomes: signaling through Ca^{2+} , calcineurin, and NF-AT. *Cell* 96:611–614.
- Escandell JM, Recio MC, Máñez S, Giner RM, Cerdá-Nicolás M, and Ríos JL (2006) Dihydrocucurbitacin B, isolated from *Cayaponia tayuya*, reduces damage in adjuvant-induced arthritis. *Eur J Pharmacol* 532:145–154.
- Escandell JM, Recio MC, Máñez S, Giner RM, Cerdá-Nicolás M, and Ríos JL (2007) Cucurbitacin R reduces the inflammation and bone damage associated with adjuvant arthritis in Lewis rats by suppression of tumor necrosis factor- α in T lymphocytes and macrophages. *J Pharmacol Exp Ther* 320:581–590.
- Garaczi E, Széll M, Jánossy T, Koreck A, Pivarcsi A, Buzás E, Pos Z, Falus A, Dobozy A, and Kemény L. (2004) Negative regulatory effect of histamine in dinitrofluorobenzene-induced contact hypersensitivity. *Int Immunol* 16:1781–1788.

- Hurtrel B, Hurtrel M, and Lagrange PH (1984) Time course and histological differences between sheep red blood cells and tuberculin DTH reactions in mice. *Ann Immunol (Paris)* **135C**:219–230.
- Jayaprakasam B, Seeram NP, and Nair MG (2003) Anticancer and anti-inflammatory activities of cucurbitacins from *Cucurbita andreana*. *Cancer Lett* **189**:11–16.
- Kuo YC, Yang NS, Chou CJ, Lin LC, and Tsai WJ (2000) Regulation of cell proliferation, gene expression, production of cytokines, and cell cycle progression in primary human T lymphocytes by piperlactam S isolated from *Piper kadsura*. *Mol Pharmacol* **58**:1057–1066.
- Meurer R, Opas EE, and Humes JL (1988) Effects of cyclooxygenase and lipoxygenase inhibitors on inflammation associated with oxazolone-induced delayed hypersensitivity. *Biochem Pharmacol* **37**:3511–3514.
- Nagai H, Ueda Y, Ochi T, Hirano Y, Tanaka H, Inagaki N, and Kawada K (2000) Different role of IL-4 in the onset of hapten-induced contact hypersensitivity in BALB/c and C57BL/56 mice. *Br. J. Pharmacol.* **129**:299–306.
- Nakae S, Komiyama Y, Narumi S, Sudo K, Horai R, Tagawa Y, Sekikawa K, Matsushima K, Asano M, and Iwakura Y (2003) IL-1-induced tumor necrosis factor- α elicits inflammatory cell infiltration in the skin by inducing IFN- γ -inducible protein 10 in the elicitation phase of the contact hypersensitivity response. *Int Immunol* **15**:251–260.
- Recio MC, Giner RM, Máñez S, and Ríos JL (2004) Anti-inflammatory activity of two cucurbitacins isolated from *Cayaponia tayuya* roots. *Planta Med* **70**:414–420.
- Ríos JL, Recio MC, Máñez S, and Giner RM, (2000) Natural triterpenoids as anti-inflammatory agents, in *Studies in Natural Products Chemistry: Bioactive Natural Products* (Atta-ur-Rahman ed) Vol. 22, pp 93–143, Elsevier, Amsterdam.

- Ríos JL, Escandell JM, and Recio MC (2005) New insight on the bioactivity of cucurbitacins, in *Studies in Natural Products Chemistry: Bioactive Natural Products* (Atta-ur-Rahman, ed) Vol. 32, pp 519–572, Elsevier, Amsterdam.
- Ross R, Gillitzer C, Kleinz R, Schwin J, Kleinert H, Förstermann U, and Reske-Kunz AB (1998) Involvement of NO in contact hypersensitivity. *Int Immunol* **10**: 61–69.
- Ross R, and Reske-Kunz AB (2001) The role of NO in contact hypersensitivity. *Int Immunopharmacol* **1**:1469-1478.
- Scott AE, Kashon ML, Yucesoy B, Luster MI, and Tinkle SS (2002) Insights into the quantitative relationship between sensitization and challenge for allergic contact dermatitis reactions. *Toxicol Appl Pharmacol* **183**:66–70.
- Serfling E, Berberich-Siebelt F, Chuvpilo S, Jankevics E, Klein-Hessling S, Twardzik T, and Avots A (2000) The role of NF-AT transcription factors in T cell activation and differentiation. *Biochim Biophys Acta* **1498**:1–18.
- Sun J, Blaskovich MA, Jove R, Livingston SK, Coppola D, and Sefti, SM (2005) Cucurbitacin Q: a selective STAT3 activation inhibitor with potent antitumor activity. *Oncogene* **24**:3236–3245.
- Thomson JA, Trout AB, and Kelso A (1993) Contact sensitization to oxazolone: involvement of both interferon-gamma and IL-4 in oxazolone-specific Ig and T-cell responses. *Immunology* **78**: 185-192.
- Wang B, Fujisawa H, Zhuang L, Freed I, Howell BG, Shahid S, Shivji GM, Mak TK, and Sauder DN (2000) CD4⁺ Th1 and CD8⁺ type cytotoxic cells both play a crucial role in the full development of contact hypersensitivity. *J Immunol* **165**: 6783–6790.
- Xu H, DiIulio NA, and Fairchild RL (1996) T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production:

interferon gamma-producing (Tc1) effector CD8⁺ T cells and interleukin (IL)-4/IL-10-producing (Th2) negative regulatory CD4⁺ T cells. *J Exp Med* **183**:1001–1012.

