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# Antihistaminic and antieicosanoid effects of oleanolic and ursolic acid fraction from *Helichrysum picardii*

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*Received September 9, 2006, accepted December 5, 2006*

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*Pharmazie 62: 459–462 (2007)* *doi: 10.1691/ph.2007.6.6704*

*Helichrysum picardii* Boiss. & Reuter is a Mediterranean vegetal species from the Asteraceae family. From the methanolic extract of the aerial flowering parts of this plant, a fraction of two pentacyclic triterpenes has been isolated. Gas chromatography revealed that the triterpene isomers ursolic and oleanolic acids comprised 69% and 29% respectively of the composition of this fraction. The triterpene isomeric fraction was tested in two phagocyte cell systems. It inhibited compound 48/80-induced histamine release from rat peritoneal mast cells in an approximately percentage of 45% at 100 μM and myeloperoxidase secretion from A23187-ionophore-stimulated rat peritoneal leukocytes in a significant manner at doses of 50 and 100 μM. Furthermore, the triterpene isomers very significantly and dose-dependently inhibited generation of the cyclo-oxygenase metabolite prostaglandin $E_2$ (41% inhibition at 50 μM) and the 5-lipoxygenase metabolite leukotriene $B_4$ (79% inhibition at 50 μM) from activated rat leukocytes. This anti-eicosanoid activity of the triterpene fraction was more potent than that produced by the pure triterpene oleanolic acid used for comparision, indicating a stronger action of the ursolic acid, the major compound of the isolated triterpene fraction. From these data, it can be suggested that the triterpene isomers oleanolic and ursolic acids present in the medicinal plant *Helichrysum picardii* contribute to the anti-inflammatory profile of this vegetal species.

## 1. Introduction

The genus *Helichrysum* Miller (Asteraceae), widely represented in the Mediterranean and Iberian flora, consists of a taxonomically complex group of plants, many of which are used in traditional medicine (Ríos et al. 1987, 1991; Czinner et al. 2000; Schinella et al. 2002).

The biological activities of these plants may be due to the presence of flavonoids, terpenes and essential oil. These compounds can be determined in some European species, such as *H. italicum, H. stoechas and H. arenarium* with anti-inflammatory and antioxidant properties (Sala et al. 2002, 2003b; Carini et al. 2001; Czinner et al. 2000). As result of that, this species are used in phytotherapy for the treatment of allergies, skin diseases and inflammatory processes.

*Helichrysum picardii* Boiss. & Reuter is an endemic species which grows in the sandy dunes from the south coasts of the Iberian peninsula. We isolated a lipohilic flavonoid from an ether extract of the flowering parts of this plant (De la Puerta et al. 1990), which demonstrated to possess anti-inflammatory and antioxidant effects (De la Puerta et al. 1999). From the same non-polar extract, a derivative of ursolic acid was identified. Moreover, other phenolic compounds, such as coumarins were identified in this species (De la Puerta et al. 1994). On the other hand, we have previously reported choleretic activity of three species of this genus (Garcia et al. 1990).

In this work, we aimed to investigate the active principles of a methanolic extract of the aerial flowering parts of *Helichrysum picardii*. In view of the important beneficial role that natural compounds from vegetal species play in cancer prevention and the maintenance of good health, the present study was designed to further investigate the anti-inflammatory mechanism of action of this Helichrysum fraction. The effect in A23187-ionophore-stimulated rat peritoneal leukocytes for interaction with eicosanoid release, granular enzyme generation and the effects on compound-48/80-induced histamine release from peritoneal mast cells of the isolated fraction have been investigated.

