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Characterization of ursolic acid as a lipoxygenase and cyclooxygenase inhibitor using macrophages, platelets and differentiated HL60 leukemic cells

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A new property of ursolic acid, lipoxygenase and cyclooxygenase inhibition, has been described in an acetone-extract of heather flowers (*Calluna vulgaris*) which could help explain the anti-inflammatory characteristics of this plant. In mouse peritoneal macrophages, human platelets and differentiated HL60 leukemic cells, ursolic acid, at 1 μM, blocks arachidonate metabolism.

Lipoxygenase; Arachidonic acid; Cyclooxygenase; Ursolic acid; *Calluna vulgaris*

1. INTRODUCTION

Although flowers of the plant, *Calluna vulgaris*, have been used for centuries in the treatment of inflammatory diseases [1, 2], its mode of action is unknown. Since arachidonic acid metabolites are important mediators of inflammation, especially the products of the lipoxygenase pathway [3, 4], we used lipoxygenase inhibition as a test to screen several heather flower extracts. This test allowed us to purify and characterize ursolic acid as the agent responsible for the inhibition of lipoxygenase activity in the acetone extract. In this paper, we describe for the first time, its biological effect on arachidonic acid metabolism in mouse peritoneal macrophages, human platelets and differentiated HL60 leukemic cells (used as a model of polymorphonuclear leukocytes) and we compare it to the classical lipoxygenase and cyclooxygenase inhibitors, NDGA, BW755C and caffeic acid.

2. MATERIALS AND METHODS

2.1. *Isolation and purification of ursolic acid*

Calluna vulgaris flowers collected in August–September 1990, in the Monédières country-side, Corrèze, France (collection data no. 101) were extracted with acetone. Dry residue was loaded on a LH_{20} Sephadex column (eluted by CH_3OH with a gradient of $CHCl_3$). Fractions inhibiting lipoxygenase activity were pooled and purified on a silicic acid column ($CHCl_3/CH_3OH$: 97/3, v/v) and analyzed by GC:MS and ^1H and ^{13}C NMR. The determination of the ursolic acid X-ray crystallographic structure is described elsewhere [5].

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2.3. *Plant lipoxygenase assay*

The preparation of potato tuber 5-lipoxygenase (5-LOX) has been described [6]. Soybean lipoxygenase was from Sigma. Activity of both enzymes was assayed with linoleic acid (Sigma) as the substrate under the conditions previously described [7, 8]. Briefly, lipoxygenase activity was tested by measuring oxygen consumption using a Clark-oxygen electrode with a Gilson oxygraph assuming a 240 μM O_2 concentration in air-saturated buffer at 25°C. The standard assay mixtures contained potato 5-LOX in 0.2 M sodium acetate (pH 5.6) or soybean 15-lipoxygenase (15-LOX) in 200 mM phosphate buffer (pH 7.4). The reaction was started by the addition of linoleic acid using Michaelis saturation conditions for each enzyme.

Caffeic acid (3,4-dihydroxycinnamic acid) and NDGA (nordihydroguaiaretic acid) were from Aldrich. BW755C (3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline) was a gift from Wellcome Research Laboratories, Langley Court, Beckenham, UK).

2.4. *Arachidonic acid metabolism by mouse peritoneal macrophages*

Resident macrophages from Swiss mice were incubated with [^{14}C]arachidonic acid as previously described [9]. Briefly, peritoneal cells from 57 mice were collected by washing the peritoneal cavity with 10 ml of ice-cold RPMI-1640 with a syringe containing 10 U/ml heparin. Peritoneal cells were then distributed and incubated at 37°C in six 175 cm^2 flasks in 50 ml of RPMI-1640 supplemented with fetal calf serum (5%), glutamine (4 mM), penicillin (200 U), streptomycin (200 μg/ml) and gentamycin (50 μg/ml). After allowing macrophages to

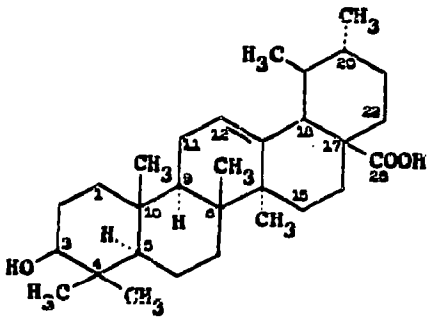

Fig. 1. Formula of ursolic acid.

