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Isolation of immunomodulatory triterpene acids from a standardized rose hip powder (*Rosa canina* L.)

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7 **Isolation of immunomodulatory triterpene acids from a standardized**
8 **rose hip powder (*Rosa canina* L.)**
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12 Short title

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15 **Immunomodulatory triterpene acids from rose hip powder**
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19
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ABSTRACT

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8 A previous published systematic review and a metaanalysis have concluded that
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10 consumption of standardized rose hip powder (*Rosa canina* L.) can reduce pain in
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12 osteoarthritis patients. Synovial inflammation has been suggested to play an important
13
14 role in the pathogenesis of osteoarthritis and to mainly involve infiltration of the
15
16 synovial membrane by macrophages. Therefore, the immunomodulatory effect of
17
18 standardized rose hip powder of *Rosa canina* L. was investigated and active principles
19
20 isolated using the Mono Mac 6 cell line as a model for human macrophages. Treatment
21
22 of Mono Mac 6 cells with the residue of a crude dichloromethane extract of rose hip
23
24 powder significantly and concentration dependently inhibited the lipopolysaccharide
25
26 induced interleukin-6 release. Through bioassay-guided fractionation the
27
28 immunomodulatory effect of the dichloromethane extract was correlated to a mixture of
29
30 three triterpene acids; oleanolic acid, betulinic acid and ursolic acid ($IC_{50} 21 \pm 6 \mu M$).
31
32 Further studies revealed that only oleanolic acid and ursolic acid, but not betulinic acid,
33
34 could inhibit the lipopolysaccharide induced interleukin-6 release from Mono Mac 6
35
36 cells when tested separately. Combination of either oleanolic acid or ursolic acid with
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38 betulinic acid enhanced the immunomodulatory effect of the two triterpene acids.
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INTRODUCTION

The dry powder of *Rosa canina* L. pseudofruits is a widely used herbal remedy against arthritis. A previously published systematic review (Chrubasik *et al.*, 2006) and a metaanalysis (Christensen *et al.*, 2008), both investigating the clinical evidence for the use of rose hip powder, concluded that consumption of powdered rose hips from *Rosa canina* L. results in a moderate reduction of pain in patients suffering from osteoarthritis. Previous attempts to isolate the active principles in rose hip powder have led to the isolation of an anti-chemotaxic galactolipid (Larsen *et al.*, 2003) as well as the characterization of cyclooxygenase (COX)-1 and COX-2 inhibitory fatty acids; linoleic acid and α -linolenic acid (Jäger *et al.*, 2008) and to the isolation of three triterpene acids (oleanolic acid, ursolic acid and betulinic acid) with COX and lipooxygenase (LOX) inhibitory activity (Wenzig *et al.*, 2008). However, the effect of these compounds cannot fully explain the clinical effect of the rose hip powder.

Osteoarthritis is characterized by a degeneration of cartilage and bone leading to loss of function of the affected joint. Synovial inflammation appears to play an important role in the pathogenesis of osteoarthritis and to involve an inflammatory infiltrate consisting mainly of macrophages (Bondeson *et al.*, 2006; Farahat *et al.*, 1993). These infiltrating macrophages secrete a variety of proinflammatory cytokines such as TNF- α , interleukin (IL)-1 β , and large amounts of IL-6 and IL-8 (Bondeson *et al.*, 2006), which are known to promote the catabolic processes in cartilage metabolism and are therefore believed to be involved in the initiation and progression of osteoarthritis (Goldring, 2000).

The aim of the present study was to characterize principles in the rose hip powder which can modulate the IL-6 release from lipopolysaccharide (LPS) activated Mono Mac 6

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4 cells. The Mono Mac 6 cell line resembles mature human monocytes (macrophages)
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6 (Zieglerheitbrock *et al.*, 1988) and produces IL-6 in a dose-dependent manner following
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8 activation with LPS (Moesby *et al.*, 1999).
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11 12 13 14 15 MATERIALS AND METHODS

16 17 **Compounds**

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19 Betulinic acid and ursolic acid was purchased from Sigma-Aldrich Co., USA.
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21 Dexamethasone (Dexamethasone – water soluble, Cell culture) was purchased from
22
23 Sigma Chemical Co., USA. Oleanolic acid was isolated from *Carophyllus aromaticus*
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25 L. in our laboratory. Purity and configuration of the isolated oleanolic acid was
26
27 confirmed by infrared spectroscopy and GC-MS analysis (data not shown).
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33 34 **Plant material**

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36 Rose hip (*Rosa canina* L., Rosaceae) powder in the form of a standardized product
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38 LitoMove® was obtained from Hyben Vital International ApS, Denmark.
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43 44 **Extraction and isolation procedures**

45 46 *Extraction*

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48 Rose hip powder (50 g) was serially extracted with petroleum ether (40 – 60 °C, VWR
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50 International, USA), dichloromethane (ChromaSolv®, Sigma-Aldrich, USA), ethyl
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52 acetate (ChromaSolv®, Sigma-Aldrich, USA) and water (1 L) by sonication for 60 min.
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54 The extracts were filtered through Whatman No. 1 filter paper and evaporated to
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56 dryness under reduced pressure. Obtained residues of the crude extracts were tested in
57
58 Mono Mac 6 cells for immunomodulating activity.
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Isolation

Rose hip powder (4000 g) was serially extracted with petroleum ether and dichloromethane (80 L) by sonication for 60 min. The dichloromethane extract was filtered through Whatman No. 1 filter paper and evaporated to dryness under reduced pressure.