## 2. Investigations and results

A non-polar fraction was isolated from the methanolic extract of the aerial flowering parts of *H. picardii,* as it is indicated in the Experimental section. This fraction was analysis by TLC and GC/MS analysis. The development of TLC silica gel with oleum reagent gave a purple spot at Rf 0.26 in the mobile phase, *n*-hexane/ethyl ether (7 : 3 v/v) and at Rf 0.73 at dichloromethane/ethyl acetate 7/3 v/v. The gas chromatogram (Fig. 1) showed two major compounds at retention time $t_R$ : 16.3 and $t_R$ : 15.1 that comprised 69% and 29% of the composition of this fraction respectively. Results were confirmed by co-injection in CG with authentic samples of oleanolic and ursolic acids.

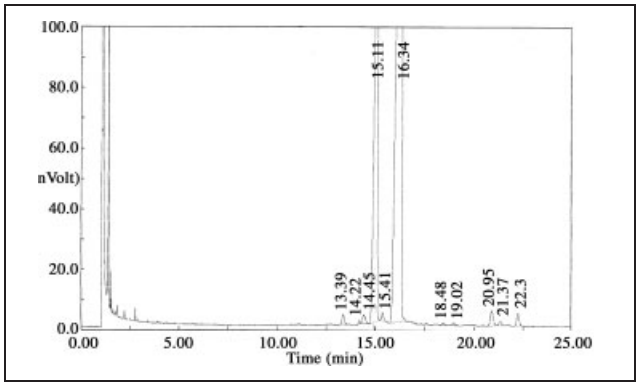
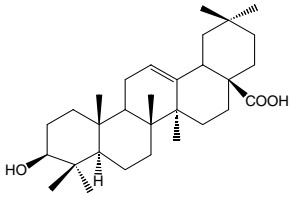
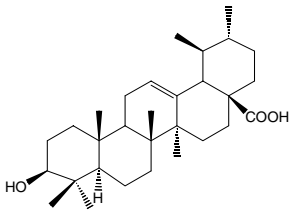



Fig. 1: Gas chromatogram of the isolated fraction from a methanolic extract of *Helichrysum picardii*




Oleanolic acid: 3β-hydroxy-olean-12-en-28-oic acid




Ursolic acid: 3β-hydroxy-urs-12-en-28-oic acid

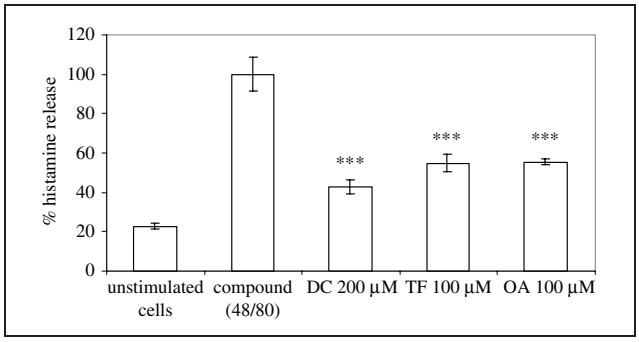



Fig. 2: Effect of the triterpene fraction (TF) from *H. picardii* on compound 48/80-induced histamine release from rat peritoneal mast cells. Oleanolic acid (OA) and Disodium cromoglicate (DC) included as reference compounds. Data are represented as de mean ± s.e. from n = 4. Statistical significance assessed using (ANOVA + Student "t" test) vs 10 μg $mL^{-1}$ of compound 48/80-treated control group

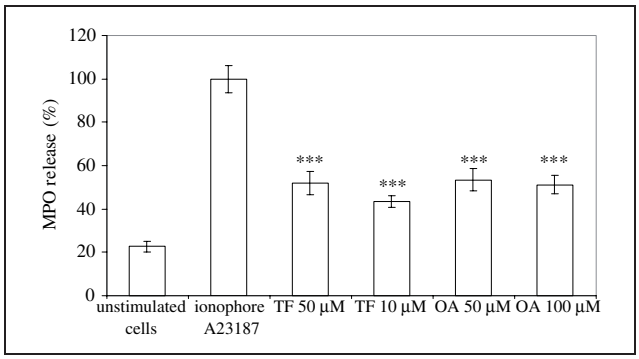



Fig. 3: Effect of the triterpene fraction (TF) from *H. picardii* on myeloperoxidase (MPO) enzyme release (expressed as $O.D_{450}$) from rat peritoneal leukocytes activated by ionophore A23187. Oleanolic acid (OA) included as reference compound. Data are represented as mean ± s.e. from n = 6. Statistical significance assessed using (ANOVA + Student t-test) vs 1 μM ionophore-A23187 treated-control group

