

## Pharmaco-Toxicological Study of *Kageneckia oblonga*, Rosaceae

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### *Kageneckia oblonga*, Antipyretic, Antiinflammatory, Analgesic Properties, Cucurbitacins

The probable antipyretic, antiinflammatory, analgesic and antioxidant properties of *Kageneckia oblonga*, Rosaceae, were investigated and the major compounds of its active extracts were isolated. The study comprised the acute toxicity of the extracts of global methanol, hexane, dichloromethane and methanol. The cytotoxicity of global methanol extract was studied in three tumoral cell lines. All the extracts exhibited the pharmacological activities under study. Methanol and dichloromethane were the most toxic extracts. From the global methanol extract, isolations were performed of prunasin, 23,24-dihydro-cucurbitacin F, and a new cucurbitacin, 3 $\beta$ -( $\beta$ -D-glucosyloxy)-16 $\alpha$ ,23 $\alpha$ -epoxycucurbita-5,24-diene-11-one. The cytotoxicity of both cucurbitacins on human neutrophils at the assayed concentrations was not statistically significant. In-vitro assays showed that both cucurbitacins can be partly responsible for the analgesic, antipyretic, and anti-inflammatory activities.

Evaluation was done of the cytotoxicity of global methanol extract, 23,24-dihydrocucurbitacin F, aqueous extracts and prunasin against P-388 murine leukaemia, A-549 human lung carcinoma and HT-29 colon carcinoma. Since global methanol extract presented a strong cytotoxicity against P-388 murine leukaemia, A-549 human lung carcinoma, and HT-29 cell lines, it is highly probable that this extract contains one or more cytotoxic compounds that could be investigated for their potential use as an agent against cancer.

## Introduction

In Chile, herbal remedies are frequently used to treat a large variety of ailments and symptoms, e.g., fever, inflammation, and pain; however, there is little information about their efficacy and lack of acute toxicity. *Kageneckia oblonga* is a native species that grows in central Chile. There are no scientific reports on either benefits or toxicity. In folklore medicine, the infusion of its aerial part is used to treat fever in spite of some evidence suggesting some degree of toxicity (Montes and Wilkomirsky, 1983). This research work explores its toxicity and determines its lethal dose 50. In addition, in relation to the probable antiinflammatory, antipyretic, analgesic and antioxidant properties of *K. oblonga*, we report the results of pharmacological assays obtained with global methanol

(GME), hexane (HE), dichloromethane (DCM), methanol, (ME), and aqueous extracts (INF). From one of the active extracts, some metabolites were isolated and evaluated through *in vitro* assays. Since fever, pain, and inflammation may be mechanistically linked through the formation of intermediates of the arachidonic acid (AA) cascade, *K. oblonga* extracts were assayed for their probable antiinflammatory and analgesic abilities (Seibert *et al.*, 1994). In addition, the antioxidant activity was also studied for its probable relation with the antiinflammatory property (Das and Maulik, 1994). GME cytotoxicity against three tumoral cell lines was investigated in search of potentially useful compounds that might help scientists doing research on cancer. For *K. oblonga*, ursolic and bentamic acids, and prunasin have been isolated in earlier studies (Fikenscher *et al.*,

1981; Cassels *et al.*, 1973) and a novel cucurbitacin glycoside has recently been isolated by our research team (Muñoz *et al.*, 2000).

## Results and Discussion

Repeated chromatography of GME, the most bioactive extract, followed by crystallization led to the isolation of the known 23, 24-dihydrocucurbitacin F, tetracyclic triterpenoid (cucurbitacin 1) and the cyanogenic glycoside named prunasin. Both compounds were identified by comparing their spectral data with those reported in literature (El-Fattah, 1994; Fang *et al.*, 1984; Majak *et al.*, 1978; Rockenbach *et al.*, 1992). No reports have been published so far on 23, 24-dihydrocucurbitacin F for *K. oblonga*.