The residue of the dichloromethane extract (13.4 g) was subjected to vacuum liquid chromatography (VLC) on a 230 g silica gel 60 (0.041 – 0.063 mm, Merck KGaA, Germany) column (70 x 230 mm). The column was eluted with 500 ml dichloromethane (fractions 1-5), followed by fractions (100 ml) of each of the following ratios of dichloromethane: methanol (ChromaSolv®, Sigma-Aldrich, USA); 99:1 (fractions 6-10), 98:2 (fractions 11-15), 97:3 (fractions 16-20), 96:4 (fractions 21-30), 90:10 (fractions 31-40), 80:20 (fractions 41-49), 40:60 (fractions 50-55) and methanol (fractions 56-57). Obtained fractions were evaluated by thin layer chromatography (TLC) (Merck silica gel 60 F₂₅₄ 0.25 mm TLC plates). TLC plates were eluted with dichloromethane: methanol 90:10 and the spots visualized by spraying with anisaldehyde-sulphuric acid R followed by heating (100 °C, 5 minutes). Fractions showing similar patterns on TLC were combined to give five fractions; DCM-A (fractions 1-18), DCM-B (fractions 19-32), DCM-C (fractions 33-35), DCM-D (fractions 36-45) and DCM-E (fractions 46-57). The combined fractions were evaporated to dryness under reduced pressure and the obtained residues were tested in Mono Mac 6 cells.

Fraction DCM-B (4.3 g) was subjected to VLC on a 120 g silica gel 60A (20 – 45 µm) column (40 x 150 mm). The column was eluted with 300 ml of toluene (fraction 1),

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4 followed by eluents (100 ml) of each of the following ratios of toluene (ChromaSolv®,
5 Sigma-Aldrich, USA): ethyl acetate; 90:10 (fractions 2-5), 85:15 (fractions 6-10), 80:20
6
7 (fractions 11-13), 70:30 (fractions 14-18), 60:40 (fractions 19-21), 40:60 (fractions 22-
8
9 26) and ethyl acetate (fractions 27-28). The fractions were evaluated by TLC. The TLC
10
11 plates were eluted with toluene: methanol 50:50 and the spots visualized as above.
12
13 Fractions showing similar patterns on TLC were combined to give five fractions; DCM-
14
15 B1 (fractions 1-5), DCM-B2 (fraction 6), DCM-B3 (fractions 7-14, 2.3 g), DCM-B4
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17 (fractions 15-23) and DCM-B5 (fractions 24-28). The combined fractions were
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19 evaporated to dryness under reduced pressure and the residues were tested in Mono Mac
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6 cells.

¹H-NMR of fraction DCM-B3

A ¹H-NMR spectrum of fraction DCM-B3 dissolved in a 1:1 mixture of CDCl₃ (99.8 atom % D, Sigma-Aldrich, USA) and CD₃OD (99.8 atom % D, Sigma-Aldrich, USA) was recorded at 300 MHz on Varian Mercury instrument.

GC-MS analysis of fraction DCM-B3

The free carboxylic acids in fraction DCM-B3 were methylated with trimethylsilyldiazomethane for GC-MS analysis. The 5 mg residue of DCM-B3 was reacted with 100 µl trimethylsilyldiazomethane (2.0 M, Sigma-Aldrich, USA) in 5 ml methanol at room temperature. After 15 minutes reaction time, 100 µl glacial acetic acid (> 99.8 %, Merck KGaA, Germany), 6 ml saturated sodium chloride (Merck KGaA, Germany) solution (0.5 g/ml) and 5 ml heptane were added. The heptane phase was

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4 analyzed by GC-MS on an Agilent 6890N Network GC System coupled to a 5973
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6 Network Mass Selective Detector. GC conditions: start 200 °C, 5 °C/min to 310 °C and
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8 310 °C for 30 minutes; column HP5MS.
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10 11 12 13 14 **LC-MS analysis of fraction DCM-B3**

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16 For LC-MS analysis, an analytical sample of fraction DCM-B3 was dissolved in
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18 methanol to give a concentration of 0.1 mg/ml. The solution was analysed by LC-MS
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20 on an Agilent 1200 HPLC system (G1316B C18 column (75mm×2.1mm)) fitted with an
21
22 Agilent G6410A triple quadrupole mass spectrometer and a Sedex 85 evaporative light
23
24 scattering detector. The analysis was performed by employing a gradient system at a
25
26 flow rate of 1 ml/min. The gradient ranged from 100 % eluent A
27
28 [water:acetonitrile:formic acid (98 %, Sigma-Aldrich, USA) (95:5:0.1)] to 50 % eluent
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30 B [water:acetonitrile:formic acid (5:95:0.043)] over 7 minutes.
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40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 **Preparation of samples**

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56 Samples were dissolved in pyrogen free dimethyl sulfoxide (DMSO) (Hybri-Max,
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58 Sigma-Aldrich, USA) to a concentration of 10 mg/ml and was further diluted to a
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60 sample concentration of 0.4 mg/ml and a DMSO concentration of 4 % with pyrogen
free water. Further dilution of samples was done with a 4 % solution of DMSO in
pyrogen free water.

