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Oleanonic acid, a 3-oxotriterpene from *Pistacia*, inhibits leukotriene synthesis and has anti-inflammatory activity

Eva M. Giner-Larza [a], Salvador Máñez [a], M. Carmen Recio [a], Rosa M. Giner [a], José M. Prieto [a], Miguel Cerdá-Nicolás [b], J. Luis Ríos [a,*]

[a] *Departament de Farmacologia, Facultat de Farmàcia, Universitat de València, Av. Vicent Andrés Estellés s / n, 46100 Burjassot, Valencia, Spain*
[b] *Departament de Patologia, Facultat de Medicina, Universitat de València, Av. Blasco Ibáñez 17, 46010 Valencia, Spain*

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Abstract

One of the best known bioactive triterpenoids is oleanolic acid, a widespread 3-hydroxy-17-carboxy oleanane-type compound. In order to determine whether further oxidation of carbon 3 affects anti-inflammatory activity in mice, different tests were carried out on oleanolic acid and its 3-oxo-analogue oleanonic acid, which was obtained from *Pistacia terebinthus* galls. The last one showed activity on the ear oedema induced by 12-deoxyphorbol-13-phenylacetate (DPP), the dermatitis induced by multiple applications of 12-*O*-tetradecanoyl-13-acetate (TPA) and the paw oedemas induced by bradykinin and phospholipase A_2. The production of leukotriene B_4 from rat peritoneal leukocytes was reduced by oleanonic acid with an IC_{50} of 17 μM. Negligible differences were observed in the response of both triterpenes to DPP, bradykinin, and phospholipase A_2, while oleanonic acid was more active on the dermatitis by TPA and on the in vitro leukotriene formation. In conclusion, the presence of a ketone at C-3 implies an increase in the inhibitory effects on models related to 5-lipoxygenase activity and on associated in vivo inflammatory processes.

Keywords: Oleanonic acid; Inflammation; Leukotriene; (*Pistacia terebinthus*); Skin; Triterpenoid

1. Introduction

Oleananes constitute what is probably the largest and most important group of triterpenes. Some have been described as anti-inflammatory agents: oleanolic acid, α-boswellic acid, erythrodiol, maniladiol, longispinogenin, β-amyrin, glycyrrhetinic acid, and many others (Ríos et al., 2000). Oleanolic acid gives its name to the wider group and is a compound fairly abundant in plants, whereas the 3-oxo homologue, oleanonic acid, is present in a limited number of natural resources. In our case, we obtained this compound from the galls of *Pistacia terebinthus*, whose methanolic extract proved to be anti-inflammatory (Giner-Larza et al., 2000). Various studies on oleanolic acid have been published. It exhibits anti-inflammatory activity in carrageenan-induced paw oedema in rats and mice and in adjuvant induced arthritis, and it suppresses the delayed sensitivity reaction induced by dinitrochlorobenzene (Ríos et al., 2000). When assayed along with other triterpenes, it significantly reduced the oedema induced by 12-deoxyphorbol-13-tetradecanoate (DPT), 12-deoxyphorbol-13-phenylacetate (DPP) and bryostatin 1 (Huguet et al., 2000). In contrast, existing literature contains few biological studies of its 3-oxo analogue oleanonic acid. One such study by Inada et al. (1995) studied the inhibitory effects of one of the oleanonic acid derivatives, 22-β-hydroxy-oleanonic acid, on TPA-induced Epstein-Barr virus early antigen (EBV-EA) activation.

In view of these antecedents, we have studied the activity of both oleanolic and oleanonic acids in different inflammatory tests to observe whether the presence of the oxo group in three has effects other than those observed with oleanolic acid, and to establish whether there is a structure–activity relationship for the punctual modification of the C-3 oxidation pattern.

2. Material and methods

2.1. Plant material

Galls from *P. terebinthus* L. (Anacardiaceae) were collected in Xàtiva (Valencia, Spain) in January 1998. The species was identified by Prof. J.B. Peris from the Departament de Biologia Vegetal and a voucher specimen, no.

* Corresponding author. Tel./fax: +34-96-386-4973.
E-mail address: riosjl@uv.es (J.L. Ríos).

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DF-8/98, is deposited in the Herbarium of the Departament de Farmacologia, Universitat de València, Spain.