The MS spectra data of the TMS ether derivatives were (70 eV): m/z (%) = 600 ($M^+$, 10), 585 ($M^+$-15, 9), 482 (28), 483 (18), 321 (14), 320 (43), 204 (17), 203 (100), 202 (59), 189 (34), 73 (45); and (70 eV): m/z (%) = 600 ($M^+$, 7), 585 ($M^+$-15,12); 482 (15), 483 (9), 321 (26), 320 (93), 297 (7), 204 (17), 203 (100), 202 (42), 189 (33), 73 (53), respectively. The peak at m/z 203 (100%) is typical of the series ursane-12-en and oleane-12-en triterpene structures. These spectral data correspond to those of the authentic samples of oleanolic and ursolid acids.

Figure 2 shows the effects of the triterpene fraction (TF) and a reference of pure oleanolic acid in histamine release from rat peritoneal mast cells. The spontaneous release of histamine from rat peritoneal mast cells was approximately 20% and the maximum histamine level of the mast cells was that of the cells treated only with 10 μg/mL of compound 48/80 (100% of release). TF and OA showed a similar effect on the histamine release, with percentages of inhibition of 45 ± 4.7 and 44 ± 1.6 respectively at 100 μM, respect to compound 48/80-stimulated cells. This inhibitory effect was slightly inferior than that produced by the reference compound DC (57 ± 1.6%).

Activation of rat peritoneal leukocytes with A23187 causes a calcium-dependent secretion of the contents of the granules, as the release of the enzyme myeloperoxidase (MPO). The results revealed a significant inhibition of MPO release ($p < 0.001$) compared to the A23187-stimulated cells, either by the TF or the pure OA at 50 and 100 μM (Fig. 3).

As shown in Fig. 4, addition of the calcium ionophore A23187 to rat leukocytes also causes the generation of nanogram amounts of eicosanoids via both cyclo-oxygenase and lipoxygenase pathways. At doses of 50 and 100 μM, the triterpene fraction produced less amount of either $LTB_4$ (Fig. 4A) or $PGE_2$ (Fig. 4B) concentrations than the pure OA used for comparison. Both doses 50 and 100 μM produced significant inhibition values against both arachidonate derivatives compared to the control ionophore-activated cells.

## 3. Discussion

The anti-histaminic and anti-eicosanoid properties of a non-polar fraction isolated from the methanolic extract of *H. picardii* have been evaluated. The gas chromatography analysis revealed that this fraction was composed mainly of ursolic acid (UA, 69%) and its isomer oleanolic acid (OA, 29%), both triterpenes with a pentacyclic structure. UA and several derivatives have previously been identified in some other *Helichrysum* extracts (De la Puerta et al. 1990; Sala et al. 2002).

The anti-inflammatory and antioxidant properties of some closely related Mediterranean species to *H. picardii*, as *H. italicum* or *H. stoechas* suggest a relationship between this activity and the presence of compounds such as terpenes (Mezzeti et al. 1970; Sala et al. 2002; Bianchi et al. 2004), flavonoids (Suzgec et al. 2005; Sala et al. 2003a; De la Puerta et al. 1999) and acetophenone derivatives (Sala et al. 2001, 2003b) present in these species.