54 55 56 57 58 59 60 **Maintenance of cell line**

The human monocytic cell line Mono Mac 6 was originally provided by Dr. Ziegler-
Heitbrock, University of Munich, Germany (Ziegler-Heitbrock et al 1988).

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4 Mono Mac 6 cells were maintained in pyrogen free RPMI 1640 (Gibco BRL, USA)
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6 supplemented with 5 % European grade fetal bovine serum (Biological Industries,
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8 Israel), 200 U/ml penicillin, 200 µg/ml streptomycin, 1 % glutamax, 9 µg/ml insulin and
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10 non-essential amino acids (Gibco BRL, USA). The cells were incubated in a humidified
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12 atmosphere containing 5 % CO₂ and 95 % air at 37 °C. Growth media was replaced
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14 twice weekly and the cells were seeded at 2x10⁵ cells/ml.
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18 The content of endotoxin in all used media and solutions was below the detection limit
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20 in our limulus amoebocyte lysate (LAL) assay (< 0.004 IU/ml) (Pedersen *et al.*, 1994).
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23 All laboratory utensils were rendered pyrogen free by heating at 180°C for 6 hours.
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26 27 28 29 **Stimulation of Mono Mac 6 cells**

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31 For stimulation, Mono Mac 6 cells were seeded at 4 x 10⁵ cells/ml and incubated for 20
32
33 – 24 hours in supplemented RPMI 1640. The cell culture was centrifuged (125 x g, 5
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35 min), resuspended in supplemented RPMI 1640 and standardized to 2x10⁶ cells/ml. A
36
37 volume of 250 µl of standardized cell suspension, 125 µl test solution (test wells) or 4
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39 % DMSO solution (control wells) and 125 µl LPS solution (200 pg/ml) (*E. coli* 055:B5,
40
41 2.6 pg/IU, Lonza, USA) were gently mixed resulting in a fourfold dilution of added test
42
43 solution. Final well concentrations of DMSO and LPS were 1 % and 50 pg/ml,
44
45 respectively. Well concentrations of tested samples appear from figure legends.
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Assay for IL-6

The amount of released IL-6 from the stimulation of Mono Mac 6 cells was determined using a slightly modified time resolved dissociation-enhanced lanthanide fluoro immunoassay (DELFLIA) sandwich immunoassay as previously described (Moesby *et al.*, 1997). In short: White FluoroNunc™ MaxiSorp™ surface microtiter plates (Nunc, Denmark) were coated with monoclonal anti-human IL-6 antibody (2 µg/ml, 100 µl/well) (MAB206, R&D Systems, UK) in phosphate buffered saline pH 8.0. After 30 min in a DELFLIA® plateshake (PerkinElmer) (950 rpm) the plates were incubated overnight at room temperature. After three washes (DELFLIA® Columbus M8/R2 platewash, Wallac) with wash buffer (0.05 % Tween 20 in phosphate buffered saline pH 7.4), non-specific binding was blocked with 300 µl of blocking buffer (1 % bovine serum albumin (A9418; Sigma), 5 % sucrose in phosphate-buffered solution pH 8.0). The plates were shaken (950 rpm) for at least 1 hour at room temperature. The wells were washed three times with wash buffer. Standards of recombinant human IL-6 (206-IL, R&D Systems, UK) and test solutions were diluted in supplemented RPMI 1640 and water (1:1). Standard and test solutions were added (100 µl/well) and shaken (950 rpm) for 2 hours at room temperature. After three washes biotinylated polyclonal goat anti-human IL-6 antibody (50 ng/ml; 100 µl/well) (BAF206, R&D Systems, UK) in Tris-buffered sodium chloride pH 7.4 with 0.1 % bovine serum albumin was added to each well. The plates were shaken (950 rpm) for 2 hours at room temperature and washed three times with wash buffer, and added DELFLIA® Eu-labelled streptavidin (100 ng/ml; 100 µl/well, PerkinElmer inc., USA) diluted in assay buffer (0.1 % bovine serum albumin, 7.866 µg/ml diethylenetriamine-pentaacetic acid, 0.2 % azide in phosphate buffered saline pH 7.75). After shaking (950 rpm) for 30 min at room temperature the

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4 plates were washed six times with wash buffer. The europium was rendered fluorescent
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6 with DELFIA® enhancement solution (100 µl/well) and the plates were shaken (1150
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8 rpm) for 10 min. The fluorescence was detected in a SpectraMax M5 (Molecular
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10 Devices) using the software SoftMaxPro 5.2. Linearity, between fluorescence and IL-6
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12 concentration, was observed within the range 5–4000 pg/ml. Each standard and test was
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14 determined in triplicate.
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21 **Evaluation of cell viability**