2.2. Animals

Groups of six female Swiss mice weighing 25–30 g and female Wistar rats weighing 180–200 g were used. All animals were fed a standard diet ad libitum. Housing conditions and experimental procedures complied with the European Union regulations on the use of animals for scientific purposes (CEE Council 86/609).

In experiments previously performed in our laboratory, it was demonstrated that the solvents used as excipients for each drug do not influence the inflammatory process when applied topically (Payá et al., 1993), i.p. or p.o. (Huguet et al., 2000).

2.3. Chemicals

Ascorbic acid, bradykinin, calcium ionophore A 23187, carbamazepine, cyproheptadine hydrochloride, 12-deoxyphorbol-13-phenylacetate (DPP), dexamethasone, dimethyl sulfoxide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), eosin, indomethacin, isoprenaline hemisulphate, hexadecyltrimethylammonium bromide (HTAB), hydrogen peroxide, formaldehyde, hematoxylin, glycogen, D-glucose, *N,N*-dimethylformamide, phospholipase A_2 from *Naja mossambica* venom, prostaglandin B_2, pyridin, pyridinium chlorochromate, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), tetramethylbenzidine, and Trypan Blue were purchased from Sigma (St. Louis, MO); ethyl phenylpropiolate and tween 80 from Fluka Chemika–Biochemika (Buchs, Switzerland); acetone and dichloromethane of analytical grade, and high performance liquid chromatography (HPLC) gradient grade methanol from Baker (Deventer, Holland); Sephadex® from Amersham Pharmacia Biotech (Uppsala, Sweden), ethanol 96°, sodium acetate and methanol of analytical grade from Panreac (Barcelona, Spain).

2.4. Isolation and identification of compound

The galls of *P. terebinthus* were dried and ground to obtain 120 g of powder which was extracted with methanol. The extractive liquid was concentrated to vacuum and extracted with hexane and dichlorometane and ethyl acetate. The dry dichlorometanic extract (9 g) was chromatographed via vacuum liquid chromatography on a silicagel column and eluted with dichloromethane to obtain 35 fractions. Fractions 16 to 19 (2.4 g) were rechromatographed by vacuum liquid chromatography, and eluted with dichloromethane to obtain 42 fractions. Fractions 13 to 22 were chromatographed by medium pressure liquid chromatography on reverse phase RP-8 and eluted with methanol/water (8:2) to obtain 19 fractions. From fractions 14–16 (85 mg), we obtained 65 mg of a pure compound by a medium pressure liquid chromatography on SiO_2 using hexane/dichloromethane (5:5) as a mobile phase, which was identified by mass spectrometry, ^{1}H and ^{13}C-nuclear magnetic resonance as 3-oxo-olean-12-en-28-oic acid or oleanonic acid.

2.5. Semisynthesis of oleanonic acid

Oleanolic acid (500 mg) was dissolved in pyridine/dichloromethane (1:3) and oxidised with 2.3% pyridinium chlorochromate by stirring for 4 h at room temperature. The reaction was then stopped with ascorbic acid, and the residue was chromatographed on Sephadex® LH-20 and eluted with methanol to purify oleanonic acid (Proksa et al., 1992).

2.6. Ethyl phenylpropiolate-induced mouse ear oedema

An oedema was induced on the right ear by topical application of 1 mg/ear (20 μl) ethyl phenylpropiolate in acetone (Brattsand et al., 1982). Oleanonic acid (1 mg/ear) and dexamethasone (0.05 mg/ear) were dissolved in acetone, and applied topically 16 h before the induction of the ear oedema. The oedema was measured by means of a micrometer (Mitutoyo) 1 h after challenge. The oedema reduction (inhibition%) was expressed as the difference between ear thickness before and after challenge while the control was treated only with ethyl phenylpropiolate.

2.7. Mouse ear oedema induced by TPA

An oedema was induced on the right ear by topical application of 2.5 μg of TPA dissolved in 20 μl acetone (Giner et al., 2000a). The triterpenes and the reference drug indomethacin were dissolved in the same volume of acetone and applied (0.5 mg/ear) on the ear simultaneously with TPA. The thickness of the ears was measured before TPA application and 4 h after, and the oedema was expressed as the increase in thickness due to the inflammatory challenge.