### Acute toxicity

GME and DCM showed toxicity which corresponded to a lethal dose (LD<sub>50</sub>) of 1.34 and 0.94 g/kg respectively. These results partially confirm the information obtained through folk medicine. Prunasin could be partly responsible of the acute toxicity of the GME (Fikenscher *et al.*, 1981). However, HE, and ME did not exhibit toxicity at the dose level assayed (2 g/kg).

### Cytotoxicity assays

GME and INF were assayed against P-388 murine leukaemia, A-549 human lung carcinoma and HT-29 colon carcinoma. In the three tumoral cell lines, GME presented the highest cytotoxicity and an inhibitory concentration (IC<sub>50</sub>) of 2.5 µg/ml, while INF showed weak cytotoxicity, (IC<sub>50</sub> = 10 µg/ml). This is the first report showing that prunasin does not exhibit cytotoxicity (IC<sub>50</sub>>10 µg/ml). Besides, 23, 24-dihydrocucurbitacin F (cucurbitacin 1) exhibits weak, non selective cytotoxicity (IC<sub>50</sub>= 5µg/ml). The weak cytotoxicity of cucurbitacin 1 may be explained by the lack of the double bond in its side chain, which, unlike cucurbitacin F, has a strong cytotoxic compound, i.e., IC<sub>50</sub>= 0.074 against KB and IC<sub>50</sub> = 0.04 µg/ml against P-388 in the cell lines (Fang *et al.*, 1984). Since GME was strongly cytotoxic against P-388 murine leukaemia, A-549 human lung carcinoma and HT-29 cell lines, it must contain cytotoxic compounds with a probable, potential use as an

agent against cancer. In a prior research, we reported that 3β-(β-D-glucosyloxy)-16α,23α-epoxycucurbita-5,24-diene-11-one (cucurbitacin 2) was not cytotoxic (Muñoz *et al.*, 2000). The cytotoxicity of cucurbitacins on human neutrophils at the assayed concentrations was not statistically significant (data not shown) and their superoxide scavenging effect was discarded by using the cell-free system hypoxanthine/xanthine oxidase.

### Antipyretic, anti-inflammatory, analgesic and antioxidant properties

Table I shows results for the pharmacological assays of the various extracts. The maximum effect of sodium naproxen (SN) was dose-dependent for the antipyretic, antiinflammatory and analgesic activities (Delporte *et al.*, 1998). It also provides the antiinflammatory dermal effect of nimesulide. Since all the extracts showed pharmacological activities, we assume that various active secondary metabolites are present. GME exhibited the strongest antipyretic, antiinflammatory and analgesic activity, even remarkably better effects than the reference drug. In addition, GME antiinflammatory activity was dose-dependent. The antiinflammatory dermal assays demonstrated that GME, HE, and ME were pharmacologically active. Since the levels of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) increased markedly after arachidonic acid (AA) application (Lloret and Moreno, 1995), we assume that some active metabolites of the extracts assayed could lower cyclo-oxygenase activity.

The antioxidant property of GME evaluated through the assay against the inhibition of xanthine oxidase (XO) was not statistically significant (19%). Table II shows the inhibitory effect of cucurbitacins 1 and 2 (Fig. 1) isolated from GME and evaluated through the production of superoxide anions and elastase in stimulated human neutrophils. Table III shows that cucurbitacins inhibited nitrite (iNOS activity index) and PGE<sub>2</sub> production (COX-2 activity index) in LPS-stimulated RAW 264.7 cells. There is evidence indicating that PGE<sub>2</sub> cause fever, inflammation and pain (Seibert *et al.*, 1994). Various biological activities have been reported for cucurbitacins, such as their high cytotoxicity; antitumoral effects (Gallily *et al.*, 1992; Gitter *et al.*, 1961; Fang *et al.*, 1984); and their antiinflammatory activity (Yesilada *et al.*, 1988;

Table I. Antipyretic (E), antiinflammatory (A), analgesic (An) and topic antiinflammatory (TA) activities of *K. oblonga*, sodium naproxen (SN) and nimesulide (NM).

