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Inhibition of Proinflammatory Cytokines and Mediators by Euphol

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ABSTRACT

The anti-inflammatory properties of Euphol (EU) a diterpene, isolated from the rhizomes of the plant *Euphorbia acaulis*, were evaluated. This study was designed to examine the effects of EU on inflammation in a rat model of pleurisy compared with a steroidal anti-inflammatory drug (DEX, dexamethasone). The rat model of pleurisy was used to evaluate the effectiveness of EU on leukocyte migration, exudation volume, proinflammatory cytokines i.e. Tumor Necrosis factor α (TNFα), Interleukin-1 (IL-1β) and Interleukin-6 (IL-6) as well as on proinflammatory mediators PGE_2, nitrite/nitrate levels, and LTB_4. Pleurisy was induced by carrageenan (cg), administered by intra pleural route (i.pl.) and the leukocyte infiltration and inflammatory parameters analyzed 4 h after pleurisy induction. EU (8mg/kg^{-1}, p.o.) treated groups caused significant decrease in the infiltration of neutrophils and exudate volume . There was an observed marked reduction in TNFα, IL-1β and IL-6 levels. Flow cytometric analysis of intracellular TNFα by leukocytes present in pleural exudates showed a significant decrease relative to the total extracellular TNFα inhibition in the pleural fluid. The proinflammatory mediators PGE_2, LTB_4 release and the nitrite/nitrate levels were remarkably deregulated which are interrelated to TNFα and IL-1β levels.

INTRODUCTION

Euphorbia acaulis (EA) (Family: Euphorbiaceae) is a shrub commonly found on the dry slopes of Himalayan range at an altitude between 3000 and 5000 ft. It is used as a remedy for joint pains and rheumatic disorders in traditional system of medicine (Mhaskar *et al.*, 2000).

Because of the medicinal value, this plant species have been of interest to several investigators of phytomedicine. *Euphorbia* species mainly contain terpenoids (diterpenoids and triterpenoids), flavanoids and its glycosides, besides other alkaloids. We are engaged in developing potent anti-inflammatory leads and in our endeavour to track down a pure molecule with possible therapeutic potential we zeroed on Euphol (EU).

We have shown that a bioactive fraction of *Euphorbia acualis* containing EU showed highly significant anti inflammatory activity in our studies (Bani *et al.*, 1999). On this basis, it was anticipated that EU should have a regulatory bearing on inflammatory parameters and hence the model of pleurisy (Saleh *et al.*, 1996) was chosen to explain its acute anti-inflammatory potential. We know cytokines have an inevitable role in the regulation of hematopoeises, mediating the differential migration, activation and proliferation of phenotypically diverse cells (Mossmann *et al.*, 1989; Garra *et al.*, 1994; Constant *et al.*, 1997).

Many cytokines are pleiotropic and exhibit interrelated functions which in turn regulate the production of other cytokines and contribute to the cytokine milieu, are often of a greater importance than the action of a single cytokine.

The analysis and quantification of cytokines in biological fluids has wide application in research and clinical laboratories and beyond helps the scientific community to clearly analyze and to further their understanding of many biological functions.

MATERIALS AND METHODS

Chemicals

EU (Isolated from *Euphorbia acaulis* IIIM, Jammu), carrageenan (Type IV), dexamethasone (DEX), MayGrunwald dye, Giemsa dye and Evans blue (Sigma Chemicals Co, USA), rat PE-labeled monoclonal TNF α antibody, FACS lysing and

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permeabilising solution, Golgi plug (BD BioSciences San Diego, CA, USA), Enzyme-linked immunosorbent assay (ELISA) for quantitative determination of rat TNFα, IL-1β, IL-6, PGE_2 and LTB_4 (R&D systems, MN, USA) were used. Other reagents and organic solvents used were of analytical grade and were obtained from different commercial sources.