2.8. Mouse oedema induced by application of DPP

The inflammatory agent (4 μg/ear) dissolved in 20 μl acetone was applied on the right ear (Huguet et al., 2000). Oleanonic acid was dissolved in the same conditions and administered simultaneously with DPP at the dose of 0.5 mg/ear. Oedema was measured 30 min after challenge and expressed as the increase in ear thickness due to the irritant agent.

2.9. Delayed type hypersensitivity induced by dinitrofluorebenzene in mouse ear

According to the method described by Góngora et al. (2000), the sensitisation phase was induced by topical application of 20 μl of 0.2% (v/v) dinitrofluorobenzene in acetone onto a shaved abdomen on days 0 and 1. Mice

were challenged on day 5 by application of 20 μl of 0.2% dinitrofluorobenzene in acetone on the inner and outer ear surfaces. Oleanolic and oleanonic acids (0.5 mg/ear) and dexamethasone (0.05 mg/ear) dissolved in acetone were applied topically (20 μl) on the ear 2, 24, 48, and 72 h after challenge. The oedema was calculated for each ear as the difference in thickness before treatment and 24 and 96 h after challenge.

2.10. Mouse ear inflammation induced by multiple topical applications of TPA

Inflammation was induced by topical application, on alternate days, of TPA (2 μg/ear) in 20 μl acetone on the inner and outer surfaces of each ear (Stanley et al., 1991). Oleanonic and oleanolic acids in acetone were applied topically (0.3 mg/ear), twice daily, for 4 days, beginning in the morning immediately after TPA application and continuing 6 h later. On the last day, the compounds were applied only in the morning. Dexamethasone was used as the reference drug (0.05 mg/ear). The mice were killed by cervical dislocation and two ear punches were taken from each animal ($n = 5$ animals). The percentage of swelling inhibition was calculated from the difference between the ear weight of each group and the acetone-only control and referred to the control treated only with TPA. Six samples placed in HTAB were frozen for the myeloperoxidase assay and four samples were placed in 4% formaldehyde for histology.

2.11. Myeloperoxidase assay

According to the methods used by De Young et al. (1989), each ear sample was mixed with sodium phosphate buffer (PBS, pH 5.4) containing 0.5% HTAB, then homogenised and decanted into a microfuge tube. After addition of a second aliquot of HTAB, the mixture was centrifuged at $11200 \times g$. The supernatant (30 μl) was added to 0.017% H_2O_2 in PBS in a 96-well microtiter plate. The reaction was started by adding 18.4 mM TMB in 8% aqueous dimethylformamide. The mixture was incubated for 3 min at 37 °C and then placed on ice. The reaction was stopped with 1.46 M NaOAc. Enzyme activity was determined colorimetrically using a Labsystems Multiskan MCC/340 plate reader set to measure absorbance at 620 nm. The percentage of myeloperoxidase activity reduction was calculated with respect to the TPA control.

2.12. Histology

Ear samples were fixed in 4% neutral-buffered formalin. Each sample was cut longitudinally into equal halves. Half of each was embedded in paraffin, cut into 3–4 μm sections and stained with hematoxylin–eosin. Epithelium thickness was evaluated using an objective ×100 and expressed as the mean ± S.D. of the number of epidermal layers from the basal to the granulous stratum, inclusive (Giner et al., 2000b).

2.13. Phospholipase A_2-induced hind–paw mouse oedema

The method was described by Neves et al. (1993). Phospholipase A_2 from *N. mossambica* (1.18 units in 25 μl saline) was injected s.c. into the right hind mouse paw. The left paw received the same volume of vehicle. Oleanolic and oleanonic acid (30 mg/kg) were injected i.p. 30 min before phospholipase A_2, and the reference drug cyproheptadine (10 mg/kg) was administered p.o. 60 min before challenge. Both the products and the reference drug were dissolved in tween 80/ethanol/water (2:2:20). The oedema was measured by means of a plethysmometer (Ugo Basile) 30, 60 and 90 min after challenge and was expressed as the difference between the right and left paw volume. The control group, was treated only with phospholipase A_2. Percentages of inhibition were referred to the control group.