Zunic M, Bahr GM, Mudde GC, Meingassner JG, and Lam C (1998) MDP(Lysyl)GDP, a nontoxic muramyl dipeptide derivative, inhibits cytokine production by activated macrophages and protects mice from phorbol ester- and oxazolone-induced inflammation. *J Invest Dermatol* **111**:77–82.

Footnotes

This work was supported by the Spanish Government (SAF2006-06726). J.M. Escandell is grateful for a fellowship from the Generalitat Valenciana, grant CTBPRB/2003/315. We are also indebted to the Centre de Transfusions de la Comunitat Valenciana (Valencia, Spain) for its generous supply of human blood.

Address correspondence: Prof. José Luis Ríos, Departament de Farmacologia, Facultat de Farmàcia, Universitat de València, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain. Phone +34 963 544 973, Fax: +34 963 544 498, E-mail: riosjl@uv.es

Legends for Figures

Figure 1. Chemical structure of dihydrocucurbitacin B.

Figure 2. A), B), and C) Effect of dihydrocucurbitacin B (DHCB) on DTH induced in mice by: A) oxazolone, expressed as percentage of inhibition of ear swelling; B) DNFB, expressed as percentage of inhibition of ear swelling; and C) SRBC, expressed as the increase in paw swelling. D) and E) Effect of dihydrocucurbitacin B (DHCB) on interleukin (IL)-1 β , IL-4, and TNF- α production in the ears of mice treated with oxazolone (D) and dinitrofluorobenzene (E). The vertical lines indicate the S.E.M. Statistically significant difference with respect to the control is expressed as ** $P < 0.01$ (Dunnett's t -test). $n = 5$ or 6 animals.

Figure 3. Oxazolone-induced DTH reaction in mice. Haematoxylin-eosin-stained section of ears. **A)** Control group treated with oxazolone only; **B)** Dihydrocucurbitacin B-treated group. Dinitrofluorobenzene (DNFB)-induced DTH reaction in mice. Haematoxylin-eosin-stained section of ears. **A)** Control group treated with DNFB only; **B)** Dihydrocucurbitacin B-treated group.

Figure 4. Effect of dihydrocucurbitacin B (DHCB, 2.5 μ M), aphidicolin (aphi, 0.75 μ M), and dexamethasone (dex, 5 μ M) on T lymphocyte proliferation and cell cycle. A) Cells were stained with propidium iodide and the DNA content analyzed by means of flow cytometry. B) Results were analyzed with the aid of the Chylcred program to determinate cell phase %. Each bar is the mean of three independent experiments. Values represent the percentage of cells in each phase of the cell cycle $\pm$ s.e.

Figure 5. Effects of dihydrocucurbitacin B (DHCB) on the cell cycle and cell proliferation at different times. Time is expressed as hours after addition of 2.5 μ M DHCB subsequent to phytohemagglutinin stimulation. Values represent the percentage of cells in each phase of the cell cycle $\pm$ standard error. A) proliferation assay. B) cell cycle analysis

Figure 6. Cytokine production in T lymphocytes treated with dihydrocucurbitacin B. A) Effects on cytokine production: (A) interleukin-4 (IL-4), (B) interferon- γ (IFN- γ), (C) interleukin-2 (IL-2), and (D) interleukin-10 (IL-10). Bars represent cytokine production in pg/ml in the presence or absence of test compounds with standard error and statistical significance. Blank (non-treated cells), control (phytohemagglutinin-stimulated cells), 10 μ M dexamethasone-

treated phytohemagglutinin-stimulated cells, and dihydrocucurbitacin B (DHCB)-treated phytohemagglutinin-stimulated cells at different concentrations (2.5–1.0 μM).

Fig. 7. A) Effects of dihydrocucurbitacin B (DHCB) on cytokine mRNA expression in T lymphocytes as detected with the aid of RT-PCR analysis. T cells were stimulated with phytohemagglutinin (PHA, lines 2 and 4) in the presence (lines 4) or absence (line 2) of dihydrocucurbitacin B (2.5 μM). Line 1 shows protein expression in cells without treatment and line 3 represents the effect of dihydrocucurbitacin B (2.5 μM) on non-stimulated T cells. B) Quantification of interleukin-4 (IL-4), interferon- γ (IFN- γ), interleukin-2 (IL-2), and interleukin-10 (IL-10) mRNA by densitometry.