Chemistry

The air-dried rhizomes of *Euphorbia acaulis* (1.5 kg) were powdered and extracted by continuous extraction with petroleum ether (60-80°C) in a Soxhlet for 48 h. Portion of the extract (100gm) was subjected to column chromatography over silica gel (3 kg, 6 cm x 90 cm). The column was eluted with petroleum ether and then with petroleum ether : ethyl acetate mixture by gradually increasing the percentage of ethyl acetate in petroleum ether. Each fraction of 150 ml was collected. Fractions were checked on TLC using chloroform: methanol (95:5) as developing solvent. Fractions eluted in petroleum ether: ethyl acetate (95:5) showed one major spot on TLC. These fractions were pooled and subjected to repeated column chromatography over silica gel using petroleum ether, petroleum ether: ethyl acetate as an eluent. Fractions showing the major compound on TLC were pooled. The residue on repeated crystallization from ethyl acetate: petroleum ether yielded a colorless compound (mpt-116°C). The compound was identified as Euphol (EU) on the basis of data reported in the literature (Chen, 1982) and is represented as Fig.1.

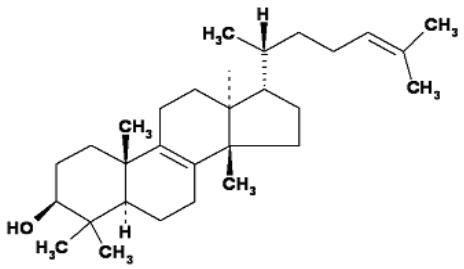

Fig. 1: Structure of Euphol (8, 24 - euphadien-3-beta-ol).

Animals

Normal male adult Wistar rats 10–12 weeks old and weighing 140–160g obtained from the animal house of the Indian Institute of Integrative Medicine, Jammu, in groups of eight were employed for the study. These were maintained at a room temperature of 22±2 ℃ with 12 h light/dark cycle and free access to pellet food (Lipton India limited) and water.

According to ethical regulations on animal research, all animals used in experimental work received humane care. All experimental procedures used in present study were in accordance with institutional guidelines for animal research (CPCSEA, 2003). The study protocols were approved by the Institutional Animal Use and Care Committee of Indian Institute of Integrative Medicine, Jammu. Drugs were prepared as a homogenized suspension in gum acacia (1%, w/v) and were a dministered orally 1 h before carrageenan injection.

Pleurisy induction

Pleurisy experiments were carried out as described (Meacock *et al.*, 1979) with carrageenan (cg) as a phlogistic agent. Cell migration, degree of exudation and proinflammatory cytokines and mediators were evaluated at 4 h of cg-(1%, i.pl.) injection. Exudates from the pleural cavity was collected and stored in a freezer (−20 °C) and determinations of the exudation (amount of Evans Blue dye) were made as accordingly. At the same time point, cytokine assays for TNFα, IL-1β and IL-6; inflammatory mediators likes nitrite/nitrate concentrations, Prostaglandin-E_2 (PGE_2) and Leukotrienes-B_4 (LTB_4) were carried out. Initially, dose response studies were carried out to optimize the EU doses to be used in the experiments by analyzing the anti-inflammatory parameters in immune response studies by *in vivo* experiments (Data not shown). Accordingly, EU at 8 $mgkg^{-1}$ p.o. was found to be most effective dose and was taken up for detailed evaluation.

Animals were randomly grouped with 8 in each set: (1) control [c]-treated with sterile saline (NaCl, 0.9%, i.pl.), (2) cg-control [cg] treated with carrageenan (1%, i.pl.), (3) cg (1%, i.pl.) plus DEX (0.5 $mgkg^{-1}$, p.o.) [cg+DEX], (4) cg (1%, i.pl.) plus EU (8 $mgkg^{-1}$, p.o.) [cg+EU], were administered 1 h prior to pleurisy induction.

Quantification of cell migration and exudation

Pleural fluid samples were collected and leukocyte counts was performed using an automatic counting machine (ABX Diagnostics, MC, France), while cytospin preparations of pleural wash were stained with MayGrunwald-Giemsa for the differential count using oil immersion objective. The degree of exudation was determined by measuring the amount of Evans blue dye extravasated in the pleural liquid as described (Bueno *et al.*, 2002). In each experimental group, animals were previously challenged (1 h) with a solution of Evans blue dye (25$mgkg^{-1}$) administered by intravenous route (i.v.) in order to evaluate the degree of exudation in the pleural cavity. On the day of the experiments, stored samples were thawed at room temperature to estimate the amount of dye by colorimetry using an ELISA plate reader (Multiskan, Thermo Electron Corporation, MA, USA) at 600 nm. The results were interpreted from a standard curve of Evans blue dye in the range of 0.01 to 50 µg/ml. In all groups, blood samples were also collected from retro orbital plexus to analyze the effect on the number of white and red blood cells.