2.14. Bradykinin-induced mouse paw oedema

In accordance with the method described by Tsurufuji et al. (1980), 25 μl of a solution containing 3 μg bradykinin in saline was s.c. injected into the right hind paw. The left paw received the same volume of saline. Oleanonic acid (30 mg/kg) was dissolved in ethanol/tween 80/ water (1:1:10), and administered i.p. 60 min before the irritant

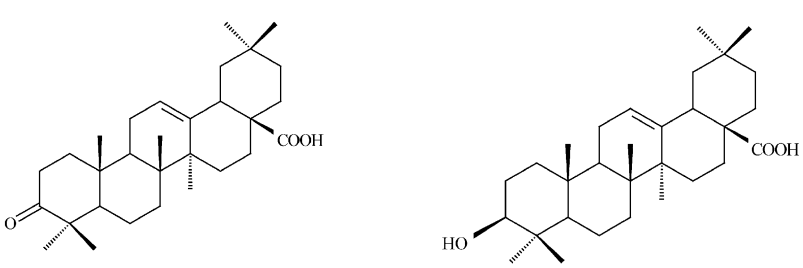

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Fig. 1. Chemical structures of oleanonic (1) and oleanolic (2) acids.

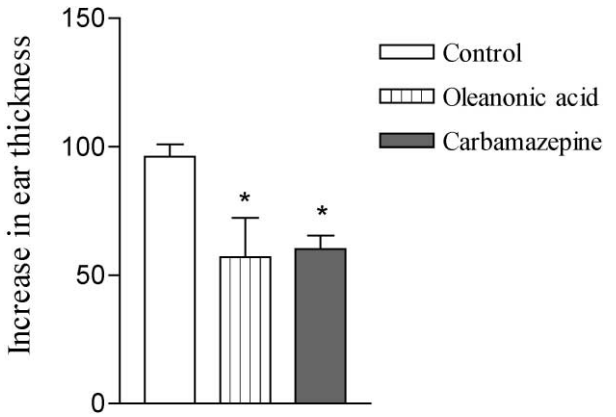

Fig. 2. Effect of oleanonic acid and carbamazepine (0.5 mg/ear) on DPP-induced mouse ear oedema, six animals. $^{*}P < 0.05$. Increase in ear thickness in μm (mean ± S.E.M.) $^{*}P < 0.05$.

agent. Isoprenaline (5 mg/kg) was dissolved in saline and administered i.p. The oedema was measured 12 min after bradykinin injection and expressed as the difference between the right and left paw volume.

2.15. Cytotoxicity assay

We used a modified MTT colorimetric assay (Mossman, 1983) to measure the cytotoxicity of the triterpenes. In short, neutrophils were exposed to the products in a microplate for 30 min, and then incubated with MTT. The coloured metabolite was dissolved in dimethyl sulfoxide in an ultrasonic bath and measured using Labsystems Multiskan MCC/340, at 490 nm. The results were expressed in absolute absorbance readings; a decrease of absorbance indicated a reduction in cell viability.

2.16. Inhibition of leukotriene B_4 production from rat polymorphonuclear leukocytes

We followed the protocol described by Safayhi et al. (1995). To obtain leukotriene B_4 formation from endogenous arachidonic acid, glycogen-elicited rat peritoneal polymorphonuclear leukocytes were stimulated by 1.8 mM Ca^{2+} and 1.9 μM calcium ionophore 23187. The cells were incubated in the presence of different concentrations of the test compounds. Separation of the 5-lipoxygenase pathway products was carried out by HPLC followed by diode array detection. A reverse phase RP-8 column was employed and eluted with methanol/water (74:26) containing 0.007% trifluroacetic acid. The results obtained from peak areas were normalised to a prostaglandin B_2 internal standard and expressed as a percentage of leukotriene B_4 production. IC_{50} value was calculated by means of the lineal regression plotted from the inhibition percentages obtained at four different concentrations between 12.5 and 100 μM.