Sample	Dose	% E		% A	% An	% TA
		0–90 min	90–180 min			
INF	0.4 ml/25 g 4 ml/kg 2 ml/kg	43.7*	91.7*	38.2*	62.8*	
GME	3 mg/ear 100 mg/kg 300 mg/kg 500 mg/kg 600 mg/kg 700 mg/kg	75.9*	100*	17.0 47.6* 74.4* 87.4*	59.9*	26.7*
HE	3 mg/ear 500 mg/kg 600 mg/kg	44.5*	43.8*	59.9*	46.9*	35.6*
DCM	3 mg/ear 250 mg/kg 600 mg/kg	100*	100*	75.7*	74.5*	22.2
ME	3 mg/ear 500 mg/kg	48*	100*	41.2*	62.6*	41.1*
SN	4 mg/kg 12.5 mg/kg 25 mg/kg	51.0*	81.1*	54.6*	70.0*	
NM	1 mg/20µl/ear					49*

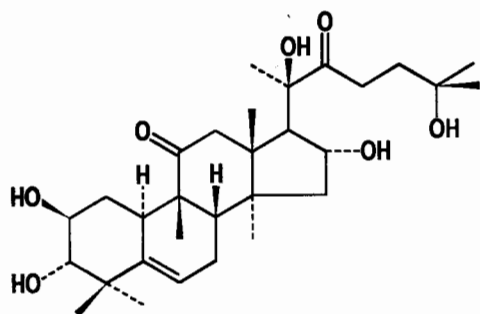
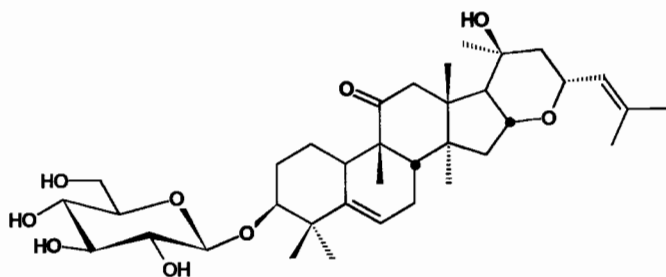
\*  $p \leq 0.05$ .

INF aqueous extract; GME global methanol extract (or crude methanol extract); HE hexane extract; DCM dichloromethane extract; ME methanol extract; SN sodium naproxen (d-2-(6-methoxy-2-naphthyl) propionic acid); NM nimesulide (4-nitro-2-phenoxy-methanesulfonamide); E antipyretic activity for the two time intervals from 0–90 to 90–180 min.

Musza *et al.*, 1994). Ursolic acid, another compound present in *K. oblonga*, has been isolated (Cassels *et al.*, 1973) and its anti-inflammatory effect has been reported (Recio *et al.*, 1995); this permits us to conclude that this triterpene may be partly responsible for the “*in vivo*” anti-inflammatory effect. On the other hand, prunasin could be partly responsible for the acute toxicity of the GME (Fikenscher *et al.*, 1981). Our results also demonstrate that both cucurbitacins contribute to the anti-inflammatory, analgesic, and antipyretic activities of the GME aerial part of *K. oblonga* and, unlike other cucurbitacins, lack cytotoxic effect. The pharmacotoxicological results obtained with the extracts demonstrate that *K. oblonga* does have the properties attributed by folk medicine and a certain degree of acute toxicity.

## Materials and Methods

Column chromatography was run using silica gel 60G (Merck 7734), LH-20 Sephadex (Pharmacy) or Amberlite XAD-2 as non ionic polymeric adsorbent (Aldrich). TLC was performed on silica gel GF254 (Merck 5554); spots were detected under UV light, or spraying sulfuric p-anisaldehyde reagent and then heating for about 5 min at 120°C. TLC was performed on 2 mm thick silica/gel F254 plates (Merck 7731). Recordings of <sup>1</sup>H and <sup>13</sup>C NMR were made in CDCl<sub>3</sub>, DMSO-d<sub>6</sub> and/or DMSO-d<sub>6</sub>+D<sub>2</sub>O at 400 MHz for <sup>1</sup>H and 100 MHz for <sup>13</sup>C; chemical shifts (internal standard TMS). Both 1D (<sup>1</sup>H, <sup>13</sup>C) and 2D (COSY, HMQC, HMBC, ROESY) experiments were performed using standard Bruker microprograms.

