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The Effects of a Triterpene Fraction Isolated from *Crataegus monogyna* Jacq. on Different Acute Inflammation Models in Rats and Mice. Leucocyte Migration and Phospholipase A_2 Inhibition

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Abstract

The plant *Crataegus monogyna* has action against cardiac insufficiency, angina and arrhythmia. The anti-inflammatory properties of the cycloartenol fraction from this plant have been investigated.

Chromatographic fractionation of the hexane extract of *Crataegus monogyna* Jacq. (Rosaceae) furnished a triterpene fraction containing cycloartenol as the main component (80·87%). The anti-inflammatory activity of the fraction was tested against hind-paw oedema induced by carrageenan in rats. At the highest oral dose (40 mg kg^{-1}) inhibition was 61·5 and 52·5% at 3 and 5 h respectively. In the mouse carrageenan peritonitis test, the triterpene fraction given orally inhibited peritoneal leucocyte infiltration (41·9, 64·7 and 89·4% at 10, 20 and 40 mg kg^{-1}, respectively). The fraction also showed weak inhibition of phospholipase A_2 (PLA_2) in-vitro.

These results suggest that the fraction containing cycloartenol as the main component exerts an important anti-inflammatory action in-vivo by reducing the oedema.

Crataegus monogyna Jacq. (Rosaceae), known as majuelo, espino majuelo, espino blanco and espinero in Spanish folklore, is a small thorny tree widely distributed on the Iberian peninsula, the Balearic Islands and in Northwest Africa (Font Quer 1990). The plant has been used for many years because of its action against cardiac insufficiency, angina and arrhythmia (Poch Noguer 1981; Furlenmeier 1984). Chromatographic fractionation of the hexane extract from this species furnished a triterpene fraction whose main component (80·87%) was identified as cycloartenol (Ahumada 1995).

The purposes of these investigations were to determine the anti-inflammatory activity of the enriched cycloartenol fraction in animal models of inflammation, to assess the suppressive effects of the fraction on leucocyte migration in the peritoneal model of acute inflammation, and to investigate, in an in-vitro assay, the inhibition on phospholipase A_2.

Materials and Methods

Materials

Aerial parts (twigs, stems and leaves) of *Crataegus monogyna* Jacq. were collected in Puerto de los Vientos (Serrania de Ronda, Málaga), during February after a cold period. A specimen (SEV 137261) was deposited at the herbarium of the Department of Vegetal Biology (Faculty of Pharmacy, University of Sevilla) and was authenticated by Professor Silvestre.

The freshly collected plant material (500 g) was extracted with hexane in a Soxhlet extractor and the hexane extract was concentrated under reduced pressure. The residue (2 g) was chromatographed on a silica gel column (60 g, 63–200 μm; Merck) and successively eluted with mixtures of hexane and diethyl ether of increasing polarity (200 mL hexane, 100 mL hexane-diethyl ether 90 : 10 v/v, 100 mL hexane-diethyl ether 80 : 20 v/v and 100 mL hexane-diethyl ether 70 : 30 v/v). The 70 : 30 hexane-diethyl ether (70 : 30 v/v) eluate yielded a crystalline residue (2·8 mg).

The composition of this crystalline fraction was determined by gas chromatography combined with mass spectrometry (Carlo Erba gas chromatograph linked to a Kratos MS 80 mass spectrometer). Separation was performed with a 25 m × 0·25 mm i.d. Chrompack CP9000 capillary column. The column temperature was maintained at 230°C for 6 min then programmed at 4° min^{-1} to 300°C; helium was used as carrier gas. Electron-impact mass spectra were acquired at 70 eV. This analysis showed the predominance of cycloartenol in the fraction. The identity of cycloartenol was confirmed by comparison of its spectroscopic properties with literature values given by Goad (1991). Cycloartenol accounted for 80·87% of the fraction.

Carrageenan-induced paw oedema

Oedema was induced in male rats, 180–200 g (Winter et al 1962). Sterile carrageenan in saline (0·1 mL, 1%) was injected into the plantar side of the right hind paws of groups of six animals. Oral doses of the triterpene fraction were selected and administered orally (10, 20 or 40 mg kg^{-1}), 1 h before injection of carrageenan. Control animals received only the excipient (Tween 80, 0·8% in saline). Paw volumes were measured by means of a plethysmometer (LI-7500, Letica) before the administration of carrageenan, and 3 and 5 h later. The increase in volume was taken as the volume of oedema. Results were compared with those obtained with the reference compound, indomethacin (25 mg kg^{-1}, 10 mL kg^{-1} in saline), administered orally.