2.17. Assay of cyclooxygenase-1 activity from human platelets

We followed the protocol described by Safayhi et al. (1995) and modified by Laufer et al. (1995) and in which the activity of cyclooxygenase-1 is measured in terms of the production of 12-hydroxyheptadecatrienoic acid (12-HHTrE). Blood platelets, obtained from healthy human donors, were separated by sequential centrifugations, and stimulated by 2.5 mM Ca^{2+} and 1.9 μM calcium ionophore 23187. The platelets were incubated in the presence of the test compound (100 μM). Separation of 12-HHTrE was achieved by HPLC followed by diode array detection. A reverse phase RP-8 column was eluted with methanol/water (74:26) containing 0.007% trifluroacetic acid. The results obtained from peak areas were normalised to a prostaglandin B_2 internal standard and expressed as percentage of 12-HHTrE production.

2.18. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. Unless otherwise stated, six animals per group were used for the pharmacological experiments in vivo.

3. Results

Following the application of spectroscopic techniques, the compound isolated from *P. terebinthus* galls was identified as 3-oxo-olean-12-en-28-oic acid, the commonly known as oleanonic acid (Fig. 1). This compound was identical to that which we obtained through oxidation of commercial oleanolic acid with Cr^{6+}.

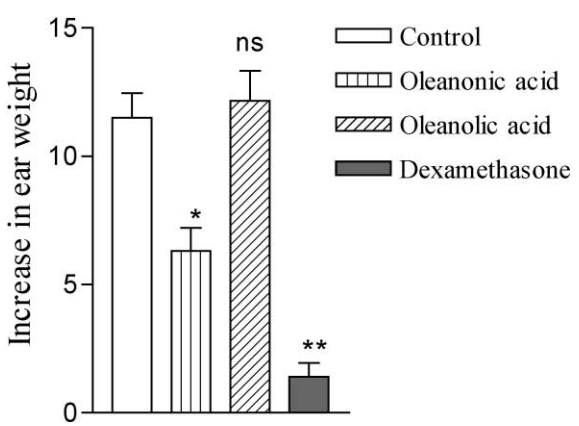

Fig. 3. Effects of oleanonic and oleanolic acids (repeatedly 0.3 mg/ear) and dexamethasone (repeatedly 0.05 mg/ear) on chronic inflammation induced by TPA in mouse ear, five animals. $^{**}P < 0.01$, $^{*}P < 0.05$, ns = not significant. Increase in ear weight in mg (mean ± S.E.M.).