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23 The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay
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25 (Mosmann, 1983) was performed to evaluate the viability of the Mono Mac 6 cells in
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27 the presence of the triterpene acid mixture [oleanolic acid: betulinic acid: ursolic acid
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29 (30:49:21)]. MTT (98 %, Sigma-Aldrich, USA) was dissolved in phosphate buffered
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31 saline (pH 7.4) at a concentration of 5 mg/ml and passed through a 0.2 µm filter
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33 (Minisart, Sartorius, Germany) to remove bacteria and insoluble residues. A volume of
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35 50 µl of cell suspension (2×10^6 cells/ml) was transferred to transparent flat bottomed 96
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37 well cell culture plates (Nunc, Denmark), in triplicate, followed by the addition of 20 µl
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39 MTT solution (5 mg/ml), 25 µl of the prepared samples of oleanolic acid: betulinic acid:
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41 ursolic acid 30:49:21 mixture (100, 75, 50, 10, 5, 2.5 and 0.25 µg/ml) or 4 % DMSO
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43 solution (negative control) and 5 µl LPS solution (1000 pg/ml). The cells were
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45 incubated in a humidified atmosphere containing 5 % CO₂ and 95 % air at 37 °C for 24
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47 hours. A volume of 100 µl lysis buffer [10 % sodium dodecyl sulfate (> 99 %, Merck
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49 KGaA, Germany) and 50 % N,N-dimethylformamide (99.5 %, Riedel-de Haën AG,
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51 Germany) in water] was added to all wells and the optical density of the wells was
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4 measured at 570 nm using a SpectraMax M5 microplate reader (Molecular Devices,
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6 USA).
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10 11 **Calculations and statistical analysis**

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13 The immunomodulatory effect of tested samples and compounds was determined as the
14 effect on IL-6 release from LPS stimulated Mono Mac 6 cells. The immunomodulatory
15 effect of tested samples and compounds was calculated as the percentage of IL-6 release
16 from treated cells (sample or compound in 1 % DMSO + LPS) compared to the IL-6
17 release from control cells (1 % DMSO + LPS). All data are presented as the mean \pm
18 standard error of the mean (SEM) where $n \geq 3$. Statistical analysis was performed by
19 student's *t*-test at a 95 % confidence interval.
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33 **RESULTS**

34 **Effect of raw extracts of rose hip powder (*Rosa canina* L.) on the IL-6 release from** 35 36 37 38 **Mono Mac 6 cells**

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40 Crude extract residues of powdered rose hips from *R. canina* L. (heptane,
41 dichloromethane, ethyl acetate and water) were tested for modulatory effect on the IL-6
42 release from LPS activated Mono Mac 6 cells. Only the residue of the dichloromethane
43 extract exhibited significant inhibitory activity at both 10 $\mu\text{g/ml}$ ($p < 0.03$) and 100
44 $\mu\text{g/ml}$ ($p < 0.0001$) in a concentration dependent manner (Table 1A). The
45 dichloromethane extract was therefore selected for bioassay-guided fractionation.
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47 Treating unstimulated cells with the crude extracts did not result in an increase in IL-6
48 release compared to cells only treated with water, which indicates that the crude extracts
49 were not contaminated with endotoxins (data not shown).
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Bioassay-guided fractionation of the dichloromethane extract of rose hip powder

The residue of the dichloromethane extract was fractionated by VLC to give five fractions (DCM-A to DCM-E) of which only fraction DCM-B inhibited the IL-6 release from LPS activated Mono Mac 6 cells at both tested concentrations (10 and 100 µg/ml) (Table 1B). Fraction DCM-B was further fractionated by VLC yielding five fractions (DCM-B1 – DCM-B5). The major activity was located to fraction DCM-B3 which exhibited inhibition of the IL-6 release from LPS activated Mono Mac 6 cells at both tested concentrations (10 µg/ml and 100 µg/ml) (Table 1C). Treating unstimulated cells with the obtained VLC fractions did not result in an increase in IL-6 release compared to cells only treated with water (data not shown).

Identification of triterpene acids in fraction DCM-B3

The ¹H-NMR spectrum of fraction DCM-B3 indicated the presence of triterpene acids (data not shown). In particular two broad singlets were characteristic for the terminal methylene group in betulinic acid (Yogeeswari and Sriram, 2005). The identity of the three compounds in fraction DCM-B3 was confirmed by GC-MS after conversion to the methyl esters. The EIMS spectra of the three compounds were superimposable with the EIMS spectra of methylated standards of oleanolic acid, betulinic acid and ursolic acid. The ratio between the three triterpene acids in fraction DCM-B3 was estimated as oleanolic acid: betulinic acid: ursolic acid (30:49:21) by GC-MS.

Effect of the triterpene acid mixture on the interleukin-6 release from Mono Mac 6 cells and cell viability

Incubation of the Mono Mac 6 cells with different concentrations (0.25 µg/ml – 100 µg/ml) of the triterpene acid mixture [oleanolic acid: betulinic acid: ursolic acid (30:49:21)] resulted in a dose-dependent inhibition of the LPS induced IL-6 release (Figure 1). The IC₅₀ value of the triterpene acid mixture was estimated to 10 ± 3 µg/ml which corresponds to 21 ± 6 µM since all three triterpene acids possess the same molecular weight. Dexamethasone, a known anti-inflammatory compound, was used as a positive control in LPS activated Mono Mac 6 cells giving an estimated IC₅₀ value 3 ± 1 nM.