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Mouse carrageenan peritonitis

Inflammation was induced by modification of the technique of Griswold et al (1987). The triterpene fraction and the reference compounds, indomethacin and prednisolone were administered orally (by intragastric probe) at doses of 10, 20 or 40 mg kg^{-1} for the triterpene fraction and 10 mg kg^{-1} for indomethacin and prednisolone, in saline, to groups of six Swiss mice. Carrageenan (0·25 mL, 0·75% in saline) was injected intraperitoneally 1 h later, and after 4 h the animals were killed by cervical dislocation and further exsanguination. Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline was then injected into the peritoneal cavity. After gentle massage peritoneal exudates were removed; total leucocytes were determined in a Neubauer chamber and the differential cell count determined by microscopic counting of Giemsa- and May-Grünwald-stained slides.

Phospholipase A_2 assay

Phospholipase A_2 was assayed by the method of Ferrándiz et al (1994). Membranes of *Escherichia coli* were labelled with [^{3}H]oleate. *E. coli* strain CECT 101 was seeded in a medium containing 1% tryptone, 0·5% NaCl and 0·6% sodium dihydrogen orthophosphate, pH 5·0, and grown for approximately 8 h at 37°C in the presence of 5 μCi mL^{-1} [^{3}H] oleic acid (spec. act. 10 Ci mmol) until growth approached the end of the logarithmic phase. After centrifugation at 2500 *g*, the membranes were washed in buffer (0·7 M Tris-HCl, 10 mM $CaCl_2$, 0·1% bovine serum albumin, pH 8·0), resuspended in saline and autoclaved for 30–45 min. The membranes were then washed, centrifuged again and frozen at −70°C. At least 95% of the radioactivity was incorporated into the phospholipids. Two secretory enzymes were assayed, *Naja naja* and bee venom enzymes, which were diluted in 10 μL 100 mM Tris-HCl, 1 mM $CaCl_2$ pH 7·5 buffer and preincubated at 37°C for 10 min with a solution (2·5 μL) of the test compound (triterpene fraction and mepacrine at 10·65 and 1·18 μg respectively) or its vehicle (methanol). Mepacrine was used as reference compound. Incubation proceeded for 10 min in the presence of 20 μL autoclaved oleate-labelled membranes and was terminated by addition of an ice-cold solution (100 μL) of 0·25% bovine serum albumin in saline to a final concentration of 0·07% w/v. After centrifugation at 2500 *g* for 10 min at 4°C, the radioactivity in the supernatant was determined by liquid-scintillation counting. Control tubes contained enzyme and the vehicle.

Statistical analysis

In in-vivo experiments results are presented as mean ± s.e.m. for each animal group (n = 6); in in-vitro assay the values are expressed as mean ± s.e.m. from three determinations. Data were analysed for significance using Student's *t*-test, and the ED50 value was calculated by linear regression analysis.

Results and Discussion

Anti-inflammatory activity in the carrageenan test was calculated according to Piña & Armijo (1978). The acute inflammation induced by carrageenan was inhibited dose-dependently by oral administration of the triterpene fraction (Table 1). The inhibition values showed that the anti-inflammatory activity decreased 5 h after treatment; 3 and 5 h after carrageenan administration the ED50 values were 24·45 and 35·10 mg kg^{-1}, respectively.

The enriched cycloartenol fraction had an anti-inflammatory effect in the carrageenan-foot oedema test, although this action was moderate compared with that of the reference compound, indomethacin. The second phase of inflammation occurred 3 to 5 h after the administration of the irritant agent and is induced by the release of bradykinin, protease, prostaglandin and lysosomal products (Vinegar et al 1987). The mode of action of this fraction could be related to the inhibition of the release of mediators involved in the second phase of inflammation.

Leucocyte aggregation at the site of inflammation is a fundamental event in the inflammatory process. Cell migration is the result of many different processes including adhesion and cell mobility. In this study we compared the effect on cell migration of indomethacin (a non-steroidal anti-inflammatory agent), prednisolone (a steroidal anti-inflammatory agent) and the triterpene fraction. The fraction resulted in marked dose-related inhibition of the leucocyte migration induced by carrageenan in mice (ED50 = 14·61 mg kg^{-1}). In this assay, the triterpene-enriched fraction was a more potent leucocyte migration inhibitor than indomethacin and the results were comparable with those obtained with prednisolone. Migration of neutrophils was also drastically reduced in this peritonitis model (Table 2). Non-steroidal anti-inflammatory agents such as indomethacin, which is a selective cyclooxygenase inhibitor, are far less effective in preventing cell infiltration into inflammatory lesions than agents that inhibit the release of arachidonic acid from membrane phospholipids (glucocorticoids such as prednisolone), and thereby suppress its conversion to eicosanoids (Bird et al 1985; Griswold et al 1987). It has also been reported that triterpenoids are able to cause a considerable reduction in the synthesis by human neutrophils of 5-lipoxygenase products (Kweifio-Okai et al 1994), and there are several studies that suggest involvement with arachidonic metabolism, activation of leucocytes and the complement cascade (Recio et al 1995).