Figure 8. A) Effects of dihydrocucurbitacin B (DHCB) on cyclin expression in human T lymphocytes as detected by chemiluminiscence. T cells were stimulated with phytohemagglutinin (PHA, lines 2 and 4) in the presence (lines 4) or absence (line 2) of DHCB (2.5 μM). Line 1 shows protein expression in cells without treatment. B) Quantification of cyclin-A₁, cyclin-B₁, cyclin-D₂, and cyclin-E₁ by densitometry.

Figure 9. Effects of dihydrocucurbitacin B (DHCB) on NF-AT in human T lymphocytes stimulated with phytohemagglutinin (PHA) as detected by electrophoretic mobility shift assays (EMSA). T cells were stimulated with phytohemagglutinin (PHA, lines 3 and 4) in the presence (line 4) or absence (line 3) of dihydrocucurbitacin B (2.5 μM). Line 2 shows cells without treatment. Line 1 = competition assay.

Figure 1

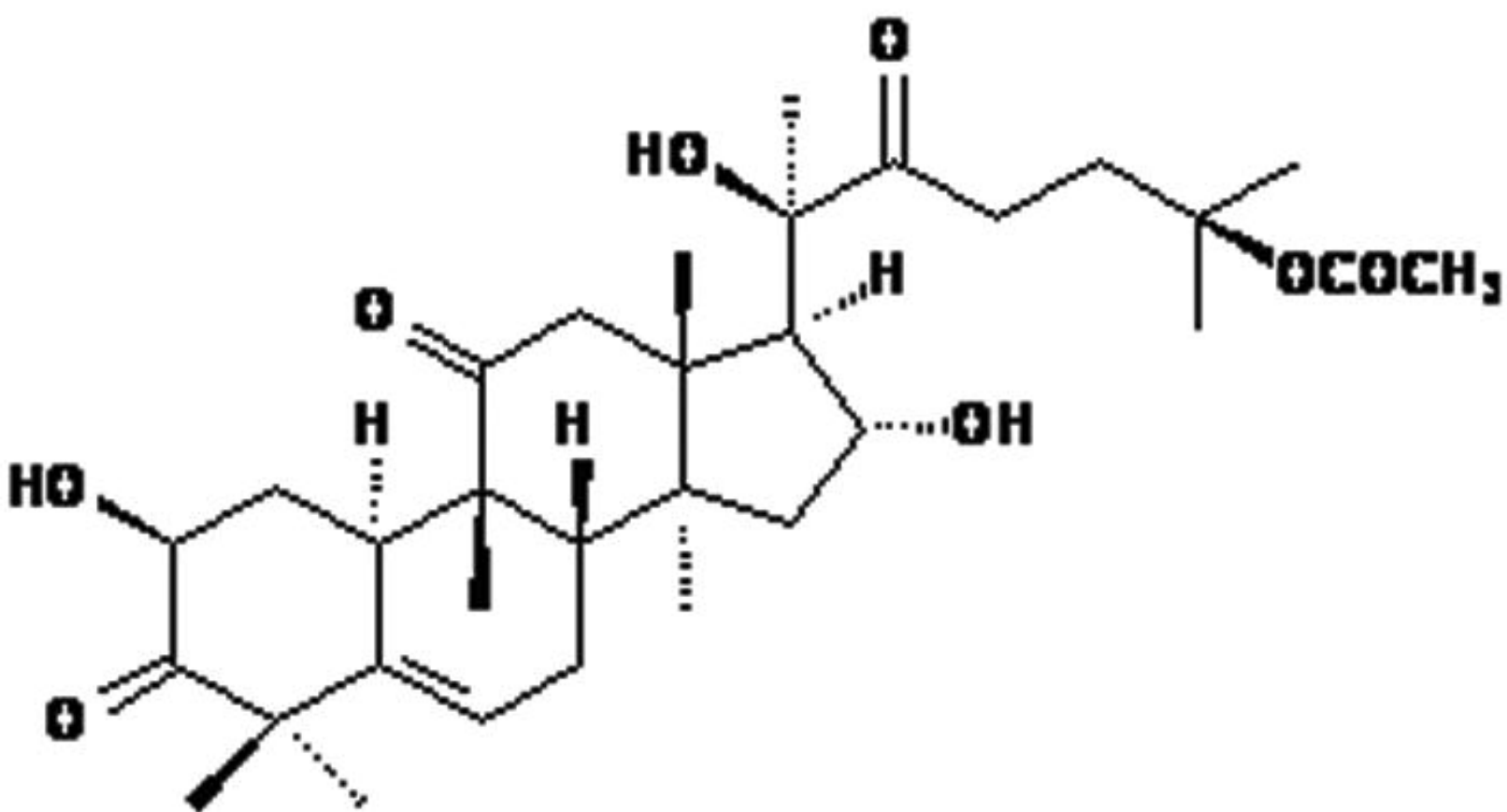


Figure 2

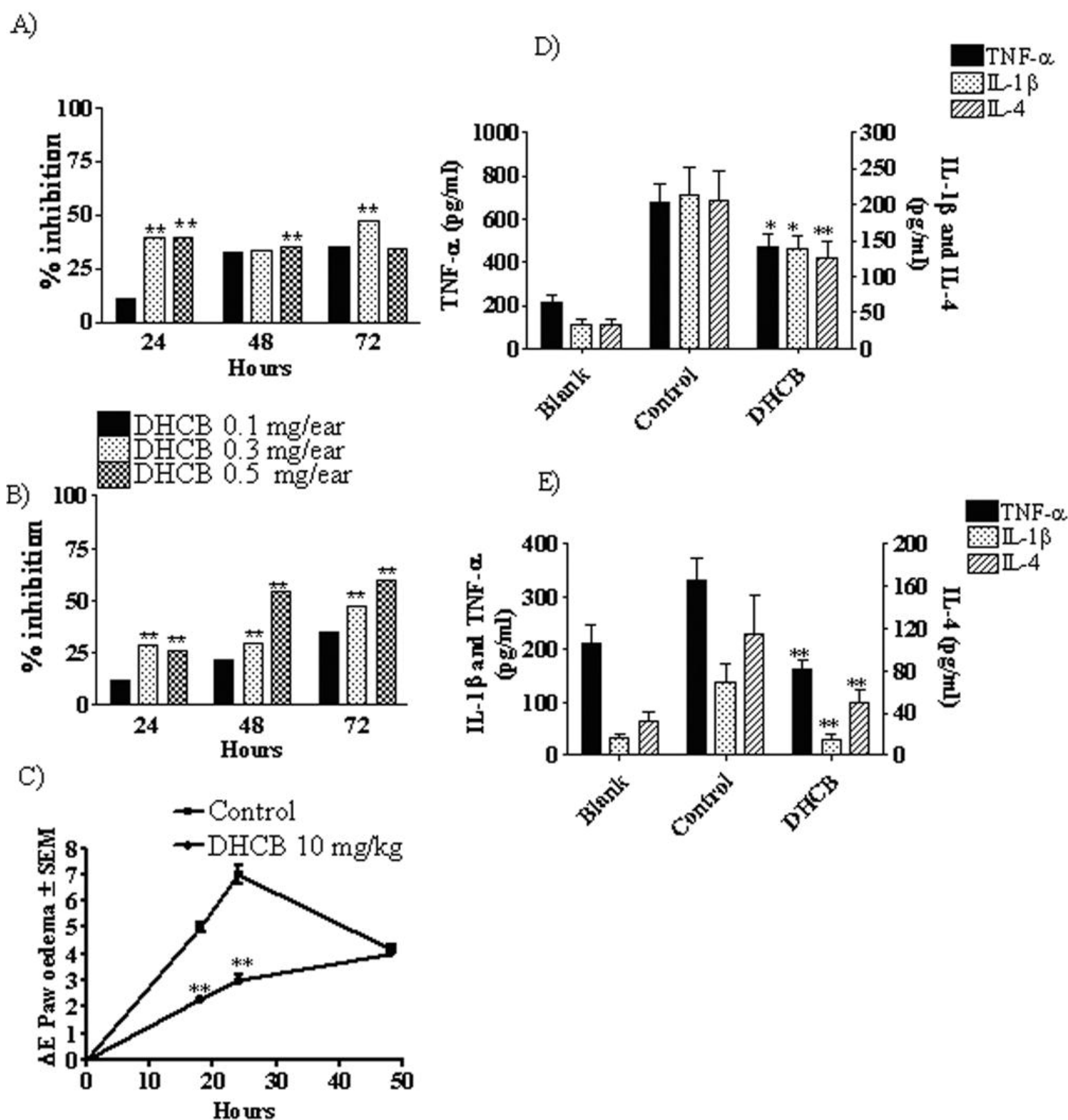
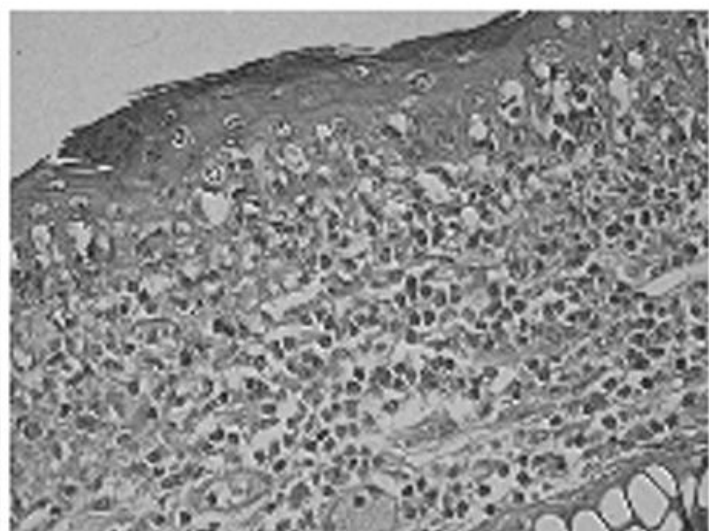
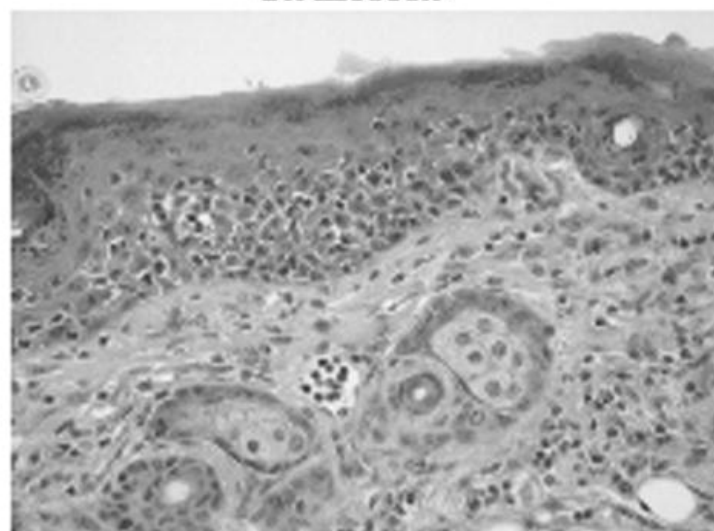