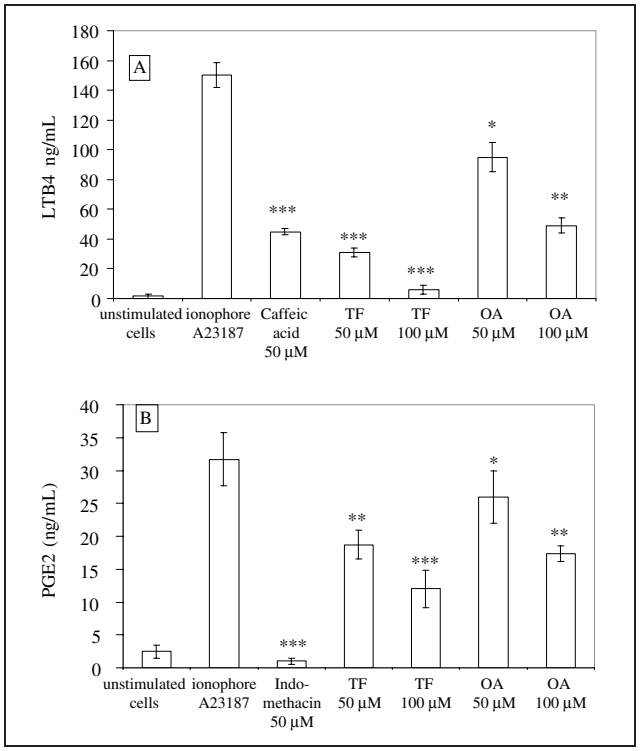



Fig. 4: Effect of the triterpene fraction (TF) from *H. picardii* on the generation of leukotriene $B_4$ (A) and $PGE_2$ (B) from rat peritoneal leukocytes activated by ionophore A23187. Oleanolic acid (OA), caffeic acid and indomethacin included as reference compounds. Data are represented as mean ± s.e. from n = 6. Statistical significance assessed using (ANOVA + Student t-test) vs 1 µM ionophore A23187 treated-control group

Concerning the active principles from *H. picardii*, we have previously reported that the lipophilic flavonoid gnaphalin, isolated from *H. picardii* exerts a potent inhibitory action on enzymes of the arachidonate cascade. Thus, gnaphalin exerted a preferential effect on the cyclo-oxygenase pathway, giving rise to reduced $TXB_2$ levels in calcium ionophore-activated leukocytes, and to a lesser extent reduced $LTB_4$ levels because of 5-lipoxygenase inhibition. Besides, free-radical scavenging properties were detected for this flavonoid (De la Puerta et al. 1999).

Related to the isolated triterpenes, it is worth noting that oleanolic acid (3β-hydroxy-olean-12-en-28-oic acid) and its isomer, ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) are distributed largely in natural plants in the forms of free acid or aglycones of a multitude of triterpenoid saponins. They have been described as effective anti-inflammatory agents and are recognised as the active principles of several therapeutically used medicinal plants (Safayhi and Sailer 1997; Recio et al. 1995). Other biological and pharmacological properties have been summarised by Liu (1995, 2005). Among the biological properties of these triterpenoids, there is a growing interest on their antitumour and chemopreventive activity (Ovesna et al. 2004; Aparecida Resende et al. 2006).

In order to study the mechanism underlying the anti-inflammatory activity of the isolated triterpene fraction, we have investigated its ability to modify certain responses related to the inflammatory process. Production of histamine by mast cells and secretion of granular enzymes and eicosanoids by neutrophils are mechanisms supporting inflammation and tissue destruction. Therefore, inhibition of cell-mediated responses could be and additional mechanism for attenuating inflammation.

Mast cell degranulation followed by histamine release plays a crucial role in the inflammatory response. In the present study, it was demonstrated that the TF significantly inhibited compound 48/80-induced degranulation and histamine release from rat peritoneal mast cells, in a similar extent to the pure oleanolic acid and the reference compound disodium cromoglycate. Ursolic acid from a chloroform extract of *Melaleuca leucadendron* has already demonstrated anti-histaminic action (Tsuruga et al. 1991). The significant inhibitory effect on the mast cells degranulation leads us to propose anti-allergic properties for this *Helichrysum* species.