Treating unstimulated cells with the triterpene acid mixture did not result in an increase in IL-6 release compared to cells only treated with water (data not shown).

Evaluating the influence of the triterpene acid mixture on the viability of the Mono Mac 6 cells revealed that the tested concentrations (0.25 µg/ml – 100 µg/ml) of the triterpene acid mixture did not affect the reduction of MTT in Mono Mac 6 cells compared to controls (Figure 1).

Individual effects of oleanolic, betulinic and ursolic acid on the IL-6 release from LPS activated Mono Mac 6 cells

The immunomodulatory effect of the three identified triterpene acids on the IL-6 release from LPS activated Mono Mac 6 cells was tested individually (Figure 2). Only oleanolic acid and ursolic acid exhibited a concentration dependent inhibition of the IL-6 release from LPS activated Mono Mac 6 cells.

Effects of combinations of oleanolic, betulinic and ursolic acid on the IL-6 release from LPS activated Mono Mac 6 cells

The three triterpene acids were tested in 1:1 combinations (oleanolic acid – betulinic acid, oleanolic acid – ursolic acid and ursolic acid – betulinic acid) for modulatory effect on the IL-6 release from LPS activated Mono Mac 6 cells (Figure 3). At a concentration of 10 µg/ml combinations of both oleanolic acid and ursolic acid with betulinic acid were able to inhibit the IL-6 release from LPS activated Mono Mac 6 cells. The IL-6 release from LPS activated Mono Mac 6 cells treated with oleanolic acid: betulinic acid (1:1) at 10 µg/ml was significantly lower than the IL-6 release from LPS activated Mono Mac 6 cells treated with oleanolic acid alone at 10 µg/ml ($p < 0.02$) (Figure 3). In the same manner, the IL-6 release from LPS activated Mono Mac 6 cells treated with ursolic acid: betulinic acid (1:1) at 10 µg/ml was significantly lower than the IL-6 release from LPS activated Mono Mac 6 cells treated with ursolic acid at 10 µg/ml ($p < 0.004$) (Figure 3). In contrast, no inhibition of the IL-6 release from LPS activated Mono Mac 6 cells treated with the 1:1 combination of oleanolic acid with ursolic acid (10 µg/ml) was observed. All three combinations were able to inhibit the LPS induced IL-6 release from Mono Mac 6 cells when tested at 100 µg/ml (data not shown).

DISCUSSION

Mono Mac 6 is a human monocytic cell line possessing characteristics of mature human monocytes including CD14 expression, phagocytosis and cytokine production upon stimulation with e.g. LPS (Neustock *et al.*, 1993; Zieglerheitbrock *et al.*, 1988). Mono Mac 6 cells produce IL-1 α , IL-1 β , IL-6 and TNF- α upon activation with LPS (Neustock

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5 *et al.*, 1993). It has further been demonstrated that the LPS induced production of IL-6
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7 in Mono Mac 6 cells is concentration dependent (Moesby *et al.*, 1999).

