Supporting Information to:
Five New Triterpene Glycosides from *Lysimachia foenum-graecum* and Evaluation of their Effect on the Arachidonic Acid Metabolizing Enzyme

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Materials and Methods for Pharmacological Experiments

Introduction to the pharmacological design
The effects of PGs and LTs in the physiopathology of inflammation are well known. However, the role of arachidonic acid (AA) metabolites in pathologies other than inflammation, such as cancer, has only recently been described. Hong et al. [1] demonstrated that COX-1 and 5-LOX were universally expressed in all cancer cell lines tested while expression of COX-2, 12-LOX and 15-LOX varied among the different cells studied. Moreover, 5-LOX inhibitors can inhibit the growth of human cancer cells by induction of apoptosis through mechanisms depending on 5-LOX metabolites [2].

In the present paper, we studied the effect of five triterpene saponins on the constitutive enzymes implicated in AA metabolism in order to establish their potential pharmacological activity.

Materials and Methods

Cytotoxicity assay [3]
Cell viability was measured spectrophotometrically by means of the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) to its formazan product. After incubation of leukocyte cells with the test compounds (at a final concentration of 100 µM, 50 µM and 10 µM) for 30 min at 37 ºC, 100 µL per well of a 0.05% MTT solution were added and incubated at 37 ºC until blue deposits were visible. The deposits were dissolved in dimethyl sulfoxide (DMSO) (100 µL per well), and optical densities were determined using a Labsystems Multiskan EX plate reader set at 490 nm. A decrease in absolute absorbance indicated a reduction in cell viability.

Leukotriene B4 (LTB4) generation by rat peritoneal leukocytes [4]
Wistar rat polymorphonuclear leukocytes (PMNL) were harvested by intraperitoneal injection of 5% glycogen in Dulbecco’s phosphate buffered saline (PBS) (1 mg/g body weight in 10 mL of PBS, 37 ºC) and washed by centrifugation (10 min, 300 × g, room temperature, twice). The cell viability of the elicited rat peritoneal PMNL was assessed before each experiment using the trypan blue exclusion test. Only harvested cells with a viability greater than 95% were employed. Leukocytes (5 × 10^6 cells) were resuspended in 1 mL PBS (with glucose, 1 g/L) and preincubated for 5 min with the test compounds (final concentration of 5 – 100 µM) at 37 ºC. The reaction was started by addition of ionophore A23187 and Ca^{2+} (1.9 µM and 1.8 mM final concentrations, respectively). After 5 min at 37 ºC, the reaction was stopped with 1 mL of cold MeOH/1 N HCl (97:3), and 500 pmol of prostaglandin B_2 (PGB_2) were added as an internal standard. Solid phase extraction (SPE) was performed with Lichrolut columns C18, 100 mg, 1 mL (Merck) attached to a 12-port vacuum manifold Visiprep (Supelco). After centrifugation (10000 × g, 5 min, 0 ºC), the samples were applied to C18 SPE columns (100 mg), which were conditioned with 1 mL of MeOH and 1 mL of water. The columns were washed with 1 mL of water and 1 mL of 25% MeOH. The 5-LOX metabolites 5(S)-HETE, LTB_4, 6-trans-leukotriene B_4 and 6-trans-12-epi-leukotriene B_4 (LTB_4 all-trans-isomers) were extracted with 300 µL of MeOH and analysed by high performance liquid chromatography (HPLC-DAD). The 5-lipoxygenase (5-LOX) products were separated on reverse phase (RP)-18 columns and quantified by RP-HPLC coupled to UV-visible photodiode-array detector. Detection wavelength was set at 274 nm for prostaglandin B_2 (PGB_2) and LTB_4, or at 235 nm for hydroxytetraenoic acids (HETEs). Percentages of inhibition were calculated from the differences in the values of drug-treated groups with those of controls.

HPLC-DAD analysis was performed on a Merck-Hitachi system (Intelligent Pump L-6200, Diode Array Detector L-7455, and Auto Sampler L-7200). Precolumn Lichrospher C18 (4 × 4 mm, 5 µm, Merck), column Lichrospher C18 (250 × 4 mm, 5 µm, Merck), and Software HSM-7000 (Merck-Hitachi) were used. Isocratic elution
Determination of cyclooxygenase-1/12-lipoxygenase (COX-1/12-LOX) activity [5]

Human platelets were obtained by diluting human buffy-coats with PBS (1:3) and centrifuging twice (10 min, 300 × g), discarding the pellets and keeping the platelet-rich supernatants. After centrifuging the resulting pellet was washed twice (10 min, 1000 × g) and finally resuspended in Hank’s balanced salt solution (HBSS) with Ca²⁺ (1 mM) and Mg²⁺ (0.5 mM). The differential counting was done by a Coulter Counter (Sysmex D-800). Semi-quantitative estimation of the platelet viability was performed by fluorescence microscopy (Nikon, Japan) staining with acridine orange/ethidium bromide solution. Aliquots of 80 × 10⁶ platelets were preincubated for 5 min with the extract at 37 °C. Then the reaction was started by addition of ionophore A23187 (1.9 µM final concentration). After 1 min the reaction was stopped with 1 ml of cold MeOH/1 N HCl (97:3), and 500 pmol of PGB₂ were added as internal standard. The platelets were subjected to the same processing as described above for rat peritoneal leukocytes. The measured metabolites were 12(S)-hydroxyheptadeca-5Z,8E,10E-trienoic acid [12(S)-HHTrE] from the COX-1 pathway and 12(S)-HETE from the 12-LOX pathway.

Elastase release/activity assays [6]

Following Barret’s protocol, 1.25 × 10⁶ PMNLs, obtained from human blood buffy coats, were suspended in Eppendorf tubes containing 0.5 mL of Ca²⁺/Mg²⁺ Hanks’ balanced salt solution (HBSS) and incubated at 37 °C with the test compounds for 5 min. Elastase release was stimulated by the addition of TPA (65 µg/mL). Fifteen min later the samples were placed on ice and centrifuged (0 °C, 10 min, 4000 g). Supernatant aliquots (200 µL) were mixed with 5 µL of 0.3% BOC in the 96-well microtiter plate, then incubated at 37 °C for 30 min, and the absorbance was measured at 414 nm.
Statistics

Percentages of inhibition of eicosanoids production are shown as mean ± SEM of three or more independent experiments, and every experiment was performed in duplicate. The inhibition of 5-LOX activity is expressed as percentages with respect to the control, which includes LTB4 and 5(S)-HETE. Inhibition of COX-1 and 12-LOX activities are expressed as percentages with respect to the control of 12(S)-HHTrE and 12(S)-HETE, respectively. For HLE release/activity assays absolute values of absorbance at 414 nm were used for statistics. Background colours of the extracts were corrected. Statistical evaluation was performed by ANOVA followed by Dunnett’s \( t \)-test for multiple comparisons using Graph-Pad InStat software.

References