Flow cytometry analysis of TNFα in pleural exudate leukocytes

The intracellular TNF α level (Saleh *et al.*, 1999) was estimated in gated population of total pleural exudates leukocytes, for c (NaCl, 0.9%), cg (cg-1%), cg+DEX (0.5$mgkg^{-1}$, p.o.) and cg+EU- (8$mgkg^{-1}$, p.o.) treated animals after 4 h of pleurisy. Golgi plug was added to the pleural exudates and (80µl) then processed by adding FACS permeabilising solution (1X) and labeled with PE-labeled TNF α monoclonal antibodies. The samples were resuspended in PBS (pH, 7.4) and acquired directly on the flowcytometer (BD-LSR, Beckton-Dickinson Biosciences, CA,

USA). A fluorescence trigger was set on the PE (FL1) parameter of the gated pleural exudates cells (Characteristically abundant with neutrophil, 10,000 events). Fluorescence compensation, data analysis, and data presentation was performed using Cell Quest Pro software (Beckton-Dickinson Biosciences, CA, USA).

Quantification of TNFα, IL-1β, IL-6, PGE_2 and LTB_4 in pleural fluids

Cell free samples of the pleural exudates obtained from c (NaCl, 0.9%), cg (cg-1%), cg+DEX (0.5mgkg^{-1}, p.o.) and cg+EU- (8mgkg^{-1}, p.o.) groups of animals, were taken for the analysis of cytokine levels by using (TNFα, IL-1β, IL-6, PGE_2 and LTB_4) commercially available kits based on sandwich and competitive ELISA technique (R&D systems, MN, USA) according to the manufacturers' instructions. All cytokine concentrations were estimated by means of colorimetric measurement at 450 nm on an ELISA plate reader (Multiskan, Thermo Electron Corporation, MA, USA) by interpolation from a standard curve.

Quantification of nitrate/nitrite concentrations

The Griess method was employed to measure NO (Nitric Oxide) as its breakdown products nitrite (NO_2) and nitrate (NO_3) (Di Rosa *et al.,* 1996). Samples obtained from c (NaCl, 0.9%), cg (cg-1%), cg+DEX (0.5mgkg^{-1}, p.o.) and cg+EU- (8mgkg^{-1}, p.o.) groups of animals were taken for estimating the levels of nitrate/nitrite were as previously described (Saleh *et al.,* 1999). Results are expressed as μM using sodium nitrite as a standard.

Safety studies

The acute oral safety studies were carried out after approval from the Institutional Animal Ethics Committee following OECD guidelines No. 423 (OECD, 1996). EU was found to be safe up to 2000 mgkg^{-1} with single dose of administration and no adverse effects or change in general behavior in treated female mice were observed.

Statistical analysis

Data are reported as mean±S.E.M (n=8). Significant differences between groups were determined by analysis of variance (ANOVA) complemented with Dunnett's tests. *p≤0.01 was considered as highest indicative of significance.

RESULTS AND DISCUSSION

Effects of EU on cellular infiltration and exudation.