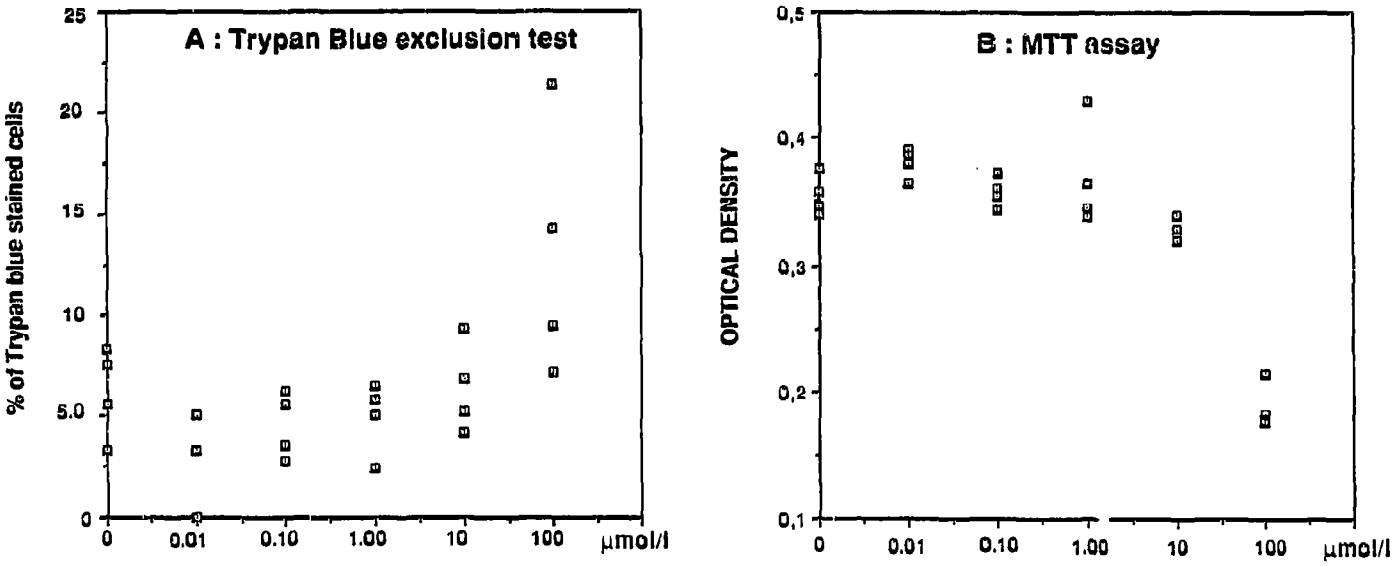

Fig. 2. Ursolic acid cytotoxicity assays. (A) The figure shows that cell viability, after 48 h treatment with 0.01–10 μM of ursolic acid, was greater than 90% as measured by the Trypan blue exclusion test as well as the reduction of MTT (B). The ursolic acid toxicity to the HL60 cells was observed only at the highest concentration used (100 μM).

adhere (2 h at 37°C), non-adherent cells were discarded and flasks were placed at 0°C on ice for 1 h. Viable macrophages (21×10^6) were recuperated, resuspended in 2 ml RPMI-1640 and incubated for 30 min with either ursolic acid (1 μM), NDGA (1 μM), BW755C (1 μM) or caffeic acid (1 μM) at 37°C in a shaking water-bath. They were then incubated with [^{14}C]arachidonic acid (0.1 μCi, Amersham) for 10 min,