Furthermore, leukocytes pre-incubated with the TF (50 and 100 µM) secreted lower amounts of myeloperoxidase enzyme from their granules (Fig. 3). The reductive effect on the secretion of both pro-inflammatory mediators could be explained by a stabilizing effect on membranes, as it has been reported that vegetal sterols, with a closely chemical structure, decrease the membrane sensitivity to the permeabalizing effects of a wide array of agents (Navarro et al. 2001).

Finally, the TF showed a potent inhibitory action on both enzymes, cyclo-oxygenase and lipoxygenase, of the arachidonic acid pathway. In fact, TF at 50 µM produced a higher inhibitory percentage of $LTB_4$ concentration (79%) than the reference caffeic acid at the same dose (70%) and at 100 µM decreases the $LTB_4$ level to almost basal values. These results agree with that of other authors where ursolic acid showed to posses an effective anti-lipoxygenase activity (Najid et al. 1992; Diaz et al. 2000). TF also produced a significant percentage of reduction on the $PGE_2$ generation (41% at 50 µM), although not to the same extent as in $LTB_4$ concentration. In both cases the anti-eicosanoid activity of the TF was more potent than that produced by the pure OA used for comparison. This could be due to a stronger action exerted by ursolic acid, the major compound of the isolated triterpene fraction.

Taken as a whole, we suggest that the anti-inflammatory activity of *H. picardii* maybe mediated by its triterpene isomers OA and UA, which may act synergistically with its flavonoids.

## 4. Experimental

### *4.1. Plant material*

The aerial parts of *Helichrysum picardii* Boiss. & Reuter (Asteraceae) were collected from the coastal sands of Cadiz (SW Spain) at the flowering period and they were identified at the Department of Vegetal Biology, University of Seville. A voucher specimen was deposited in their Herbarium (ref. Prof. Silvestre).

### *4.2. Extraction and identification*

The air-dried flowering parts of *Helichrysum picardii* was successively extracted with water and methanol in a Soxhlet apparatus. The methanolic solution was evaporated under reduced pressure yielding a dry residue of a 10% (w/w).

The methanolic dry residue (10 g) was chromatographed on a silica gel column and eluted using different proportion of solvents of increasing polarity as mobile phases: *n*-hexane, ethyl acetate and methanol. The fractions were subjected to thin layer chromatography (TLC) and those showing identical behaviour were joined.

From the fraction corresponding to the *n*-hexane/ethyl acetate (8 : 2 v/v) eluate a white power precipitated. Plates of silica gel TLC developed with *n*-hexane/ether (7 : 3 v/v) and dichloromethane/ethyl acetate (7 : 3 v/v) as mobile phases was performed with that product. After silanization of this product with hexamethyldisilazane and trimethylchlorosilane in pyridin, the TMS derivatives were separated and analysed by CG/MS. That was performed on a Carlo Erba gas chromatograph linked to a Kratos MS80 mass spectrometer equipped with a NBSLIB2 data system using cross-linked 5% phenyl-methyl-silicone (OV-5 capillary column, 25 m × 0.25 mm × 0.23 µm). Samples were run under at programmed temperature 230 °C (6 min) to 300 °C at 4 °C/min.

### 4.3. Reagents and solutions for bioassay

Calcium ionophore A23187, glycogen, dimethylsulfoxide (DMSO), compound 48/80, *o*-phthlalaldehyde (OPT), bovine serum albumine (BSA), disodium cromoglycate, toluidine blue, heparin sodium and phosphate buffer were supplied by Sigma Chemical Company and NaOH and HCl by Panreac.

