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Phytochemical study and immunomodulatory activity of *Fraxinus excelsior* L.

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Objectives *Fraxinus excelsior* L. (FE) is traditionally used to treat inflammatory and pain disorders. This study aimed to identify the constituents of FE leaves and evaluate the effects of its *n*-hexane (FEH), ethyl acetate (FEE), methanol (FEM) extracts and constituents on the viability of THP-1 cells and their ability to release pro-inflammatory cytokines.

Methods THP-1 cell viability was assessed using an MTT assay. The immunomodulatory activity was evaluated by measuring tumour necrosis factor-alpha (TNF-α) and interleukin 12 (IL-12) released by lipopolysaccharide-stimulated THP-1 cells using enzyme-linked immunosorbent assays.

Key findings Triterpenes, tyrosol esters, alkanes, phytyl and steryl esters, pinocembrin and bis(2-ethylhexyl)phthalate were isolated from FE. The tyrosol esters showed no significant effect on THP-1 cell viability. FEH, FEE, FEM, and pinocembrin, ursolic acid, oleanolic acid had IC_{50} values of 56.9, 39.9, 124.7 µg/ml and 178.6, 61.5 and 199.8 µM, respectively. FE extracts, ursolic acid, oleanolic acid and pinocembrin significantly reduced TNF-α/IL-12 levels. The tyrosol esters did not significantly affect TNF-α/IL-12 production.

Conclusions FE was able to reduce pro-inflammatory cytokine production indicating a mechanistic focus in its use for inflammation and pain. Further investigations are warranted to unravel the mode of action of the tested constituents and discover other potentially active compounds in FE extracts.

Keywords: *Fraxinus excelsior*; cell viability; immunomodulatory activity

Introduction

The innate immune response, mediated by cells like macrophages, monocytes, natural killer cells and neutrophils, is defined as a rapid, non-specific, first line of defence of the body against a variety of foreign invaders.[1] In the presence of an infectious agent, specific immune cell receptors, called pattern recognition receptors, bind to pathogen-associated molecular patterns or damage-associated molecular patterns released from infected cells. This activates signalling pathways leading to the release of mediators such as cytokines that promote a controlled inflammatory response which results in the efficient clearance of the pathogen.[2, 3] Among these cytokines, tumour necrosis factor-alpha (TNF-α) and interleukin 12 (IL-12) are two major pro-inflammatory molecules produced in response to the presence of bacterial components such as lipopolysaccharide (LPS) on the cell surface on Gram negative bacteria.[3, 4] Many studies, however, have highlighted that the overproduction of such cytokines is linked to various chronic inflammatory disorders including atherosclerosis, diabetic mellitus, neurodegenerative and inflammatory bowel diseases, psoriasis, multiple sclerosis and rheumatoid arthritis.[4–6]

Plants have previously been identified as a promising source for the discovery of compounds able to interfere with cytokine production, including TNF-α and/or IL-12.[6, 7] *Fraxinus excelsior* L. (FE; Oleaceae), also called common ash, is a deciduous tree found in Europe and south-western Asia.[8] This species is used traditionally for a range of ailments including skin and nervous disorders, diabetes, burns, tuberculosis, peritonitis, constipation, snake/insect bites, intestinal worms, malaria, dropsy, syphilis, gynecologic diseases, and as an aphrodisiac.[9, 10] Its leaves are used to treat arthritis, rheumatism, gout, minor articular pain, fever, neuralgia, bad breath, obesity, biliary calculus, urinary complaints, and for their anti-aging, hypercholesterolemic, hypotensive, cathartic, diuretic, and laxative properties.[11–18] Previous phytochemical analyses on *F. excelsior* demonstrated that it contained coumarins, secoiridoids, phenylethanoids, flavonoids, benzoic and cinnamic acid derivatives, indole-3-acetic acid and jasmonate derivatives, terpenoids and β-sitosterol.[11, 19–29] Previous pharmacological investigations demonstrated the antihypertensive, antihypertriglyceridemic, antidiabetic, antihyperglycemic, anti-inflammatory, antirheumatic, anti-obesity, diuretic, antimalarial, antioxidant, anti-tyrosinase, anti-elastase, anti-collagenase, antibacterial, antiproliferative and anti-inflammatory activity of *F. excelsior*.[18, 30–39]

To the best of our knowledge, its effects on THP-1 cell viability and TNF-α/IL-12 production have never been reported. The purpose of this investigation was to isolate phytochemicals from *F. excelsior* leaves and screen its extracts/constituents for immunomodulatory activity, specifically targeting TNF-α and IL-12 as these are directly involved

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in mediating inflammation, in an effort to validate the traditional use of this plant in inflammatory and pain disorders.

Materials and Methods

General procedures

Vacuum liquid chromatography (VLC), thin layer chromatography (TLC) and column chromatography (CC) were performed using silica gel 60 H, silica gel 60 F_{254} plates and silica gel 60 (0.063–0.200 mm) (Merck, Germany), respectively. The TLC plates were visualised under short and long wave UV light, sprayed with anisaldehyde-sulfuric acid reagent then heated at 110°C for 3 min. Lipophilic Sephadex LH20-100, *p*-anisaldehyde, pinocembrin (95% purity), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), LPS from *Escherichia coli*, L-glutamine, potassium chloride, sulfuric acid and Tween 20 for molecular biology were from Sigma-Aldrich (UK). Oleanolic acid (98.4% purity), ursolic acid (98% purity), Triton X-100, sodium chloride, KH_2PO_4 and Na_2HPO_4 were from Fisher Scientific (UK). Streptavidin-HRP conjugate (enzyme-linked immunosorbent assay [ELISA] grade) and 3,3′,5,5′′- tetramethylbenzidine (TMB) stabilized chromogen were from Invitrogen (USA).