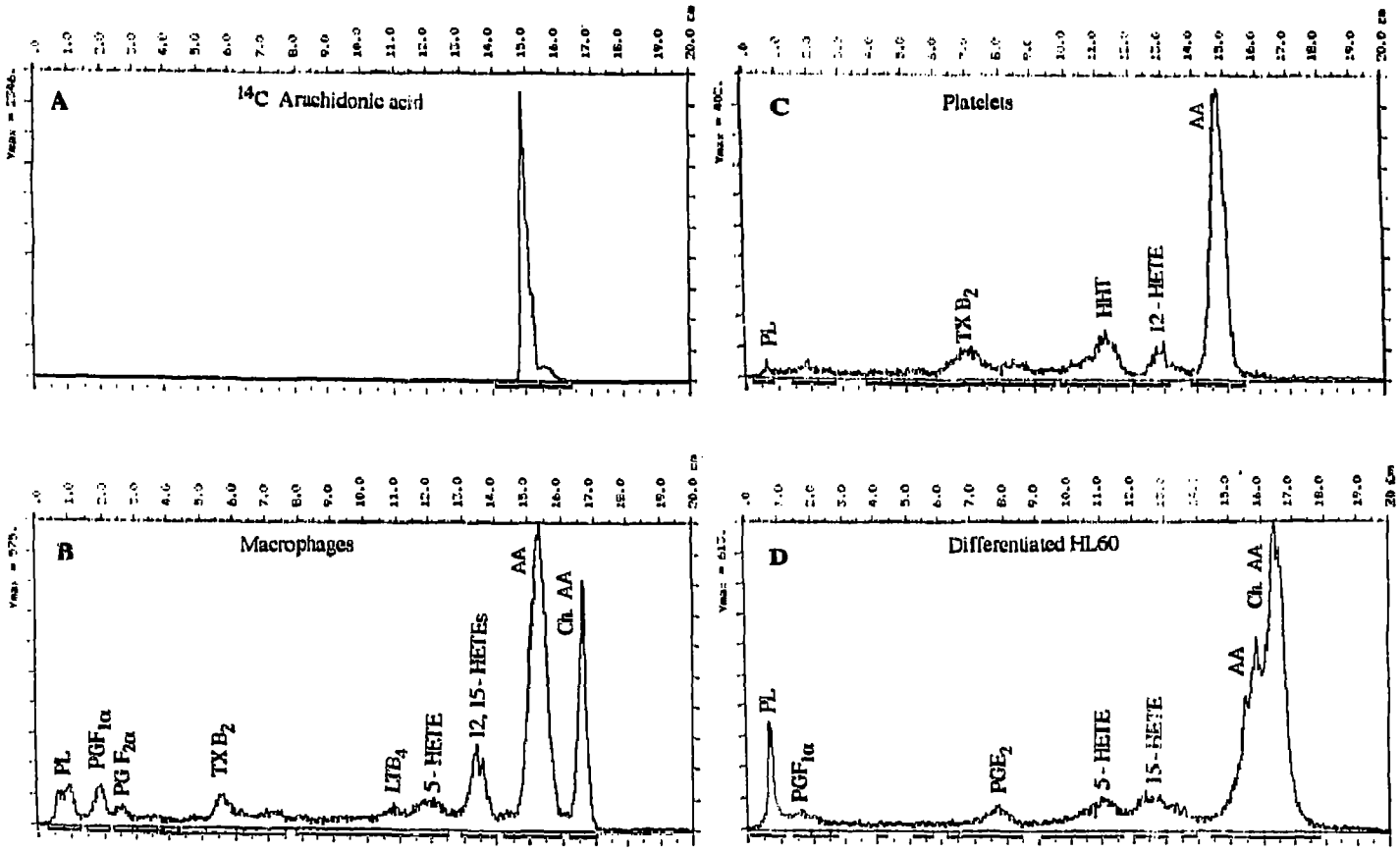

Fig. 3. Arachidonic acid metabolism by mouse peritoneal macrophages (B), human platelets (C) and leukemic HL60 cells (D). (A), arachidonic incubated without cells (control); PL, phospholipids; AA, arachidonic acid; Ch.AA, cholesterol-arachidonate; TX, thromboxane; PG, prostaglandins; HETEs, hydroxyeicosatetraenoic acids; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid.

the suspension acidified to pH 3 (citric acid, 2 M) and extracted twice with 5 ml of diethyl ether and once with 5 ml of ethylacetate. Lipid extracts were evaporated to dryness under a nitrogen stream and submitted to thin-layer chromatography (Silica gel 60 (20 × 20 cm, 0.25 mm), Merck). An aliquot (200 μl) of this extract was applied to a TLC plate under a nitrogen stream, together with a known standard solution, containing 5-HETE, 12-HETE, 15-HETE, prostaglandins (PGA_2, PGD_2, PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$), and thromboxane B_2 (TxB_2). The solvent system [10] consisted of ethyl acetate/isooctane/acetic acid/water (11:5:2:10, v/v, organic phase). After visualization of standards with iodine vapour, the radioactive metabolites were detected using a Berthold scanner.