23,24-dihydrocucurbitacin F  
(Cucurbitacin 1)

3β-(β-glucosyloxy)-16α,23α epoxycucurbitacin-5,24-diene-11-one (Cucurbitacin 2)

Table II. Inhibitory effect of cucurbitacins 1 and 2 on human neutrophils.

	Elastase release		Chemiluminescence	
	nmol <i>p</i> -nitrophenol/ml supernatant×min	% Inhibition	Chem. Units	% Inhibition
Non-stimulated cells	0.84 ± 0.08	---	2817 ± 71	---
Stimulated cells	58.84 ± 0.48	---	12841 ± 356	---
Cucurbitacin 1 (10 μM)	33.12 ± 0.24	42.9 ± 5.1**	5396 ± 309	46.2 ± 3.1**
Cucurbitacin 2 (10 μM)	46.16 ± 0.24	20.5 ± 4.3**	7126 ± 430	27.1 ± 4.4**

Results are expressed as mean ± S. E. M. ( $n = 6-12$ ). \*\*  $p < 0.01$  with respect to the stimulated control group. Elastase activity expressed as a rate (nmol *p*-nitrophenol/ml supernatant×min). Chem. units = chemiluminescence units.

#### Plant material

The aerial part of *Kageneckia oblonga* R. et P., Rosaceae was collected in Lagunillas, Santiago, Chile, in May and later identified by Dr. Carla Delporte. Voucher specimens were kept in the Herbarium of Escuela de Química y Farmacia, University of Chile (SQF: 22144a).

#### Extraction and isolation

Air-dried and powdered vegetal material (1 kg) was extracted using methanol at room temperature. A rotary evaporator was used to remove methanol and obtain dry 120 g GME in the vacuum. An aqueous extract (INF) was prepared from dried and ground vegetal material, adding boiling water to a weighed amount to obtain 20%

Table III. Effect of cucurbitacins 1 and 2 on iNOS and COX-2 activities in LPS-stimulated RAW 264.7 cells.

	NO <sub>2</sub> <sup>-</sup>		PGE <sub>2</sub>	
	ng/ml	% Inhibition	ng/ml	% Inhibition
Non-stimulated cells	154 ± 7		7.3 ± 0.6	
Stimulated cells	727 ± 64	-----	33.5 ± 1.3	-----
Cucurbitacin 1 (10 μM)	363 ± 8	36.7 ± 1.3**	14.7 ± 0.5	44.0 ± 2.1**
Cucurbitacin 2 (10 μM)	429 ± 8	25.2 ± 1.3**	18.9 ± 0.7	28.0 ± 2.7**

Results are expressed as mean ± S. E. M. ( $n = 6-12$ ). \*\*  $p < 0.01$  with respect to the stimulated control group. iNOS inducible nitric oxide synthase; COX-2 cyclo-oxygenase-2; RAW 264.7 macrophage cell line; LPS lipopolysaccharide of *Escherichia coli*.

and 40% (w/v) aqueous extracts. A new amount (4.7 kg) of plant material was successively extracted at room temperature hexane, CH<sub>2</sub>Cl<sub>2</sub> and MeOH, yielding 176 g HE, 211 g DCM and 1.123 g ME respectively after removing the corresponding solvents in vacuum. The dry extracts were used to perform pharmacological and toxicological assays. A smaller amount (59.4 g) of GME, the most bioactive extract, was dissolved in MeOH-H<sub>2</sub>O (1:9 v/v) mixtures. The solution was chromatographed on an Amberlite column with MeOH-H<sub>2</sub>O (1:9, 9:1v/v) mixtures, and finally MeOH. mixtures. Five fractions (I-V) were collected. Fraction IV was chromatographed again on a Sephadex LH-20 column with hexane-CHCl<sub>3</sub>-MeOH (2:1:0.5v/v) mixtures as eluent. Fractions of 20 ml were collected and monitored by TLC. Fractions 26-29 contained a cucurbitacin mixture. Solvent evaporation afforded a residue (1.5 g) that was subjected to MPLC on silica gel, eluting with 9:1 CHCl<sub>3</sub>-MeOH mixture. Fractions of 15 ml were collected and monitored by TLC. Fractions 22-25 containing cucurbitacin 1 were combined (20 mg) and purified by flash CC over silica gel with CHCl<sub>3</sub> - MeOH (7:1 v/v) as eluent. Further purification by preparative TLC using a mixture of CHCl<sub>3</sub> - MeOH (4:0.5 v/v) gave 6.3 mg pure cucurbitacin 1. Fraction V was rechromatographed on a Sephadex LH-20 column with hexane-CHCl<sub>3</sub>-MeOH (2:1:1 v/v/v) mixtures as eluent. Fractions of 20 ml were collected and monitored by TLC. Fractions 26-40 that contained a compound that was later identified as prunasin was purified by flash CC over silica gel with ethyl acetate - MeOH (1:1 v/v) as eluent giving 2.5 g this compound.

