Supporting Information to

*In Vitro* 12(S)-HETE and Leukotriene Metabolism Inhibitory Activity of Sesquiterpenes of *Warburgia ugandensis*

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**In vitro 12(S)-HETE assay**

For the 12(S)-lipoxygenase assay peripheral venous blood from healthy volunteers was drawn into trisodium citrate-containing (9:1, v/v) blood collection tubes (BD Vacutainer Systems; PLymouth, UK). Platelet-rich plasma (PRP) was collected by centrifugation at 200 g for 10 min at 20 °C. PRP was further centrifuged at 1200 g for 15 min at 20 °C and the platelet pellet was washed twice with PBS buffer (Fluka; Buchs, Switzerland) containing 1 mM EDTA (Fluka). Thereafter, the platelets were resuspended in PBS to a final concentration of 0.9 × 10^8 platelets/mL. 970 µL of platelet suspension were preincubated at 37 °C for 7 min in the presence of 2 mM reduced glutathione (Sigma; Steinheim, Germany) with test solutions or controls. Test samples were dissolved in absolute EtOH (final EtOH concentration of 1% in the assay mixture). The suspensions were incubated with 33 µM arachidonic acid (Sigma) for another 7 min at 37 °C. The reaction was stopped by 2 M HCl and by cooling with ice. 12(S)-HETE was quantified by 12(S)-HETE-EIA using a Correlate-EIA™-12(S)-HETE-kit (96-well, Assay Designs, Ann Arbor, MI, USA). Before quantification, the samples were centrifuged at 2000 g for 15 min at 4 °C. 12(S)-HETE concentrations were calculated in relation to a 12(S)-HETE-standard (Sigma). The results are means of at least two and in most cases three single experiments. Limits of quantification of 12(S)-HETE were 146 pg/mL for EIA. Positive control measurements were performed with baicalein (Aldrich; Milwaukee, WI, USA).

**In vitro LTB₄ assay**

*Principle:* Activated neutrophile granulocytes with 5-LOX activity were incubated with a defined concentration of test sample and arachidonic acid. After stopping the enzymatic reaction by addition of formic acid and centrifugation to remove cellular fragments, the produced LTB₄ was quantified in the supernatant by means of an LTB₄ EIA Kit (Cayman Chemical; Ann Arbor, MI, USA).

*Isolation of human neutrophile granulocytes:* 30 mL of venous human blood from healthy voluntary donors were collected with a Vacutainer™ (BD; Plymouth, UK) system containing a 0.219 M pre-analytical citric acid solution. The blood was immediately transferred to a falcon tube containing 20 mL of sedimentation solution (1% NaCl, 6% dextran T-500) and left to separate for 60 min at 4 °C. While most of the dense erythrocytes sink into the dextran layer, the lighter blood fractions remain in the upper layer which was then removed and centrifuged at 1600 rpm at 4 °C for 10 min to concentrate the leukocytes, the plasma
supernatant was discarded, the pellet resuspended in 10 mL of a wash buffer (7.4% CaCl₂ dihydrate p.a.; 0.1 % anhydrous D-glucose; 0.2% MgCl·6 H₂O; 0.04% KCl; 1.75% Tris p.a.; with the pH adjusted to 7.6 with 1 N HCl. After centrifugation at 1400 rpm at 4 °C for 10 min and removal of the supernatant the resulting pellet was resuspended in 10 mL of hypotonic lysis buffer (0.17% NH₄Cl; 0.2% Tris; pH 7.2) and gently shaken for 5 min at room temperature to destroy remaining erythrocytes. The suspension was submitted to another centrifugation at 1400 rpm at 4 °C for 5 min. The pellet was resuspended in 10 mL of wash buffer and then centrifuged at 1400 rpm at 4 °C for 15 min. The resulting pellet which now mainly contains neutrophile granulocytes was resuspended in 2 mL of Tris buffer (1.75% Tris p.a., 0.9% NaCl, pH 7.4), tested for vitality, quantified and then diluted to a cell concentration of 5000 cells/µL with Tris buffer.

*Cell vitality test:* 50 µL of cell suspension and 10 µL of (0.4 %) trypan blue solution (Sigma Chemical Co.; Steinheim, Germany) were mixed on a glass object carrier and examined with a light microscope at 1000-fold magnification. Dead cells appear larger and darker due to absorption of trypan blue. The vitality of the cells must be over 95%.

*Determination of cell concentration:* 10 µL of granulocyte cell suspension were dyed using 990 mL aqueous TÜRKS solution (Merck; Darmstadt, Germany) and quantified in a “Neubauer” chamber (Assistent; Sondheim, Germany) as described by the producer.

When using blood from more than one donor, the cells were never pooled. Each test-plate contained cells from only one person. The activity of cells from different people was shown to always be quite similar, whereas the cell concentration varied significantly making an exact adjustment of the cell concentration in the assay a vital issue.

Samples were dissolved in an appropriate amount of ethanol p.a. to be added to the test mixture resulting in screening concentrations of 40 µM to 5 µM for pure test substances and 20 µg/mL for crude extracts. The commercially available specific 5-LOX inhibitor zileuton (Sequoia Research Products Ltd.; Pangbourne, UK) was used as a positive control. The incubation mixture in a 96-well flat bottom microtitre plate (Bibby Sterilin; Staffordshire, UK) consisted of 265 µL leukocyte suspension, with 75.5 µM CaCl₂, 0.152 µM eicosatetraenoic acid (ETYA) as an inhibitor of the 12-LOX pathway, 0.642 µM Ca ionosphere A 23 187 and 4.43 µM arachidonic acid, the substrate of the 5-LOX pathway,
along with 5 μL of each plant extract or inhibitor dissolved in EtOH p.a. or 5 μL of EtOH p.a. in control wells. After incubation for 10 min at 37 °C in darkness, the reaction was stopped by addition of 20 μL 10% formic acid. The microtitre plate was centrifuged for 15 minutes at 1400 rpm to separate free LTB₄ from cellular particles before the supernatant was diluted 50-fold and applied to a Leukotriene B₄ EIA Kit (Cayman Chemical; Ann Arbor, MI, USA). The competitive enzyme immunoassay was used according to the producer’s instructions. The plate was incubated for 18 hours at 4 °C in the dark. After the EIA kit had been emptied and rinsed five times with Cayman EIA wash buffer it was developed with 200 μL Ellmans reagent in each well on an orbital shaker (MS 1 Minishaker, IKA Works; Wilmington, NC, USA) for 150 min at room temperature in the dark. Hereby Ellmans reagent which contained the substrate for AChE gained a distinct yellow colour with an intensity directly proportional to the amount LTB₄ tracer bound to the well and thus inversely proportional to the amount of free LTB₄ present in the well after incubation, thus enabling quantification of LTB₄ biosynthesis inhibition by measuring the absorption at 412 nm using a photometric ELISA plate reader (Tecan RAIN BOW, Tecan, Grödig, Austria) and processing with easyWIN-Fitting 4.0a (Tecan). The inhibition is expressed in percent in relation to a control experiment using only the solvent ethanol. The final ethanol concentration in the control wells of 1.89 % had no cytotoxic effect on the cells as could be shown by determination of the cell vitality by dying with trypan blue solution after the incubation to mark dead cells. At this concentration ethanol shows no inhibition of leukotriene biosynthesis. This was proven by using 5 μL of test buffer instead of EtOH and comparing the LTB₄ concentrations.

Each active substance was tested at least three times at the concentrations 40 μM, 20 μM, 10 μM and 5 μM and was quantified in duplicate.