2.5. *Arachidonic acid metabolism by human platelets*

Human platelets were prepared from healthy donors who had received no aspirin-like compounds during the preceding 4 weeks. Blood was collected in 0.15 volume of anticoagulant, citrate dextrose, and centrifuged for 20 min at 300 × g at room temperature; the platelet-rich plasma (PRP) was recovered. After addition of EDTA to a final concentration of 1 mM, the PRP was cooled to 0°C and centrifuged at 2,000 × g for 20 min. Arachidonic acid metabolism was analyzed as described above for macrophages.

2.6. *Arachidonic acid metabolism by leukemic cells*

HL60 cells were maintained in liquid culture RPMI-1640 medium supplemented with 10% fetal bovine serum, 4 μM L-glutamine, 100 μg/ml streptomycin and 100 U/ml of penicillin (Gibco). For differentiation studies, cells from log phase cultures were diluted to 10^5 cells/ml and cultured for 5 days. Cells were induced to granulocytic differentiation by the addition of DMSO (Merck) at the time of plating at a final concentration of 1.3%. The arachidonic acid metabolism was analyzed as described above for macrophages.

2.7. *MTT assay*

The MTT colorimetric assay was made as described by Mosmann [13]. This test is based upon the selective ability of living cells to reduce the yellow soluble salt, MTT (3-[4,5-dimethymthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), to a purple-blue insoluble formazan precipitate. Experiments were performed in quadriplicate in a 24-well culture plate (Falcon). MTT (Sigma) was dissolved in PBS at 5 mg/ml. After 48 h incubation of HL60 with ursolic acid, stock MTT solution (200 μl per 2 ml medium) was added to all wells of an assay, and plates were incubated at 37°C for 4 h. After addition of 2 ml of 0.04 N HCL in isopropanol, the optical density of the medium was read at 570 nm.

3. RESULTS AND DISCUSSION

In an effort to find naturally occuring anti-lipoxygenase compounds, several organic or aqueous heather flower extracts were screened for their ability to inhibit lipoxygenase activity. Among them, the acetone extract was further analyzed. Thus, we purified and characterized ursolic acid (Fig. 1), and determined its X-ray crystallographic structure: this latter showed us that ursolic acid adopts chair conformations [5]. Fig. 2 shows that cell viability, after 48 h treatment with 0.01–10 μM of ursolic acid, was greater than 90% as measured by the Trypan blue exclusion test (Fig. 2A) as well as the reducton of MTT (Fig. 2B). The ursolic acid toxicity to the HL60 cells was observed only at the highest concentration used (100 μM).