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9 In the present study, the residue of a crude dichloromethane extract of powdered rose
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11 hips from *Rosa canina* L. has been found significantly and concentration dependently to
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13 inhibit the IL-6 release from LPS activated Mono Mac 6 cells. Through bioassay-guided
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15 fractionation, this effect has been correlated to a mixture of oleanolic acid, betulinic
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17 acid and ursolic acid (30:49:21). The estimated IC₅₀ value of the triterpene acid mixture
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19 is 21 ± 6 µM. In the same assay, the estimated IC₅₀ value of dexamethasone, is 3 ± 1
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21 nM. The reduction of MTT in Mono Mac 6 cells is not affected in the tested
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23 concentration range of the triterpene acid mixture, compared to controls (Figure 1).
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28 When tested individually, only oleanolic acid and ursolic acid exhibits a concentration
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30 dependent inhibition of the IL-6 release from LPS activated Mono Mac 6 cells (Figure
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32 2). However, this inhibitory effect is less potent than what was observed for the mixture
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34 of oleanolic acid, betulinic acid and ursolic acid (30:49:21). At a concentration of 10
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36 µg/ml the mixture of the three triterpene acids exhibited a 45 % reduction of the IL-6
37
38 release from LPS activated Mono Mac 6 cells, while no reduction of the IL-6 release
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40 was observed for neither oleanolic acid nor ursolic acid at this concentration (compare
41
42 Figure 1 and 2). In contrast, betulinic acid is unable to inhibit the IL-6 release from LPS
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44 activated Mono Mac 6 cells in concentrations up to 100 µg/ml (Figure 2). The greater
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46 potency of the mixture of oleanolic acid, betulinic acid and ursolic acid (30:49:21)
47
48 prompted an investigation of the three possible 1:1 combinations of the three triterpene
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50 acids: oleanolic acid – betulinic acid, ursolic acid – betulinic acid and oleanolic acid –
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52 ursolic acid (Figure 3). Mixtures of oleanolic acid – betulinic acid (1:1) and ursolic acid
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54 – betulinic acid (1:1) exhibits a concentration dependent inhibition of the IL-6 release
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4 from LPS activated Mono Mac 6 cells, while only the highest tested concentration (100
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6
7 $\mu\text{g/ml}$) of oleanolic acid-ursolic acid (1:1) inhibited the IL-6 release from LPS activated
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9 Mono Mac 6 cells (data not shown). The IL-6 release from LPS activated Mono Mac 6
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11 cells treated with 10 $\mu\text{g/ml}$ of the combination of oleanolic acid and betulinic acid (1:1)
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13 is significantly lower than the IL-6 release from LPS activated Mono Mac 6 cells treated
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15 with 10 $\mu\text{g/ml}$ oleanolic acid alone (Figure 3). Analogously, the IL-6 release from LPS
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17 activated Mono Mac 6 cells treated with 10 $\mu\text{g/ml}$ of the combination of ursolic acid
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19 and betulinic acid (1:1) is significantly lower than the IL-6 release from LPS activated
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21 Mono Mac 6 cells treated with 10 $\mu\text{g/ml}$ ursolic acid (Figure 3). Thus, a combination of
22
23 betulinic acid with either oleanolic acid or ursolic acid enhances the inhibitory effect of
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25 oleanolic acid and ursolic acid on the IL-6 release from LPS activated Mono Mac 6
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27 cells.
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33 All three triterpene acids possess a pentacyclic carbon skeleton (Figure 4). The
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35 difference between oleanolic acid and ursolic acid relates to methyl group 29 which is
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37 located at C-20 in oleanolic acid, and at C-19 in ursolic acid. Ursolic acid and oleanolic
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39 acid both possess a skeleton containing five cyclohexane rings, of which ring A, B and
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41 C are trans annulated and ring D and E are cis annulated. Both compounds also possess
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43 an endocyclic double bond between C-12 and C-13. In contrast, the skeleton of betulinic
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45 acid consist of four cyclohexane rings and one cyclopentane ring with all rings being
46
47 trans annulated and a double bond between C-20 and C-29. It is likely that the different
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49 abilities of the three triterpene acids to affect the IL-6 release from Mono Mac 6 cells
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51 stems from structural differences at ring E and the position of the double bond.
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56 Expression of genes for IL-6 and other proinflammatory cytokines (e.g. IL-1, IL-8,
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58 TNF- α .) are known to be regulated by transcription factor nuclear factor- κB (NF- κB)
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4 (May and Ghosh, 1998). Briefly, NF- κ B is found in the cytosol of unstimulated cells in
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6 an inactive state complexed to an inhibitor molecule (I κ B). Stimulation of the cell
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8 results in phosphorylation of I κ B through the action of I κ B α kinase. Phosphorylation of
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10 I κ B promotes its degradation which releases the active NF- κ B, which in turn
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12 translocates to the nucleus and induces gene expression (May and Ghosh, 1998). Levels
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14 of mRNA for IL-6 and the above mentioned proinflammatory cytokines have been
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16 reported to be significantly increased two (Song *et al.*, 2009) and four hours (Schwarz *et*
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18 *al.*, 2000) after LPS stimulation of human peripheral blood mononuclear cells.

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21 All three triterpene acids indentified in this study have been shown to inhibit NF- κ B
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23 activation of various cell lines. In this way oleanolic acid has been reported to inhibit
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25 the activation of NF- κ B in the murine macrophage cell line RAW 264.7 (Wu *et al.*,
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27 2004). Ursolic acid was shown to inhibit the activation of NF- κ B in a leukemic cell line
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29 by preventing the phosphorylation of I κ B (Shishodia *et al.*, 2003). In another study,
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31 ursolic acid was found to inhibit the NF- κ B pathway in a rat C6 glioma cell line (Huang
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33 *et al.*, 2009). It has been proposed that ursolic acid activates the glucocorticoid receptor
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35 in the cytosol (Cha *et al.*, 1998) and thereby suppress the activation of NF- κ B (Barnes
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37 and Adcock, 2009). Dexamethasone, a synthetic glucocorticoid is also known to
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39 activate the glucocorticoid receptor (Cha *et al.*, 1998) and it may thus be speculated that
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41 ursolic acid possess a mode of action similar to glucocorticoids. Betulinic acid has been
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43 identified as an inhibitor of NF- κ B activation in epithelial cells (HCT 116), Caco-2 cells
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45 (colon carcinoma) and in H1299 cells (lung adenocarcinoma) and this effect has been
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47 correlated with the ability of betulinic acid to inhibit the function of I κ B α kinase
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49 (Takada and Aggarwal, 2003). However, this does not fully support the findings in the
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4 present study, where it was observed that betulinic acid was not able to inhibit the IL-6
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7 release from LPS activated Mono Mac 6 cells.

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9 Based on the above findings, we suggest that the immunomodulatory activity of the
10
11 triterpene acid mixture observed in the present study may be explained by preventing
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13 phosphorylation and degradation of I κ B and interaction with the glucocorticoid
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15 receptors, and thus indirectly inhibiting the NF- κ B mediated IL-6 gene expression in
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18 LPS activated Mono Mac 6 cells.