Table.1 shows, EU (8mgkg^{-1}, p.o.,) caused a significant decrease in leukocyte migration (% of inhibition: 43.0±4.3) (P≤0.01). This reduction is attributed to inhibition of neutrophil influx (% of inhibition: 56.1±3.9) (P≤0.01). EU did inhibit mononuclear cells but not as significantly (% of inhibition: 31.6±6.6) (P≤0.001) when compared to total leukocytes and neutrophils. The degree of exudation (Evan's blue assay) and the exudate volume (by subtracting the lavage volume taken from the total exudate volume) was reduced less significantly (% of inhibition: 20.8±3.6 and 23.7±3.6 respectively) (P≤0.001) (Table.1). DEX, a standard drug (0.5 mgkg^{-1}, p.o.) caused a significant inhibition of leukocytes (% of inhibition: 47.5±5.8), neutrophils (% of inhibition: 54.6± 3.2), mononuclear cells (% of inhibition: 55.5±3.2) (P≤0.01). DEX inhibited mononuclear cells significantly when compared to EU. The degree of exudation and exudation volume was reduced significantly by DEX (% of inhibition: 38.4±7.8 and % of inhibition: 46.0±3.6 respectively) (P≤0.01, Table.1) when compared to EU. EU (8mgkg^{-1}, p.o.) and DEX (0.5 mgkg^{-1}, p.o.) did not cause much change in the blood's white or red cells in this model of pleurisy (Data not shown).

Effects of EU on TNF α intracellular levels in leukocytes of pleural exudates

EU (8mgkg^{-1} p.o.), significantly decreased intracellular TNFα levels of pleural exudate neutrophils (% of inhibition: 51.3±3.9) while DEX showed 44.4±4.2 % inhibition (P≤0.01) (Fig. 2).

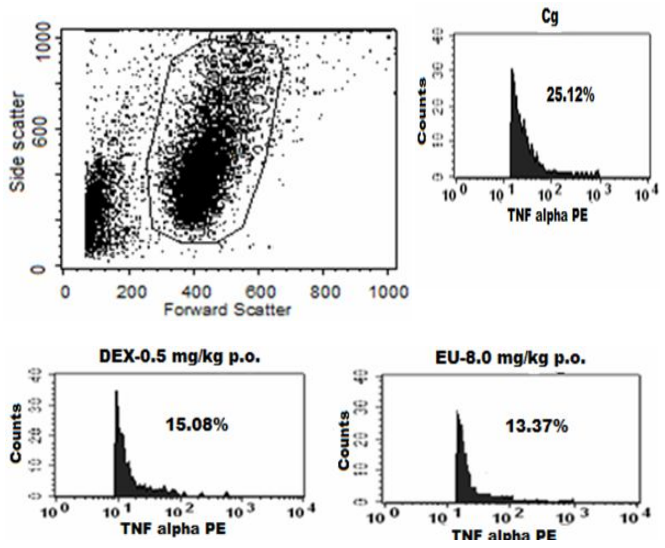

Fig. 2: Effects on intracellular TNFα secretion, by EU-8mgkg^{-1}, p.o. administered 1 h before carrageenan administration on the gated total leukocytes from pleural exudates of rats. The histogram plots of of percentage gated population of one representative rat of each group of TNFα secretion of c (NaCl, 0.9%), cg (cg-1%), cg+DEX (0.5mgkg^{-1}, p.o.) and cg+EU (8mgkg^{-1}, p.o.) groups respectively where values represent mean ± S.E.M (n=8). Asterisks indicate significant difference of DEX and EU-treated vs. cg, under specified conditions (*p< 0.01).

Effect of EU on proinflammatory TNF α IL-1β, IL-6, PGE_2 and LTB_4 levels

EU (8mgkg-1 p.o.) and DEX (0.5 mgkg-1 p.o.), significantly decreased the TNF α (% of inhibition: 46.0±2.9, Fig. 3A), IL-1β (% of inhibition: 50.1±1.9, Fig. 3B), IL-6 (% of inhibition: 37.1±2.3, Fig. 3C), PGE_2 (% of inhibition: 54.1±1.7, Fig. 3D) (P≤0.01) and LTB_4 levels (% of inhibition: 18.8±1.8, Fig. 3E) (P≤0.001) respectively. DEX also significantly inhibited TNFα, IL-1β, IL-6, PGE_2 and LTB_4 to 52.2±2.0%, 57.2±2.0%, 39.9±2.6%, 50.0±1.3% (Fig.3A-D, P≤0.01) and 31.2±2.3 % (Fig.3E, P≤0.001) respectively. DEX showed marked reduction of LTB_4 when compared to EU.