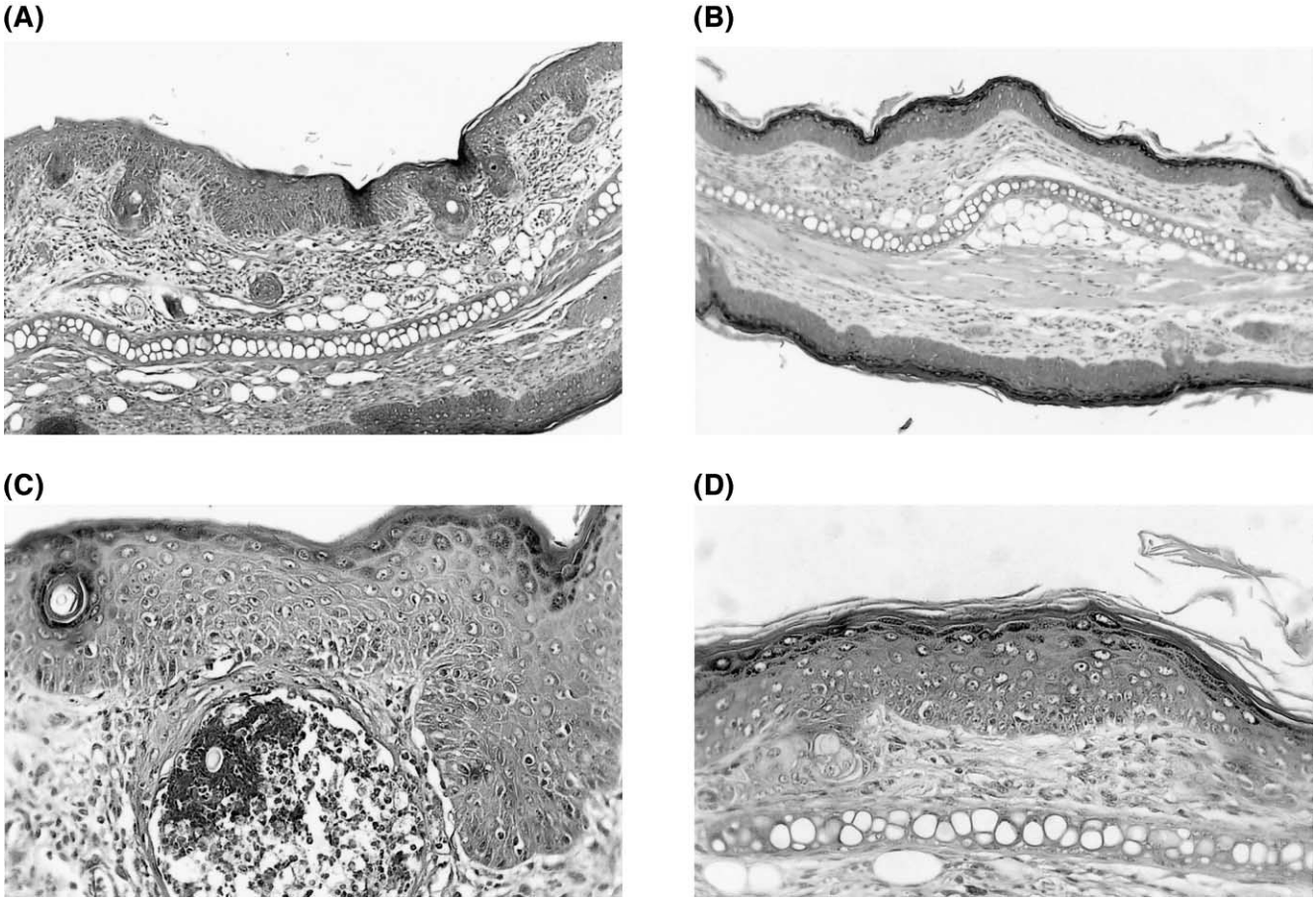

Fig. 4. Ear tissues after repeated application of TPA. (A) Control, ×10. (B) Ear treated with oleanonic acid, ×10. (C) Control, ×40. (D) Ear treated with oleanonic acid, ×40.

Oleanonic acid exerted no activity on the oedema induced by application of ethyl phenylpropiolate after a pre-treatment of 16 h. In the TPA ear oedema test, it showed a non-significant 28% inhibition. However, when assayed on the ear oedema induced by DPP, oleanonic acid reduced the swelling by 40%, an effect similar to that of the standard carbamazepine (Fig. 2). In the mouse model of delayed hypersensitivity induced by dinitrofluorobenzene, oleanonic acid was ineffective at both 24 and 96 h, while oleanolic acid reduced non-significantly the oedema at 96 h by 32%.

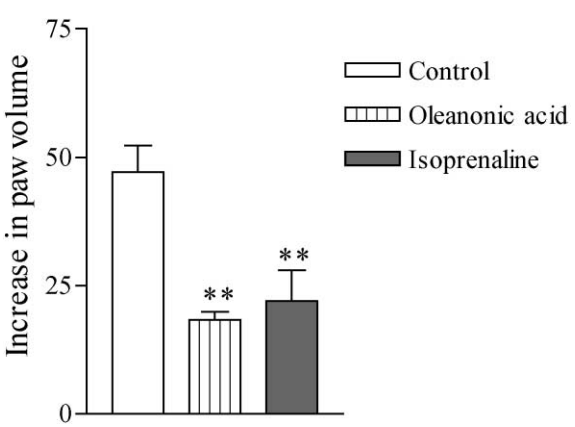

Fig. 5. Effect of oleanonic acid (30 mg/kg i.p.) and isoprenaline (5 mg/kg i.p.) on bradykinin-induced mouse paw oedema, six animals. $^{**}P < 0.01$. Increase in paw volume in μl (mean ± S.E.M.).