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20 In conclusion, a mixture of oleanolic acid, betulinic acid and ursolic acid which possess
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22 inhibitory activity on the IL-6 release from LPS activate Mono Mac 6 cells was isolated.
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24 Comparison of the inhibitory activity of the three triterpene acids and the inhibitory
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26 activity of the three possible pairwise combinations indicate that combination of either
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28 oleanolic acid or ursolic acid with betulinic acid can enhance the immunomodulatory
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30 activity of the two triterpene acids. The presence of the three triterpene acids is likely to
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32 contribute to the observed clinical effect of the standardized rose hip powder (Lito
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34 Move®) in patients suffering from osteoarthritis. However, the pathology of
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36 osteoarthritis is complex and therefore more research is needed in order to fully
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38 understand the underlying mode of action of this phytomedicine.
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Table 1. Effect of extracts and VLC fractions of *Rosa canina* (L.) on the LPS induced IL-6 release from Mono Mac 6 cells

	Released IL-6 relative to control (%)	
	10 µg/ml	100 µg/ml
A: Extracts of <i>Rosa canina</i>^a		
Heptane	62 ± 12	74 ± 25
Dichloromethane (DCM)	63 ± 17	12 ± 3
Ethyl acetate	123 ± 29	17 ± 7
Water	114 ± 16	172 ± 32
B: VLC fractions of dichloromethane extract^b		
DCM-A	205	308
DCM-B	30	9
DCM-C	133	3
DCM-D	89	5
DCM-E	124	88
C: VLC fractions of DCM-B^b		
DCM-B1	97	18
DCM-B2	104	112
DCM-B3	36	4
DCM-B4	91	3
DCM-B5	174	163

Effect of the crude rose hip extracts and vacuum liquid chromatography (VLC) fractions at 10 µg/ml and 100 µg/ml on the IL-6 release from LPS (50 pg/ml) stimulated Mono Mac 6 cells. The results are presented as the percentage effect of each concentration relative to control treatment (1 % DMSO, 50 pg/ml LPS). ^a Measurements done in three replicates (n = 3) data shown as mean + SEM, ^b Only one measurement performed (n = 1). IL-6, interleukin-6; LPS, lipopolysaccharide.

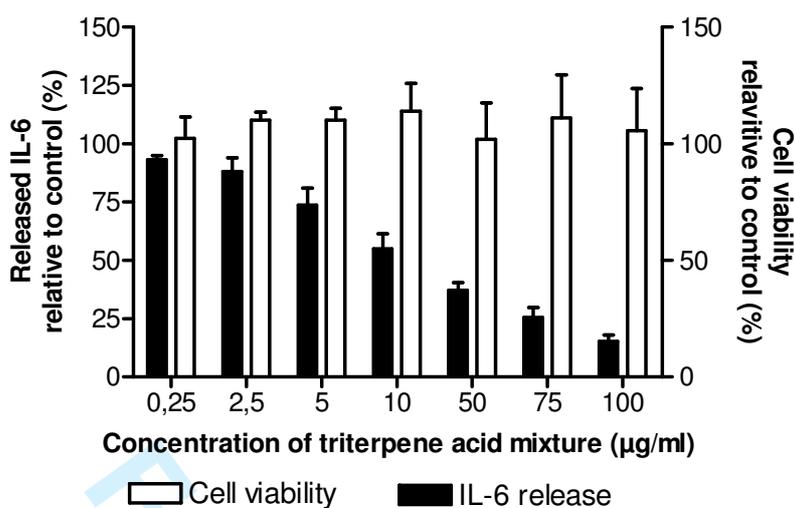


Figure 1. Effect of the triterpene mixture (oleanolic acid: betulinic acid: ursolic acid 30:49:21) on the IL-6 release from and the cell viability of LPS stimulated (50 pg/ml) Mono Mac 6 cells. The bars show the percentage effect of each concentration compared to control treatment (1 % DMSO, 50 pg/ml LPS). The presented data was obtained in triplicate and shown as mean + SEM. IL-6, interleukin-6; LPS, lipopolysaccharide.