Table. 1: The parameters were analyzed 4 h after the administration of carrageenan (1%) in the rat pleural cavity for Total leukocyte count (TLC); Neutrophils (NEU); Mononuclear cells (MNC); Evans Blue (EB); Exudate Volume (EV). [a] Treatment by oral route,1h before carrageenan injection. [b] Treatment by Intrapleural route, with no test drug administration. Values represented as **%↓** indicate percentage inhibition. Values are statistically significant as mean ± S.E.M (n=8, *P≤0.01, **P≤0.001).

Group	Dose($mgkg^{-1}$)	TLC($\times10^6$)	NEU($\times10^6$)	MNC($\times10^6$)	EB(µl/ml)	EV(ml)
	4h	**4h**	**4h**	**4h**	**4h**	**4h**
c [a]	-	11.65±0.41	10.00±0.29	8.01±0.31	2.52±0.07	0.29±0.04
cg [b]	-	79.48±1.37	65.36±1.60	73.86±3.17	9.53±0.06	1.39±0.36
cg+DEX [a]	0.5	41.72±0.96 **47.50%↓***	29.67±0.52 **54.60%↓***	32.80±0.82 **55.59%↓***	5.88±0.04 **38.42%↓***	0.75±0.03 **46.04%↓***
cg+EU [a]	8	45.26±0.55 **43.05%↓***	28.68±0.59 **56.11%↓***	16.20±1.18 **31.69%↓****	7.54±0.38 **20.88%↓****	0.95 **23.77%↓****