Figure 3

Oxazolone

DNFB

A)



B)

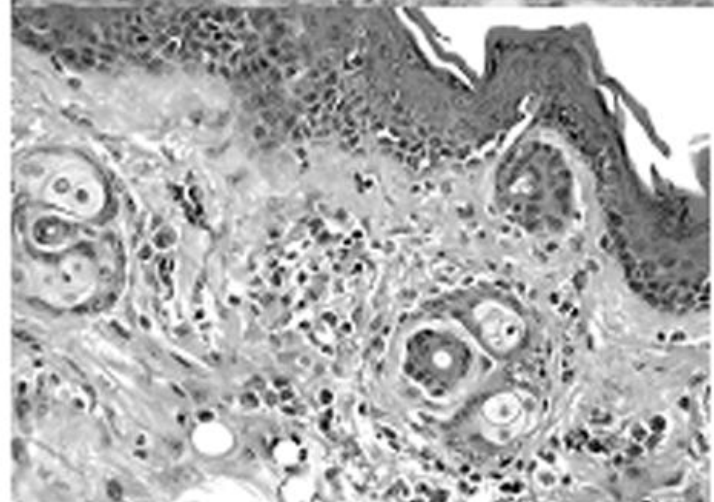
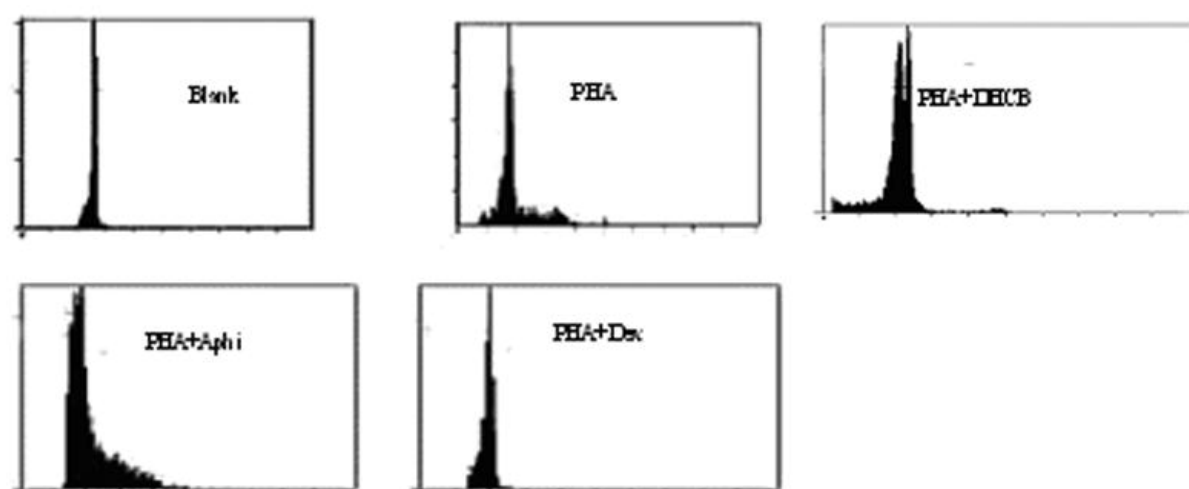
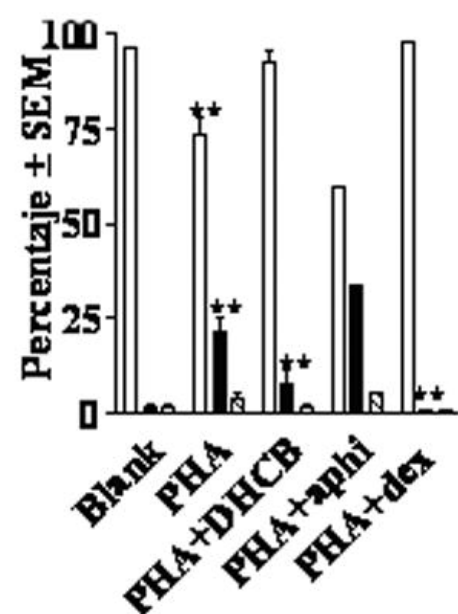