### *In vivo assays animals*

Pirbright guinea pigs (220-300 g) of both sexes and female New Zealand rabbits (2-3 kg) were used for the antiinflammatory and antipyretic studies respectively. CF-1 mice of either sex (20-25 g) were used to assess the analgesic effect and acute toxicity. Animals under standard conditions from the Chilean Public Health Institute were fasted overnight before the day of the experiments.

### *Acute toxicity*

For each dose, groups of 10 mice of both sexes were allowed free access to water. GME, HE, DCM and ME, suspended in saline gum arabic, 5%, were orally administered via gastric catheter. They were weighed daily for a week to detect physiological alterations. LD<sub>50</sub> was calculated by the Morgan Scoring method (Morgan, 1992).

### *Antipyretic activities*

For each per os dose, antipyretic activity was determined using five rabbits and 5 control ones. Pyrexia was induced by i.v. injection of 13 ng/kg *Escherichia coli* endotoxin. Their rectal temperatures were recorded for 180 min with an Ellab Pyrogentester (model Z12DP) after pyrogen injection. The mean areas under the curve (AUC) of temperature vs time for each pyrogen-treated animal with and without previous oral administration of the samples were compared. The antipyretic effect (E) was calculated according to the following equation:

$$\% E = [1 - \text{AUC}(\text{pyr} + \text{sample}) / \text{AUC}(\text{pyr})] \times 100$$

where AUC (pyr+sample) represents the mean AUC after plotting temperature vs time in minutes for the sample-assayed rabbits, and AUC(pyr) is the corresponding mean AUC for the animals treated only with pyrogen. These mean AUCs were calculated for the two time intervals: from 0–90 to 90–180 min. The active principles with fast absorption and elimination could be present in the samples under study, showing an effect only in the first 90 min of the assay. However, the active principles could have a slow absorption and the effect would only be seen after 90 min (Delporte *et al.*, 1998).

#### Antiinflammatory activity

For each per os dose, the antiinflammatory activity was evaluated in groups of 10 to 15 guinea pigs and 20 control ones, using the  $\lambda$ -carrageenan-induced paw edema described by Backhouse *et al.* (1994). Paw volume was measured with an Ugo Basile plethysmometer (model 7150), and 3 h after injecting 0.1 ml of sterile saline ( $\lambda$ -carrageenan, 1%). Antiinflammatory activity (%A) was evaluated as:  $\%A = [(\%Ic - \%Is)/\%Ic] \times 100$

where %Ic is the mean inflammation reached in the control group receiving only the vehicle ( $34.0 \pm 2.3\%$  paw volume increase), and %Is corresponds to the mean inflammation in the sample-treated animals, expressed as:

$$\%I = [V_f - V_i/V_i] \times 100$$

where  $V_f$  and  $V_i$  are final and initial paw volumes respectively, mean %I over all the animals used in each test.