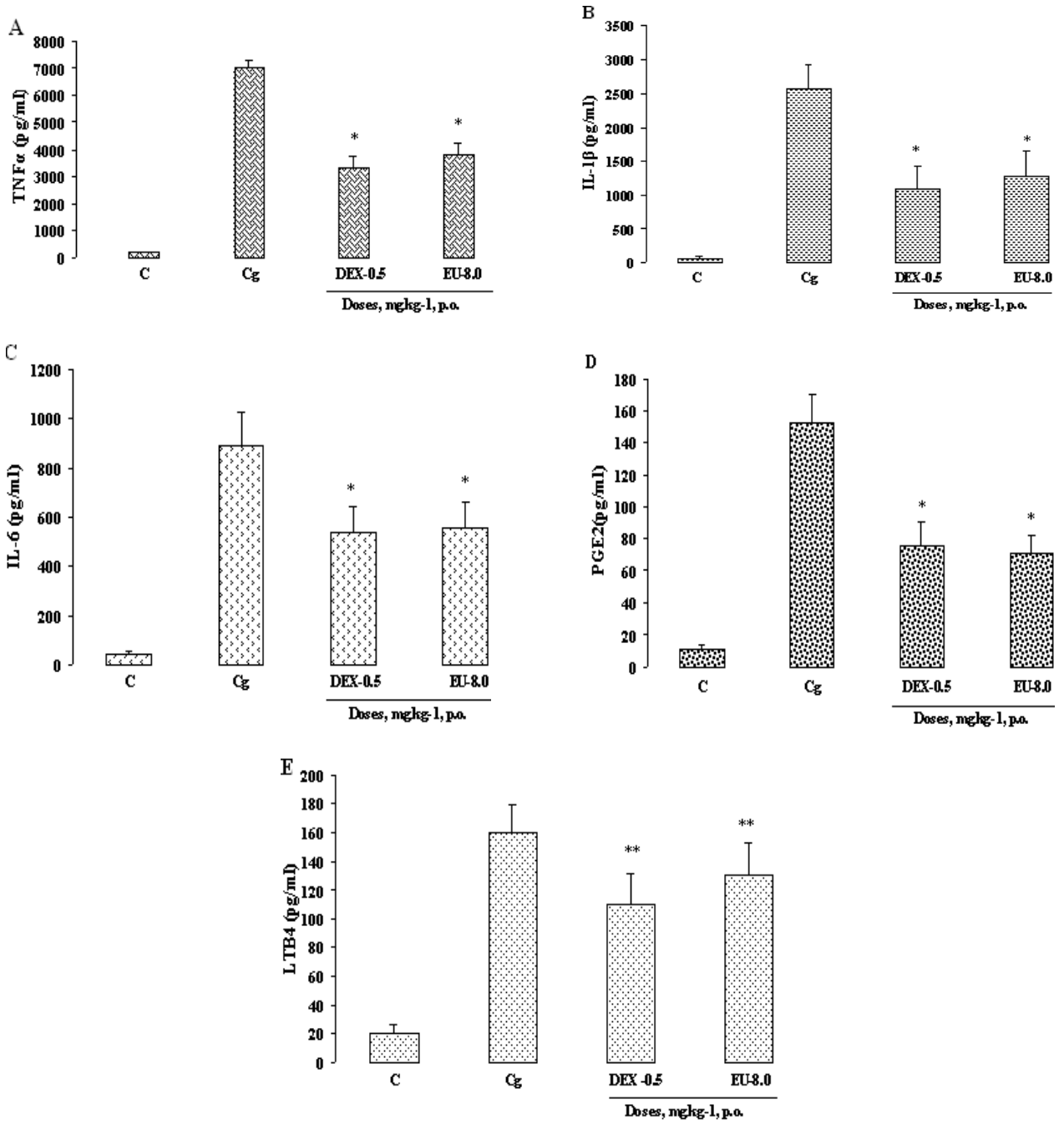

Fig. 3: Effects of EU ($8mgkg^{-1}$, p.o.) administered 1 h before carrageenan administration on TNFα (A), IL-1β (B), IL-6 (C), PGE_2 (D) and LTB_4 (E) levels on the rat model of pleurisy by ELISA. The histogram plots show the levels of respective cytokines and mediators of, c (NaCl, 0.9%), cg (cg-1%), cg+DEX ($0.5mgkg^{-1}$, p.o.) and cg+EU ($8mgkg^{-1}$, p.o.) groups respectively. Values are represented as mean ± S.E.M (n=8) Asterisks indicate significant difference of DEX and EU-treated vs. cg, under specified conditions (*p< 0.01).

Effects of EU on nitrite/nitrate concentrations

EU (8 $mgkg^{-1}$, p.o.) caused a marked reduction in nitrite/nitrate concentrations (% of inhibition: 46.3±2.9, Fig. 4) ($P \leq 0.01$) when compared with cg-treated animals. DEX (0.5 $mgkg^{-1}$ p.o.) also significantly inhibited nitrite/nitrate concentrations (% of inhibition: 42.6±3.2) ($P \leq 0.01$).

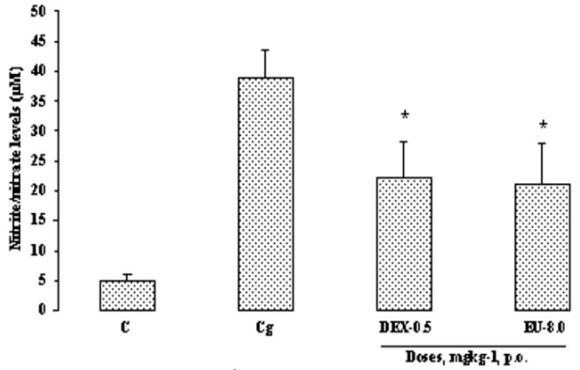

Fig. 4: Effects of EU (8$mgkg^{-1}$, p.o.) administered 1 h before carrageenan administration on the rat model of pleurisy on nitrite/nitrate levels of c (NaCl, 0.9%); cg (cg-1%), cg+DEX (0.5$mgkg^{-1}$, p.o.) and cg+EU (8$mgkg^{-1}$, p.o.) groups respectively. Values are represented as mean ± S.E.M (n=8). Asterisks indicate significant difference of DEX and EU-treated vs. cg, under specified conditions (*p< 0.01).

DISCUSSION

The anti-inflammatory effects of EU have been studied on known conventional models (Yasukawa *et al.,* 2000; Singh *et al.,* 1989) and hence the study with acute inflammatory condition was required to understand its possible mechanism. Various studies have shown that pleural inflammatory response induced by carrageenan (cg) constitutes an interesting experimental model, characterized by two distinct phases: an early phase characterized by the release of chemical mediators such as histamine, bradykinin and prostaglandins followed by a phase that involves mainly leukotrienes (Vinegar *et al.,* 1982).