Figure 4

A)



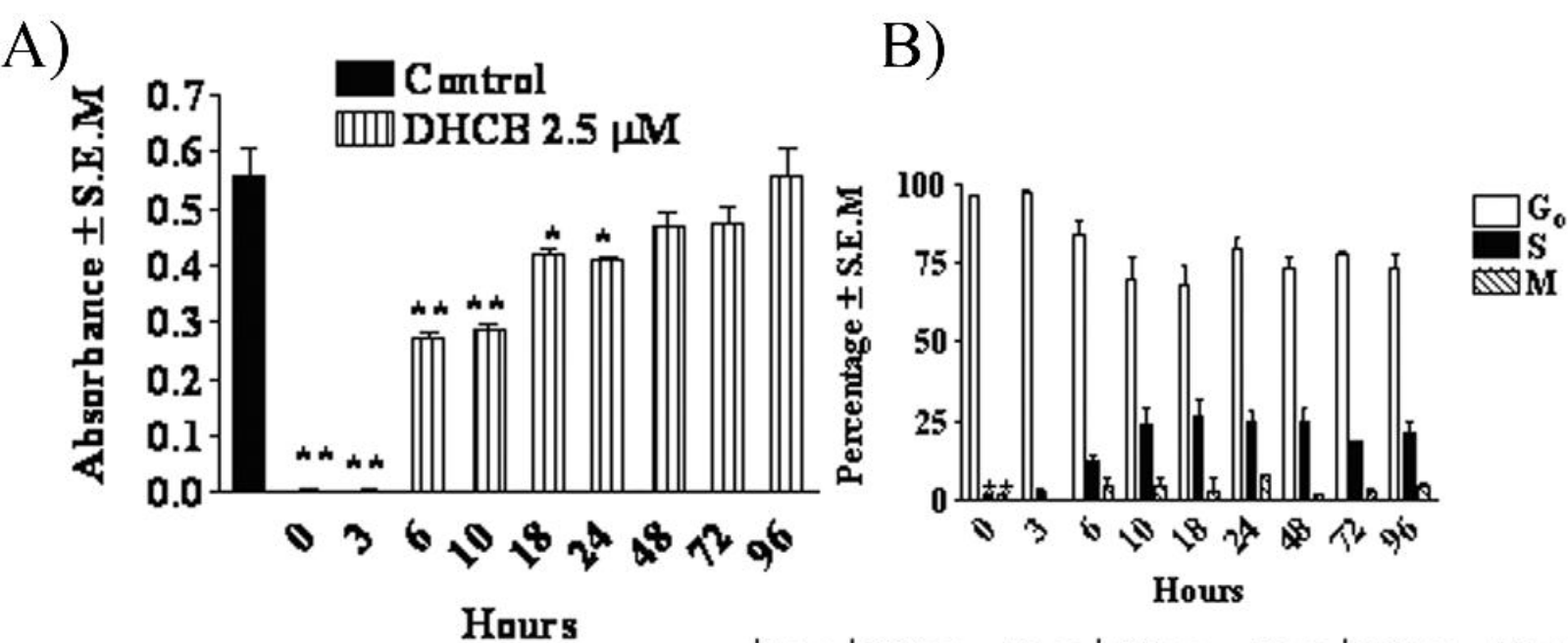
B)



□ G₀
 ■ S
 ▨ M

	G ₀ phase	± Error	S Phase	± Error	MPhase	± Error
Blank	96.0	0.5	1.9	0.2	1.8	0.2
PHA	73.0	5.2	21.3	3.8	4.2	1.5
PHA+DHCB	92.8	2.5	7.6	3.6	1.6	0.3
PHA+Aphi	60.0	0.3	34.0	0.8	5.0	0.2
PHA+dex	98.0	0.5	0.7	0.4	1.2	1.5

Figure 5



hours	G ⁰ Phase	$\pm$ Error	S Phase	$\pm$ Error	M Phase	$\pm$ Error
0	96.00	0.50	1.90	0.20	1.80	0.20
3	97.00	1.00	2.65	1.20	0.15	0.10
6	83.70	5.20	12.60	1.70	4.25	3.00
10	70.00	6.70	24.00	5.20	4.60	2.60
18	67.90	6.10	26.50	5.60	3.20	3.80
24	79.50	3.60	25.00	3.70	8.20	0.10
48	73.00	3.60	25.00	3.90	1.60	0.60
72	77.50	1.50	18.50	0.50	3.00	1.00
96	73.00	5.20	21.30	3.80	4.20	1.50