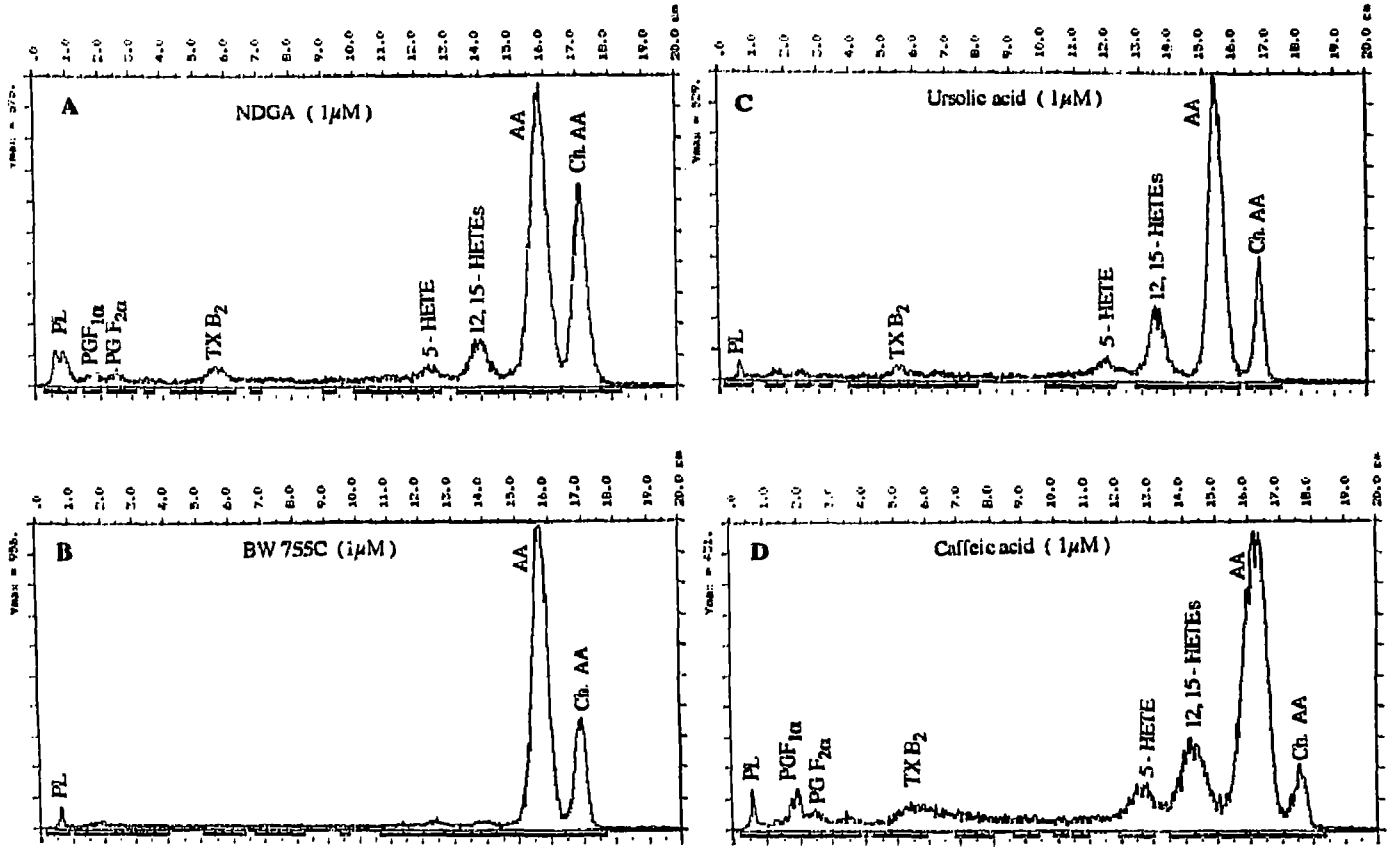

Fig. 4. Effects of NDGA (A), BW755C (B), ursolic acid (C) and caffeic acid (D) on mouse peritoneal macrophages arachidonic acid metabolism. PL, phospholipids; AA, arachidonic acid; Ch.AA, cholesterol-arachidonate; TX, thromboxane; PG, prostaglandins; HETEs, hydroxyeicosatetraenoic acids.