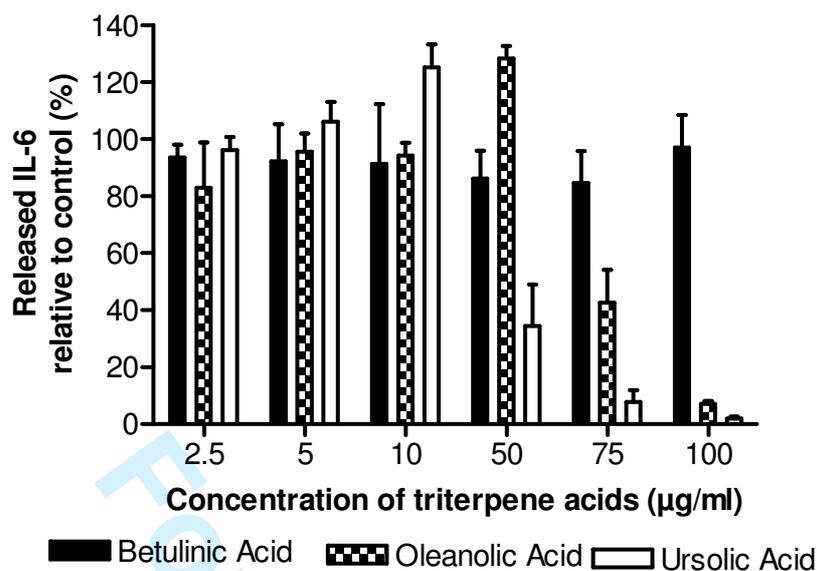


Figure 2. Dose dependent effect of various concentrations of oleanolic acid, betulinic acid and ursolic acid on the IL-6 release from LPS stimulated (50 pg/ml) Mono Mac 6 cells. The bars show the percentage effect of each concentration compared to control treatment (1 % DMSO, 50 pg/ml LPS). The presented data was obtained in triplicate and shown as mean + SEM. IL-6, interleukin-6; LPS, lipopolysaccharide.

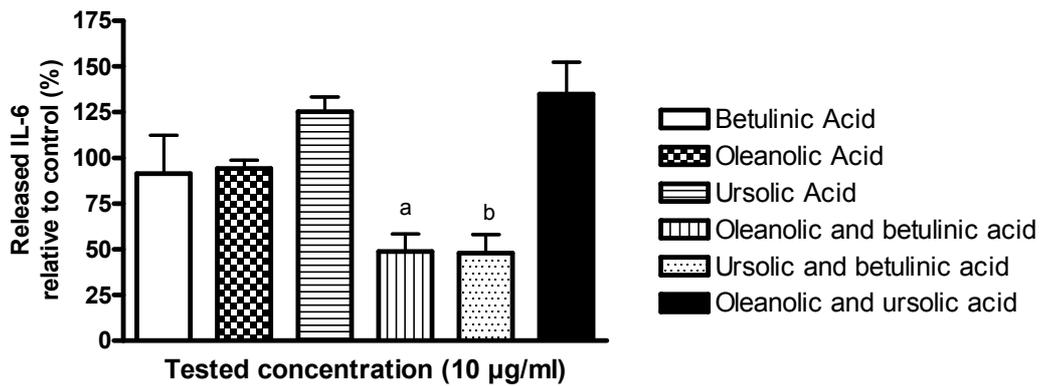
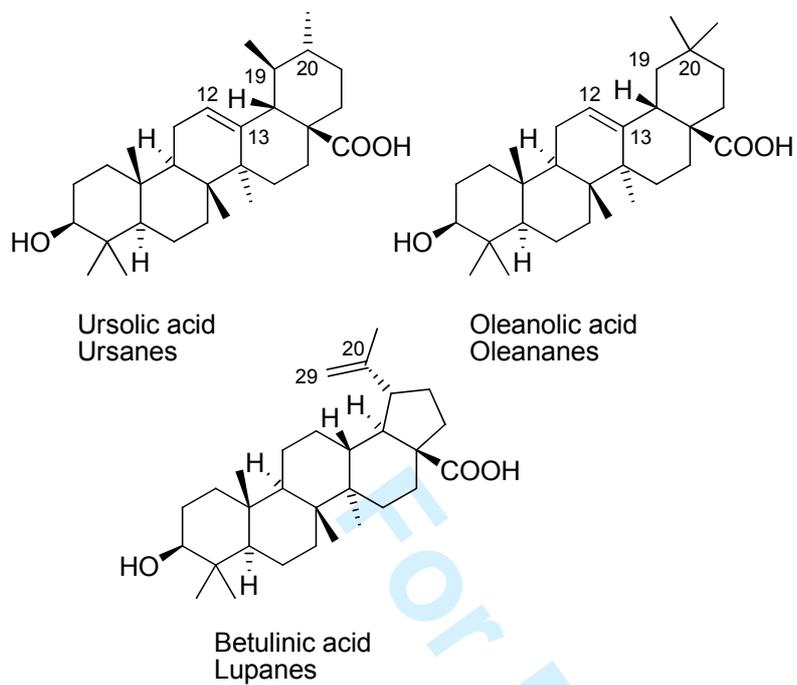


Figure 3. Effect of oleanolic acid, betulinic acid, ursolic acid tested and in pairs (oleanolic acid and betulinic acid, oleanolic acid and ursolic acid and ursolic with betulinic acid) on the IL-6 release from LPS stimulated (50 µg/ml) Mono Mac 6 cells. The bars show the percentage effect of each sample at a concentrations of 10 µg/ml compared to control treatment (1 % DMSO, 50 µg/ml LPS). The presented data was obtained in triplicate and shown as mean + SEM. ^a significantly lower than the effect of 10 µg/ml oleanolic acid ($p < 0.02$), ^b significantly lower than the effect of 10 µg/ml ursolic acid ($p < 0.004$). IL-6, interleukin-6; LPS, lipopolysaccharide.



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Figure 4: Constitutions of ursolic acid, oleanolic acid and betulinic acid