#### Topic antiinflammatory activity

Eight mice were treated with the sample and after 5 minutes they received 2 mg arachidonic acid dissolved in 20  $\mu$ l acetone. Ten control subjects received only AA at the same concentration. Both the sample and the AA were applied to the inner (10  $\mu$ l) and outer (10  $\mu$ l) surfaces of the right ear. The left ear received only the acetone. Mice were sacrificed by cervical dislocation and a 6 mm diameter section of the right and left ears were cut and weighed (Lloret and Moreno, 1995). Dermal antiinflammatory activity was evaluated according to the following equation:

$$\%TA = [W_c - W_s/W_c] \times 100$$

where  $W_c$  and  $W_s$  are the difference mean values of the weights of the right and the left ear sections of the control and the treated animals respectively.

#### Analgesic activity

For each per os dose of the sample under study, the analgesic activity was evaluated in groups of 8 mice and 16 control subjects, using a intraperitoneal injection of 0.5 ml of 0.6% acetic acid. The analgesic effects were calculated by comparing the number of abdominal writhes of the treated and the control group, which only received the vehicle (Davies *et al.*, 1997). The number of abdominal writhes of each mouse was counted for 30 min, beginning 5 min after acetic acid administration.

The following equation was used to calculate the mean pain percentage:  $\%P = [C_{\text{sample}} / C_{\text{control}}] \times 100$

where C sample is the mean writhes reached in sample-treated animals and Ccontrol ( $41.6 \pm 3.79$ ) is the mean writhes reached in control animals which received only the vehicle.

The analgesic effect, An, was calculated according to the following equation:  $\%An = 100 - \%P$

In antipyretic, antiinflammatory and analgesic assays, the extracts (GME, INF, HE, DCM and ME) were orally administered 1 h before  $\lambda$ -carrageenan, endotoxin injection or acetic acid by means of an intragastric catheter, suspended in saline gum arabic. For antiinflammatory and analgesic activities, the drug-induced changes were statistically estimated using the Wilcoxon test for independent data and the same test for dependent data was used to estimate the antipyretic activity (Hollander and Wolfe, 1973). The effects were significant for  $p \leq 0.05$ . The  $S_m (SD/\sqrt{n})$  values were calculated for mean %Ic and %Is values (Ic and Is are the mean inflammation reached in the control and in the sample-treated guinea pigs respectively), for the mean areas under the curve, for the mean writhes constriction and for the mean weight ears in treated and untreated animals in each assay.

Sodium naproxen, obtained from Laboratorios Saval, Chile, was used as a reference drug and was suspended in the same vehicle;  $\lambda$ -carrageenan was obtained from Sigma. *E.coli* endotoxin was obtained from the Chilean Health Institute. For the dermal antiinflammatory activity, nimesulide from Laboratorio Chile was the reference drug used at the dose of 1 mg/20  $\mu$ l/ear.

*In vitro* assays

## Cytotoxicity assays

A screening procedure was used to assess the cytotoxicity of GME, INF, cucurbitacin 1 and the compound later identified as prunasin against the following cell lines: P-388 (lymphoid neoplasm from DBA/2 mouse, ATCC CCL-46), A-549 (human lung carcinoma, ATCC CCL-185), HT-29 (human colon carcinoma, ATCC HTB-38). Cells were seeded into 16 mm wells (multi-dishes) (NUNC 42001) at concentrations of  $1 \times 10^4$  (P-388),  $2 \times 10^4$  (A-549) (HT-29) cells/well, respectively, in 1 ml aliquots of MEM 10FCS medium containing the compound to be assessed at the concentrations assayed. In each case, a set of control wells was incubated in the absence of sample and counted daily to ensure the exponential growth of cells. After four days at 37 °C, under a 10% CO<sub>2</sub>, 98% humid atmosphere, P-388 cells were observed through an inverted microscope and the degree of inhibition was determined by comparison with the control, whereas A-549 and HT-29 were stained with crystal violet before examination (San Feliciano *et al.*, 1993).