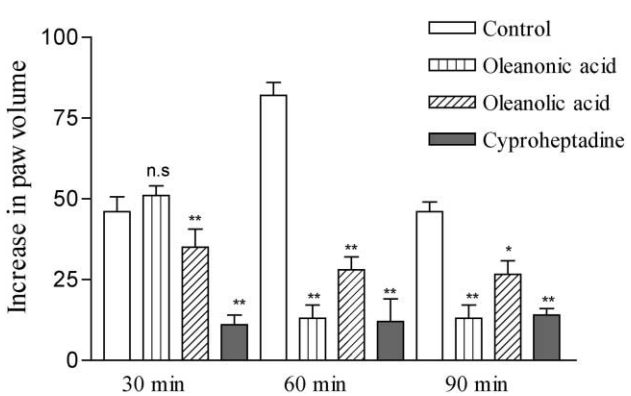

Fig. 6. Effects of oleanonic and oleanolic acids (30 mg/kg i.p.) and cyproheptadine (10 mg/kg p.o.) on *N. mossambica* phospholipase A_2-induced mouse paw oedema, six animals. $^{**}P < 0.01$, $^{*}P < 0.05$, ns = not significant. Time values in abscissa indicate the interval between phospholipase A_2 injection and oedema measure. Increase in paw volume in μl (mean ± S.E.M.).

In the TPA model of chronic inflammation induced by multiple applications, oleanonic acid showed a significant effect, with 45% inhibition. In contrast, oleanolic acid was inactive (Fig. 3). Both inhibited the neutrophil infiltration measured as myeloperoxidase activity by 84% and 67%, respectively. The inhibition observed for dexamethasone on the swelling and myeloperoxidase activity was around 90%. The histological study of ears treated only with repeated doses of TPA showed an extensive diffusive inflammatory lesion with microabscesses affecting dermis and epidermis. The main infiltrating cells in the skin were neutrophils and epithelial thickness was 6.6 ± 1.0 cells. In the tissues treated only with the solvent acetone, epithelial thickness was 2.1 ± 0.5 and no signs of lesion or leukocyte infiltration were detectable. The multidose treatment with oleanonic acid reduced both the intensity and extension of the damage produced by TPA, as this was localized in the dermis, where the main infiltrating cells were lymphocytes, and where fibrosis was observed. In this case, epithelium thickness was 4.4 ± 0.7 cells. The ears treated with dexamethasone showed minimal inflammatory lesions and sometimes none at all, and the epithelium thickness was 4.3 ± 0.7 cells (Fig. 4).

The paw oedema induced by bradykinin was significantly reduced (61%) by oleanonic acid, whereas isoprenaline had a slightly lower effect (52%, Fig. 5). Both oleanolic and oleanonic acid also reduced the paw oedema induced by phospholipase A_2; the latter showing its strongest effect at 60 min, with an 84% inhibition, and maintaining activity at 90 min. Oleanolic acid also had its maximum effect at 60 min, vanishing at 90 min, while the activity of cyproheptadine was uniform along the experiment, ranging 80–90% inhibition (Fig. 6).

Oleanonic acid was considered non-cytotoxic, since it gave a polymorphonuclear leukocyte viability higher than 95%. The production of leukotriene B_4 from the cells from the rat peritoneal cavity was considerably reduced by oleanonic acid and with a dose–effect dependence, which led to a IC_{50} value of 17 μM (Fig. 7).

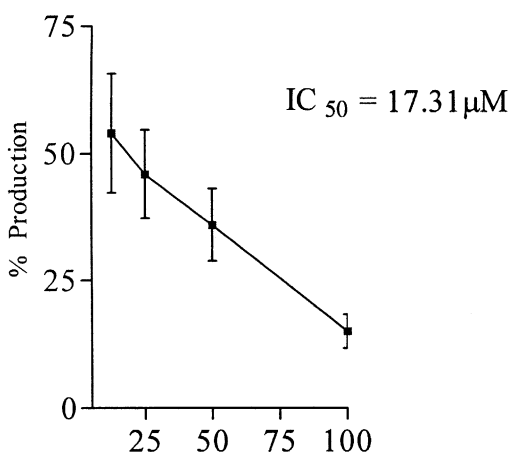

Fig. 7. Dose–response relationship for the inhibition by oleanonic acid of leukotriene B_4 production from rat peritoneal polymorphonuclear leukocytes stimulated by calcium ionophore A 23187. Concentrations of oleanonic acid (μM) in abscissa. IC_{50} = inhibitory concentration 50%. For each of the doses, the graph represents mean ± S.E.M.

Later, oleanonic and oleanolic acids, at doses of 100 μM, were tested for their inhibitory effects on cyclooxygenase-1 from human platelets. Oleanonic acid reduced by 78% the production of 12-HHTrE, whereas oleanolic acid exerted no significant inhibition.