Figure 6

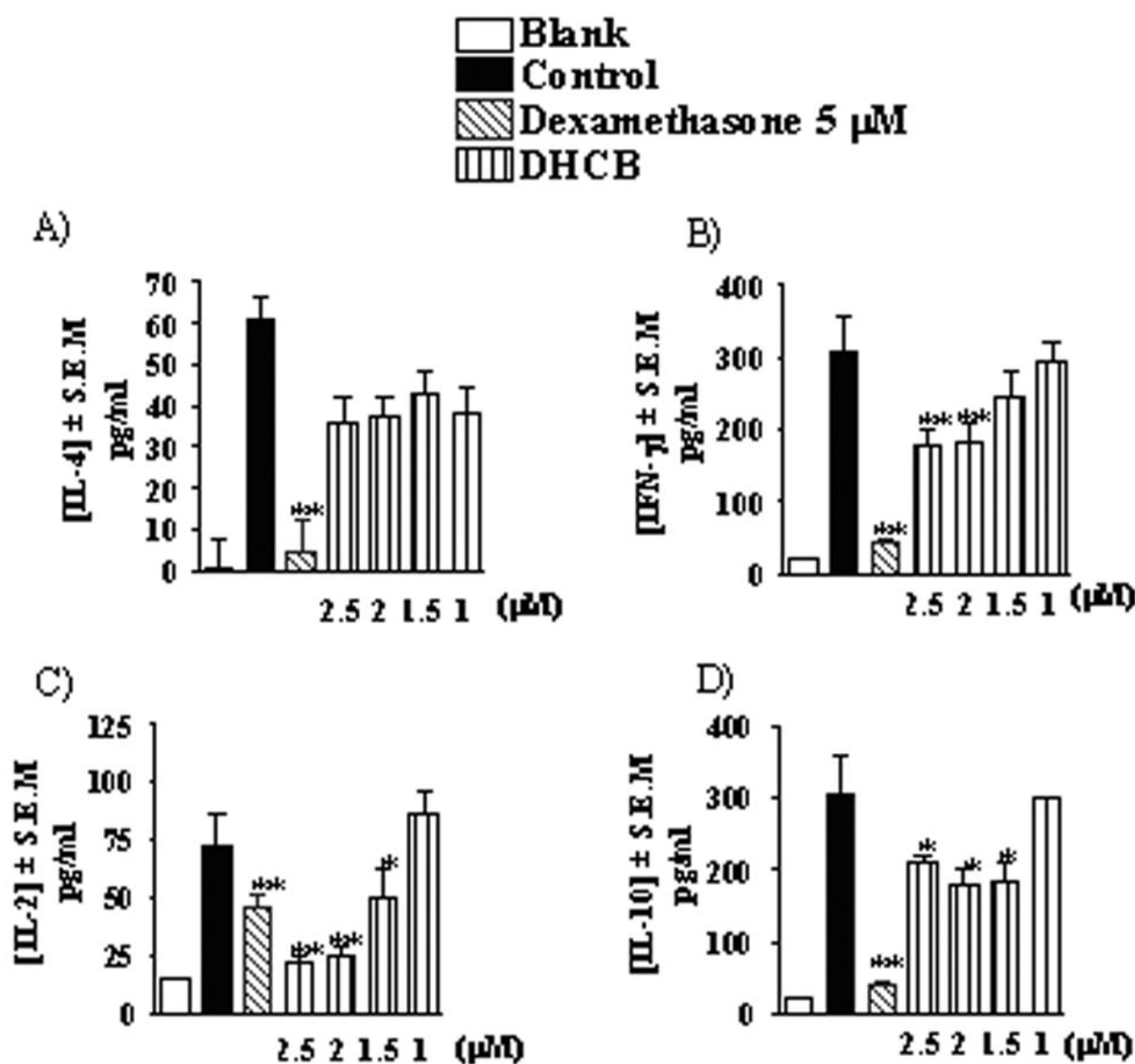
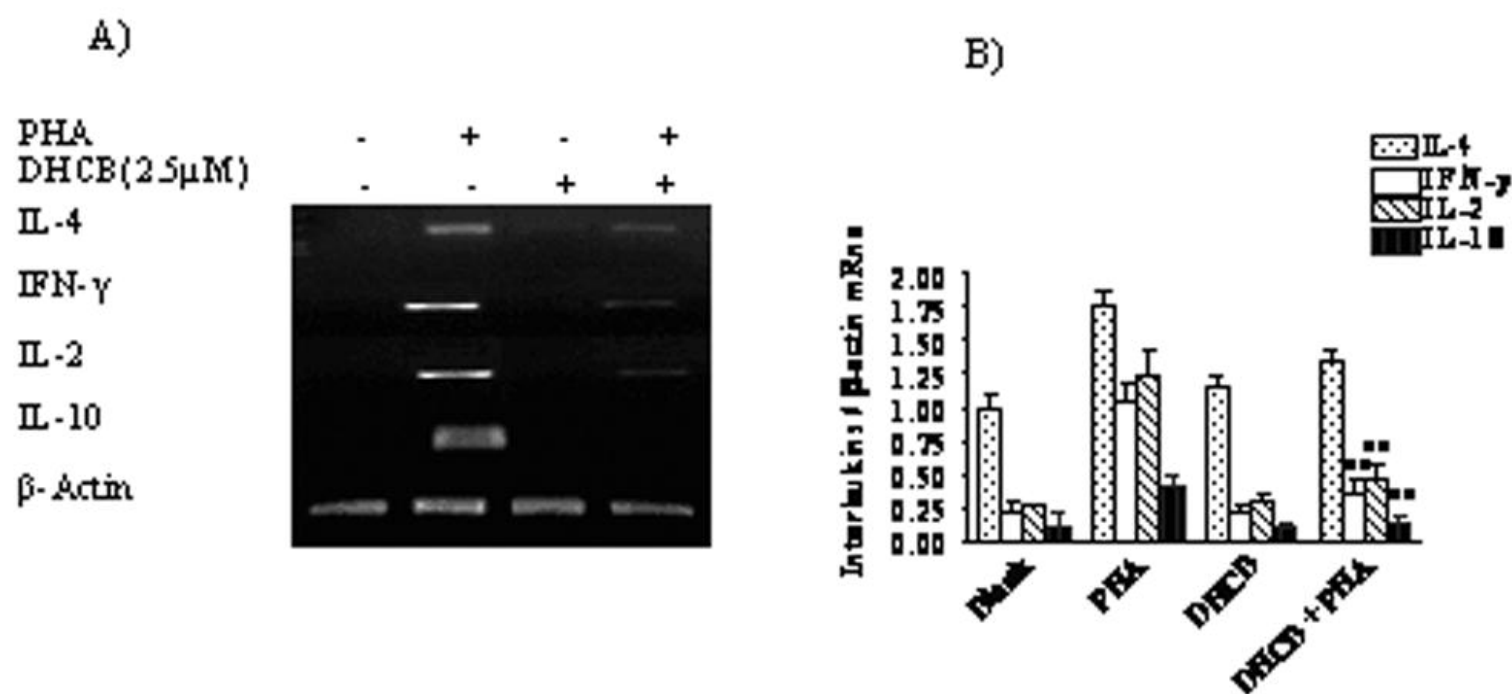