The presence of elevated levels of PLA_2 in inflamed tissues and in a variety of experimental models suggests a direct role in inflammation (Potts et al 1992). The release of arachidonic acid from phospholipids is a rate-limiting step for the formation of arachidonic acid metabolites in polymorphonuclear leucocytes and depends mainly on PLA_2 activity (Meade et al

Table 1. Effect of the triterpene fraction from *Crataegus monogyna* on carrageenan-induced hind-paw oedema in rats.

Treatment (mg kg^{-1})	Anti-inflammatory activity	
	3h	5h
Triterpene fraction		
10	41·09 ± 1·33*	29·61 ± 3·21
20	47·33 ± 1·80*	38·01 ± 2·51
40	61·45 ± 0·86*	54·00 ± 1·42†
Indomethacin		
25	90·30 ± 6·62‡	78·41 ± 6·22‡

Results are mean ± s.e.m., n = 6. *$P < 0·05$, †$P < 0·01$, ‡$P < 0·001$ compared with the control group. ED50 values were 24·45 ± 4·40 and 35·10 ± 4·40 mg kg^{-1} at 3 and 5 h, respectively.

Table 2. Effect of the triterpene fraction from *Crataegus monogyna* on leucocyte migration in peritoneal exudates.

Treatment ($mg\,kg^{-1}$)	Leucocytes ($10^5\ mL^{-1}$)	Neutrophils ($10^5\ mL^{-1}$)	Leucocyte inhibition (%)	Neutrophil change (%)
Control	$4{\cdot}25 \pm 0{\cdot}45$	$2{\cdot}57 \pm 0{\cdot}14$	–	–
Indomethacin				
10	$2{\cdot}81 \pm 0{\cdot}24$*	$0{\cdot}97 \pm 0{\cdot}07^{\ddagger}$	33·89	−62·26
Prednisolone				
10	$0{\cdot}78 \pm 0{\cdot}15^{\ddagger}$	$0{\cdot}06 \pm 0{\cdot}01^{\ddagger}$	81·65	−97·56
Triterpene fraction				
10	$2{\cdot}47 \pm 0{\cdot}17^{\dagger}$	$0{\cdot}65 \pm 0{\cdot}07^{\ddagger}$	41·49	−74·49
20	$1{\cdot}50 \pm 0{\cdot}09^{\ddagger}$	$0{\cdot}25 \pm 0{\cdot}04^{\ddagger}$	64·70	−90·16
40	$0{\cdot}45 \pm 0{\cdot}05^{\ddagger}$	$0{\cdot}11 \pm 0{\cdot}007^{\ddagger}$	89·41	−95·81

*$P < 0{\cdot}05$, $^{\dagger}P < 0{\cdot}01$, $^{\ddagger}P < 0{\cdot}001$ compared with the control group. ED50 was $14{\cdot}61 \pm 3{\cdot}20\ mg\,kg^{-1}$.

Table 3. Effect of triterpene fraction on *Naja naja* and bee venom phospholipase A_2.

Enzyme	Compound (μg)	Inhibition (%)
Naja naja PLA_2	Triterpene fraction (10·65)	$34{\cdot}12 \pm 2{\cdot}40$*
	Mepacrine (1·18)	$68{\cdot}11 \pm 3{\cdot}17^{\dagger}$
Bee venom PLA_2	Triterpene fraction (10·65)	$12{\cdot}35 \pm 1{\cdot}12$
	Mepacrine (1·18)	$65{\cdot}38 \pm 2{\cdot}97^{\dagger}$

*$P < 0{\cdot}05$, $^{\dagger}P < 0{\cdot}01$ compared with the control group.

1986). To investigate the mechanism underlying the anti-inflammatory effects of the cycloartenol enriched fraction, we tested its influence on two secretory phospholipases. The triterpene fraction was able to inhibit *Naja naja* venom PLA_2 (34·12%); the effect on bee venom PLA_2 was only 12·35% (Table 3). The results were lower than those obtained with the reference inhibitor, mepacrine.

In summary, the anti-inflammatory action of the cycloartenol-enriched fraction would seem to be essentially similar to that of the parent compound because the fraction can inhibit peritoneal leucocyte infiltration. We did not, however, observe a direct relationship between its anti-inflammatory activity and the inhibition of PLA_2 activity, because the drastic decrease of polymorphonuclear leucocyte migration at the site of inflammation was not only a consequence of inhibition of PLA_2. Further studies will be required to determine the exact mechanism of action and to establish if cycloartenol was the principal responsible for this action.

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