4. Discussion

In a previous report, Recio et al. (1995) tested oleanolic acid on ethyl phenylpropiolate-induced ear oedema, and found that it lacked activity, as observed for oleanonic acid in the present study. We can therefore assume that the action of these compounds is unlike that of glucocorticoids, as the latter were the only compounds among the usual anti-inflammatory drugs to be active in this test. In addition, none of these compounds showed activity on the contact hypersensitivity model of oedema by dinitrofluorobenzene, which suggests that there was no interference with the expression of cell-mediated immunity.

In the previous study by Huguet et al. (2000), it was established that, among a representative list of triterpenoids, only oleanolic and betulinic acid were active against the oedema induced by DPP, a protein kinase C activator with marked neurogenic character. In the present study, we have demonstrated that oleanonic acid has results very similar to those yielded by oleanolic acid, proving that the oxidation of C-3 in oleanolic acid does not change the activity. Similarly, oleanolic and oleanonic acid showed equivalent activity on the paw oedema induced by bradykinin. When compared with other triterpenes of various structures, both compounds exhibited high-range effects in this test, for which little relationship was found between structure and activity, possibly due to the large sum of mechanisms that participate in the effect of bradykinin (Huguet et al., 2000).

One of the most salient properties of oleanonic acid was the high inhibition on the phospholipase A_2-induced oedema, 60 and 90 min after the stimulus. The mechanism underlying this effect may be a direct inhibition of the enzyme, but also a blockage of the mastocyte degranulation, which in turn leads to release of serotonin and other vasoactive amines (Cirino et al., 1989). The participation of serotonin, and histamine, in the oedema is evident by the strong effect of the mixed antagonist cyproheptadine. The differences in the time profile of the effects of oleanolic and oleanonic acids on the oedema induced by phospholipase A_2 could be due to pharmacokinetic aspects. It is possible that the structural difference between both acids prolongs the elimination time, and, consequently, the effect of the 3-keto derivative.

One of the possible reasons for the more marked effect of oleanonic acid, on the chronic model of TPA, compared with that of oleanolic acid, is the specific inhibition of

some mediators, such as leukotrienes, which are generated in large quantities in chronic inflammation. In this context, it should be remembered that, in the model of acute oedema induced by TPA, an efficient reference drug is indomethacin, a cyclooxygenase inhibitor, whereas in chronic inflammation the reference drug is a glucocorticoid, dexamethasone, although other kinds of products such as 5-lipoxygenase inhibitors are also active (Stanley et al., 1991). Histological examinations of ears subjected to repeated treatment with TPA, both alone and with oleanonic acid or dexamethasone, establish that oleanonic acid is an efficient inhibitor of neutrophil affluence into the skin, which would constitute a basis for its anti-inflammatory cutaneous effect. This compound tended to modify the original lesion, converting it in a milder chronic form, which was indicated by fibrosis and lymphocyte infiltration. It produced an antiproliferative effect on the epithelial layer similar to that of dexamethasone at the applied dose. The restriction in the leukocyte influx to the dermis was biochemically confirmed by a diminished activity of myeloperoxidase levels in the homogenised tissues. It should be noted that although oleanolic acid also reduced myeloperoxidase activity, and consequently the leukocyte infiltration, its anti-inflammatory activity in terms of swelling was weaker.

In relation to the previous knowledge on the possible inhibitory activity of oleanolic of 5-lipoxygenase, Prof. Safayhi confirmed, in a personal communication, that the highest of the four tested doses (100 μM), showed only a slight inhibition approximately, 30%. In contrast, the more powerful effect of oleanonic acid in this system, suggests that it acts through a mechanism related to the inhibition of 5-lipoxygenase, either directly or interfering with some of the mechanisms that participate in the complex activation of this enzyme. Our preliminary data on platelet cyclooxygenase inhibition suggest that oleanonic acid also acts by reducing prostaglandin synthesis, although further experiments should follow to determine both its potency and effectiveness in inflammatory cells.

To conclude, it would seem from the results obtained for oleanolic and oleanonic acid, that the presence of a keto group at C-3 augments the anti-inflammatory activity in certain models closely related with the activation of 5-lipoxygenase, and to a lesser extent, in those models involving phospholipase A_2 activity.

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