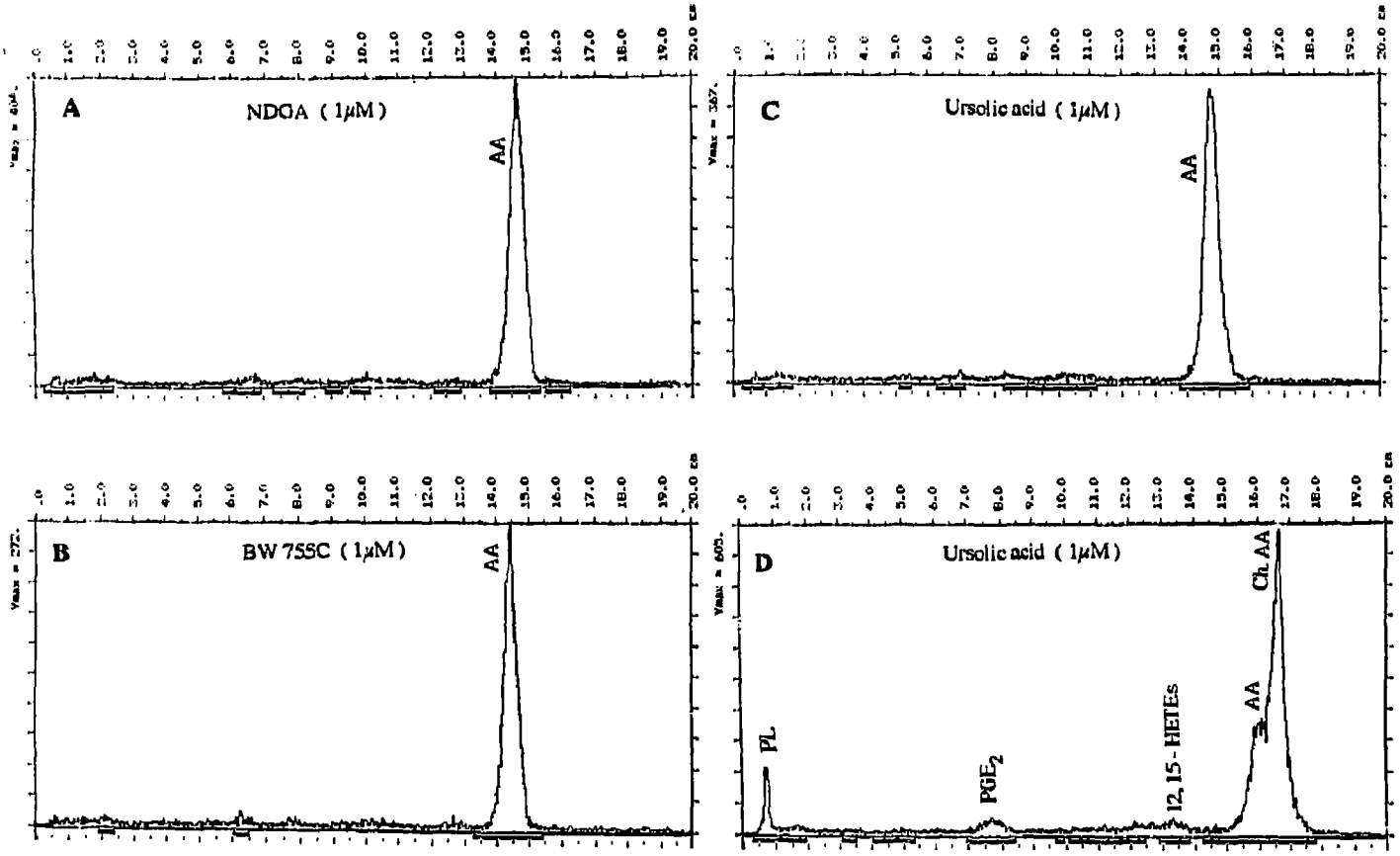

Fig. 5. Effects of NDGA (A), BW755C (B) and ursolic acid (C) on human platelet (A,B,C) and ursolic acid (D) on differentiated HL60 cells (D) arachidonic acid metabolism. PL, phospholipids; AA, arachidonic acid; Ch.AA, cholesterol-arachidonate; TX, thromboxane; PG, prostaglandin; HETE, hydroxyeicosatetraenoic acid; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid.

Using radiochromatographic procedures and blood cells, we investigated the direct effects of ursolic acid on arachidonic acid metabolism in comparison with classical lipoxygenase and cyclooxygenase inhibitors. The TLC profiles obtained for [^{14}C]arachidonic acid metabolism by blood cells are shown in Fig. 3. The 12- and 15-HETEs reference standards could not be separated with the TLC system used. Addition of 1 μM ursolic acid to the reaction mixture decreased the formation of the lipoxygenase and cyclooxygenase products in all tested cells. In macrophages, ursolic acid (1 μM) inhibited the 5-LOX metabolism of arachidonic acid to a much greater extent than that of 12- and 15-LOX (respectively, 80 and 60%, Fig. 4). Cyclooxygenase activity was inhibited by 38%. The comparison with the classical lipoxygenase inhibitors (phenolic antioxidants such as NDGA, BW755C and naturally occuring phenolic caffeic acid) showed that ursolic acid was significantly less inhibitory than NDGA and BW755C on the formation of LOX and CO (cyclooxygenase) products in macrophages, but it was more effective than caffeic acid (Fig. 4). In contrast, the effect of ursolic acid and BW755C on the formation of LOX and CO products in platelets was more pronounced than that of NDGA (Fig. 5). Ursolic acid also inhibited arachidonic acid metabolism in differentiated HL60 leukemic cells (used here as a model of granulocytes): the level of 5-HETE was diminished by 86%, 15-HETE by 59% and prostaglandins by 50% (Fig. 5).

Ursolic acid has never been related to lipoxygenase biochemistry before. Its effect appears to be dependent upon cell type (macrophage, platelet or granulocyte), but it affected the level of arachidonic acid metabolites in all tested cells.

During preparation of this manuscript, Ying et al. [11] described the inhibition of human leucocyte elastase by ursolic acid. Since elastase is a lysosomal proteinase stored in leucocytes and responsible for tissue destruction in rheumatoid arthritis [12] and since arachidonic acid metabolites are among the mediators associated with perpetuating inflammatory processes, ursolic acid could be considered as a potential naturally occuring anti-inflammatory agent. Our results, combined with those of Ying et al. [11], probably demystify the belief in certain aspects of the use of *Calluna vulgaris* in folk medicine [1, 2].

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