Figure 7



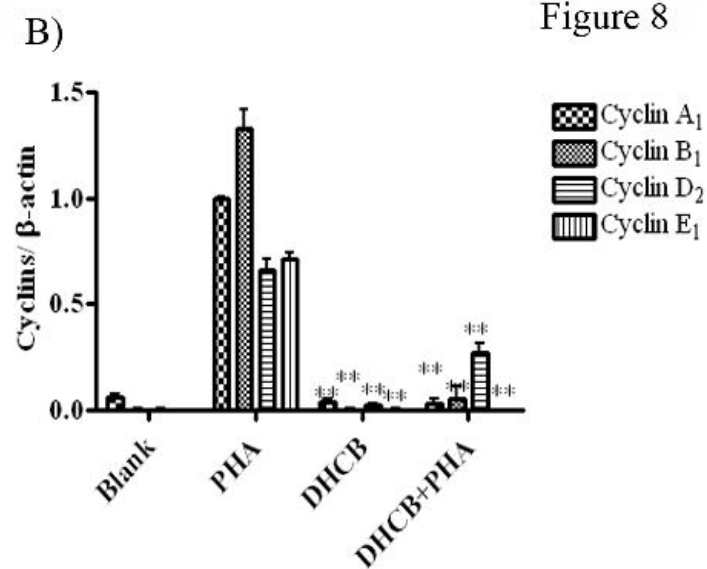
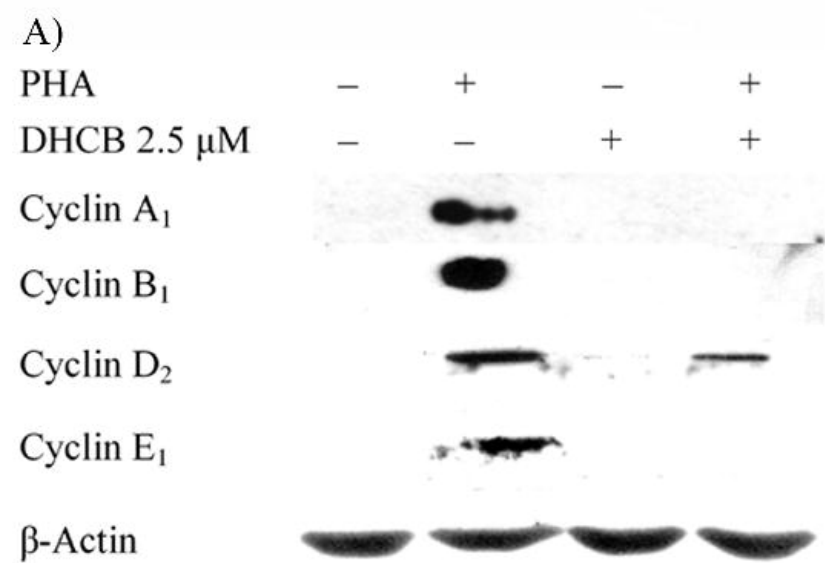


Figure 8

Figure 9