## Xanthine oxidase activity

Both xanthine and xanthine oxidase (XO) from cow's milk were purchased from Sigma Co. and the standard inhibitor allopurinol was obtained from Laboratorios Saval, Chile; GME was evaluated at 50 µg/ml and those having an inhibition >50% were further tested for IC<sub>50</sub> determination (Noro *et al.*, 1983; Schmeda-Hirschmann *et al.*, 1992). The inhibition of XO activity using xanthine as the substrate was spectrophotometrically measured in relation to the amount of uric acid, which was determined at 290 nm using a UNICAM spectrophotometer. The assayed mixture consisted of 1.0 ml of test solution, 2.9 ml of phosphate buffer (Na<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>; pH = 7.5) and 0.1 ml of enzyme solution. After preincubation of the mixture at 25 °C for 15 min, the reaction was initiated by adding 2.0 ml of substrate solution. This assayed mixture was incubated at 25 °C for 30 min. This reaction was stopped by adding 1.0 ml of 1 N HCl, the absorbance was measured. The inhibition percent of xanthine oxidase activity (%I) was calculated as:

$$\%I = (A-B) - (C-D) / (A-B) \times 100$$

where A is the activity of XO without test material (total uric acid); B, the blank of A without XO; C, the enzyme activity with test material (residual uric acid); and D, the blank of C without the enzyme.

The IC<sub>50</sub> determination of allopurinol was 0.035 µg/ml (0.267 µM). For xanthine oxidase activity, the drug-induced changes were statistically estimated using Wilcoxon test for independent data (Hollander and Wolfe, 1973). Effects were significant when  $p \leq 0.05$ . The Sm (SD/√(n)) values were calculated for mean %I<sub>c</sub> and %I<sub>s</sub> values (I<sub>c</sub> and I<sub>s</sub> are the mean of inflammation reached in the control and sample-treated animals respectively), the mean areas under the curve, the mean writhes constriction and the mean weight ears for treated and untreated animals in each assay.

*In vitro* assays of cucurbitacins 1 and 2

*Cytotoxicity assays:* the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Gross and Levi., 1992) was used to assess the possible cytotoxic effects of test compounds on human neutrophils. Leukocytes were obtained and purified as previously described (Bustos *et al.*, 1995).

*Chemiluminescence: neutrophils* ( $2.5 \times 10^6$ /ml) were mixed with luminol (40 µM) and stimulated with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) 1 µM. The chemiluminescence was recorded in a Microbeta Trilux counter (Wallac, Turku, Finland) after 7 min, previously determined as the time of maximal production (Terencio *et al.*, 1998). Superoxide anions were also generated by the hypoxanthine/xanthine oxidase system (Betts, 1985).

*Elastase release by human neutrophils: neutrophils:* ( $2.5 \times 10^6$ /ml) were preincubated with assayed compounds or the vehicle for 5 min and then stimulated with cytochalasin B (10 µM) and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 10 nM) for 10 min at 37 °C. After centrifugation at 400×g at 4 °C for 5 min, supernatants were incubated with *N*-*tert*-butoxy-carbonyl-L-alanine *p*-nitrophenyl ester (200 µM) for 20 min at 37 °C (Barrett, 1981). The extent of *p*-nitrophenol release was measured at 414 nm in a microtiter plate reader and expressed as a rate.

**Nitrite ( $NO_2^-$ ) and  $PGE_2$  production:** the mouse macrophage cell line Raw 264.7 was cultured in DMEM (Dulbecco's Medium M) medium containing L-glutamine 2 mM, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 10% fetal bovine serum. Macrophages were removed from the tissue culture flask using a cell scraper, centrifuged at 800 g for 10 min. Cells were resuspended at a concentration of  $2 \times 10^6$ /ml and cultured in 96-well culture plate. Macrophages were co-incubated with the assayed compounds and *Escherichia coli* LPS (serotype 0111:B4) (10  $\mu$ g/ml) at 37 °C for 18 h. Nitrite concentration as reflection of NO release was assayed fluorometrically (Misko *et al.*, 1993). The amount of nitrite was obtained by extrapolation from a standard curve with sodium nitrite as a standard.  $PGE_2$  levels were assayed by radioimmunoassay (Moroney *et al.*, 1988). The results are presented as mean  $\pm$  SEM. The level of statistical significance was determined by analysis of variance (ANOVA), followed by Dunnett's *t*-test for multiple comparisons.

For *in vitro* assays,  $PGE_2$  was purchased from Amersham Iberica, (Madrid, Spain). The rest of reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

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