In this line of view, our studies showed that EU exerts an acute anti-inflammatory effect in the early phase (4 h) and the observations characterized a marked inhibitory profile upon cell migration, regarding both, besides less significant inhibition of exudation. Studies show that the anti-inflammatory drug dexamethasone (DEX) is known to act by inhibiting the transcriptional activation of Activating Protein-1 (AP-1) and NF-kB (Nuclear Factor-kappa B) factors that are linked to the activation of early cytokine genes (Fulvio *et al.,* 2002; Yamamoto *et al.,* 2001; Baeuerle *et al.,* 1994). In a way we could arbitrarily extrapolate the mechanism of EU's action by comparing with DEX but with few reservations. It is known widely that the expression of several genes involved in the immune and inflammatory response is regulated at the transcriptional level, which was found to play an important role in carragenan induced pleurisy (Fulvio *et al.,* 1999).

Neutrophils are involved in processes like phagocytosis, release of proteolytic enzymes, generation of active free radicals, synthesis of cytokines, chemokines and lipid mediators causing inflammation. Recent studies have demonstrated that the recruitment of leukocytes into an inflamed area is mediated by various peptides, and other factors which in turn amplify the inflammatory response by their effects on macrophages and lymphocytes (Oda *et al.,* 1992). In addition specific adhesion molecules and mediators are needed for the diapedesis of leukocytes, which again depends on the presence of proinflammatory cytokines like TNFα, IL-1β, and IL-6 etc (Tomlinson *et al.,* 1994; Butcher 1993).

In this context, EU inhibited significantly extracellular TNFα and IL-1β levels which are potent triggers involved in leukocyte migration (Crofford *et al.,* 1997; Yamamoto *et al.,* 1995) similar to DEX. There was a concordant decrease in the intracellular TNFα in total pleural exudate leukocytes consisting of about 83% of neutrophils, which were the most representative cells of carrageenan pleurisy at 4 h (Torres *et al.,* 2000). Observations showed that EU inhibited cell migration by decreasing the influx of neutrophils. We also observed a significant inhibition of IL-6 in parallel with TNFα and IL-1β reduction which could have contributed for the perpetuation of the inflammatory processes. Several proinflammatory cytokines such as IL-1β, TNFα and IL-6 are involved in kinin induction which has a key role in recruitment of neutrophils in the model of pleurisy induced by carrageenan (Pesquero *et al.,* 2000; Marceau *et al.,* 1998; Sardi *et al.,* 2002).

Analysis of EU's effects also showed significant inhibition of nitrite/nitrate levels further supporting their inhibitory effects upon neutrophils. Studies have found that any increase or decrease in the amounts of nitrite/nitrate levels was highly correlated to a concomitant increase or decrease in PGE_2 (Hermann *et al.,* 1990; Maloney *et al.,* 1998). Various studies have proven that proinflammatory factors like TNFα and chemotactic peptides induce neutrophils to synthesize PGE_2 (McAdam *et al.,* 2000) which is synthesized and preceded by increased COX-2 expression at early time points (between 1.5 to 3 h). Hence, distinct effects of EU were observed in relation to other inflammatory parameters such as PGE_2, and LTB_4 levels. This decrease was in accordance with marked reduction of infiltrated leukocytes and neutrophils but with less significant reduction in mononuclear cells.

EU might also have decreased the reciprocity between nitrite/nitrate levels and PGE_2 and hence would have in turn could have decreased the degree of an inflammatory reaction. Above all, LTB_4, a known neutrophil chemotactic protein was reduced less significantly by EU compared to PGE_2. Altogether, these observations indicated that a different pathway is activated by EU, where predominant regulation was more specifically towards neutrophils and its effector mechanisms like release of proinflammatory cytokines and mediators like TNFα, IL-1β, PGE_2, and nitrite/nitrate levels respectively. Also the decrease in TNFα and IL-1β at 4 h of pleurisy suggested that in this model, EU might have exerted its anti-inflammatory effects via other mediator pathways in addition to Nitric Oxide.

CONCLUSION

EU showed important remarkable anti-inflammatory property with overall associated decrease of proinflammatory cytokines and mediators release. These preclinical studies evidently confirm EU's potential as lead molecule with a therapeutic promise.

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