### 4.4. Animals

Male Wistar rats, 8–10 week-old and weighing 250–300 g, were housed at $24 \pm 2$ °C with $60 \pm 20$ % relative humidity, on a 12 h light-dark cycle. Rats were given free access to a diet of standard chow and water. All the experiments were performed according to the guidelines for the ethical treatment of animals of the European Union on Animal Care (CEE Council 86/609).

### 4.5. Preparation of rat peritoneal mast cells

Peritoneal mast cells were isolated as previously reported (Wang and Teng 1990; Quilez et al. 2004), mixed rat peritoneal cells were collected by peritoneal lavage with heparinized Tyrode solution and were purified by centrifugation through a 30% BSA density gradient. Purified mast cells were washed and resuspended in Tyrode's solution with glucose and 0.1% of BSA. The concentration of mast cells was adjusted to $1 \times 10^6$ cells/mL for the experiments using toluidine blue 0.05% in saline.

### 4.6. Effect of the isolated fraction on histamine release from rat peritoneal mast cells

The mast cell samples (0.5 mL) in triplicate were prewarmed at 37 °C for 10 min with the test isolated fraction (TF at 100 μM) or its vehicle dimethylsulphoxide (DMSO). Disodium cromoglycate (DC) at 200 μM was used in these experiments as reference compound. Pure oleanolic acid (OA) was included for comparison. The histamine release reaction was triggered by the addition of 2 μL of compound 48/80 at a final concentration of 10 μg/mL for an incubation time of 20 min at 37 °C. The reaction was stopped by the addition of ice-cold Tyrode's solution and the mixture was centrifuged for 10 min at 1000 g.

Histamine in the supernatant was determined fluorimetrically (Hakanson and Ronnberg 1974) after condensation with *o*-phthalaldehyde (1% methanol). The emitted fluorescence was measured in a fluorescence microplate reader (Spectra Fluor Tecan) ($\lambda_{excitation}$ 360 nm, $\lambda_{emission}$ 450 nm). To estimate the spontaneous release of histamine, exactly the same procedure was followed without adding samples or compound 48/80. The release percentage of histamine was expressed as percent of total of the maximum level (cells stimulated with compound 48/80).

### 4.7. Preparation of rat peritoneal mixed leukocytes

Leukocytes containing approximately 85% polymorphonuclear leukocytes (PMNs) and 15% mononuclear cells were prepared as in (Moroney et al. 1988; De la Puerta et al. 1999) from male Wistar rats and resuspended in complete Hanks balanced salt solution (HBSS) at $2.5 \times 10^6$ cells/mL containing 1.26 mM $Ca^{2+}$ and 0.9 mM $Mg^{2+}$. Cell viability based on trypan blue exclusion was greater than 95%.

### 4.8. Effect of the isolated fraction on eicosanoid and myeloperoxidase release from rat peritoneal leukocytes

Triplicate aliquots of 0.5 mL leukocytes were preincubated at 37 °C for 10 min with the test fraction (50 and 100 μM) or its vehicle DMSO. After this, 5 μL of calcium ionophore A23187 was added in DMSO to give a final concentration of 1 μM for a further 10 min of incubation. The cells were pelleted by centrifugation at 2500 g for 10 min at 4 °C, and the supernatants were frozen and retained for analysis of $TXB_2$ or $LTB_4$ by enzyme-immunoassay (Kits obtained from Cayman) or used for measurement of secreted myeloperoxidase (MPO) activity.

MPO release was determined in the above supernatants in triplicate by measuring the rate of oxidation of *o*-dianisidine following the method of Bradley et al. (1982) modified for reading the absorbance in a microplate reader. The OD was read at 1 min and thereafter at timed intervals at 450 nm at room temperature with intermittent shaking. The release percentage of MPO was expressed as percent of total of the maximum level (cells stimulated with calcium ionophore A23187).

### 4.9. Statistical analysis

Results are expressed as means ± standard error of the mean. Differences between untreated and treated cells were tested using one-way analysis of variance followed by Student's t-test for single comparisons.

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