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III AV500 spectrometer, a Bruker Avance AV400 or a Bruker Avance III AV600. All samples dissolved in $CDCl_3$ were transferred to Norell NMR tubes (5 mm) from Sigma-Aldrich (UK). Samples in low amounts (<5 mg) were transferred to Shigemi or Norell microbore tubes. $CDCl_3$ was used as an internal standard using residual solvent peaks as references. All data were processed with MestReNova software (version 14). High resolution mass spectra were recorded at the National Mass Spectrometry Facility, Swansea University (UK). Positive and negative ion mode nano-electrospray experiments were recorded on an LTQ Orbitrap XL. Atmospheric solids analysis probe analyses were conducted using a Time-Of-Flight (TOF) mass spectrometer on Waters Xevo G2-S. Gas-chromatography/electron impact-mass spectrometry (GC/EI-MS) analysis was performed on Waters GCT Premier (TOF). The structures of all compounds were elucidated from their NMR/MS spectra and by comparing with published data.

Extraction of plant material

Dried powdered FE leaves (500 g from G. Baldwin & Co, UK – batch number BN0016) were extracted with *n*-hexane followed by ethyl acetate then methanol (5 l each) using a Decon® sonicator for 1 h at 25°C. The extracts were filtered using a qualitative filter paper. The filtrates were rotary evaporated at <40°C to obtain a crude *n*-hexane extract coded FEH (3.9 g, 0.78% w/w), an ethyle acetate extract FEE (7.7 g, 1.54% w/w) and a methanol extract FEM (31.8 g, 6.40% w/w). All extracts were kept in sealed glass vials and stored at –20°C.

Fractionation of extracts and isolation of constituents

VLC was performed on FEH (3.9 g), eluting with *n*-hexane, EtOAc-*n*-hexane mixtures of increasing polarity (0–100% EtOAc in hexane (v/v) with increments of 5–10%) and finally mixtures of EtOAc and MeOH (up to 50% MeOH in EtOAc). The collected fractions (F1–F10) were gathered based on their similar TLC profiles. Fraction F1, eluted with 1–2% EtOAc in *n*-hexane, gave a single spot on TLC and was characterised as **1** (15.5 mg). Fraction F7 (421 mg), eluted with 40% EtOAc in *n*-hexane, was subjected to gel filtration (5% *n*-hexane in dichloromethane [DCM], then 100% DCM) to yield **2** (2.7 mg).

FEE (7.7 g) was subjected to VLC, eluting with *n*-hexane, EtOAc-*n*-hexane mixtures of increasing polarity (0–100% EtOAc in *n*-hexane (v/v) with increments of 5–10%) and finally mixtures of EtOAc and MeOH (up to 50% MeOH in EtOAc). The fractions (F1–F12) were gathered based on their similar TLC profiles. Fraction F3 (258 mg), eluted with 10% EtOAc in *n*-hexane, was further subjected to CC on silica gel (*n*-hexane, then *n*-hexane-EtOAc mixtures of increasing polarity) to afford **3** (4 mg) and **4** (20 mg). Fraction F5 (168 mg), eluted with 20% EtOAc in *n*-hexane, was subjected to gel filtration (5% *n*-hexane in DCM and 100% DCM) to afford **5** (10.2 mg) and **6** (6.8 mg). Fraction F9 (330 mg), eluted with 60% EtOAc in *n*-hexane, gave a single spot on TLC and was characterised as **7** (107 mg). Fraction F12 (1.105g), eluted with 10-20% MeOH in EtOAc, was subjected to gel filtration (100% DCM, then 5% and 10% MeOH in DCM) to afford **8** (3.5 mg).

Cell culture

Human monocyte leukaemia THP-1 cells (ECACC 88081201 from the European Collection of Authenticated Cell Cultures, UK) were grown in RPMI 1640 medium (Corning, USA) supplemented with 10% foetal bovine serum (Biosera, UK) and 1% L-glutamine in 75 cm^2 culture flasks under 5% CO_2 at 37°C and 100% humidity. After 2–3 days (upon reaching ≈ 80% confluence), the cells were removed from the flasks and centrifuged at 400 g for 6 min at 22°C. The medium was discarded and the cells resuspended into fresh RPMI-1640 medium without phenol red (Gibco, UK) or RPMI-1640 medium for the cell viability and immunomodulatory assays, respectively. Cell counts were performed using a haemocytometer to obtain a seeding density of approximately 5×10^5 and 1×10^6 cells/ml for the cell viability and immunomodulatory assays, respectively.

Cell viability assays

Viability of THP-1 cells at various concentrations

THP-1 cells (1.7×10^6 cell/ml) were serially diluted in RPMI-1640 without phenol red medium in a 96-well microtitre plate. After incubation at 37°C with 5% CO_2 and 100% humidity for 20 min, the cells were treated with 12 µL MTT in phosphate buffered saline (PBS) (5 mg/ml). After 2–3 h of re-incubation, the purple formazan crystals were solubilized in 10% sodium dodecyl sulfate (SDS) (150 µl), then the plates were re-incubated for 24 h. A microplate reader (Spectra Max 119, Molecular Devices, USA) was used to measure absorbance values at λ 570 nm. Each concentration was assayed in quadruplate and at least two independent experiments were conducted.

Viability of THP-1 cells in the presence of FE extracts/constituents

This was performed using an MTT assay with some modifications.[40–42] THP-1 cells were seeded at a density of 5×10^5 cells/ml in 96-well microtitre plates in RPMI-1640 without phenol red medium. Stock solutions of FE

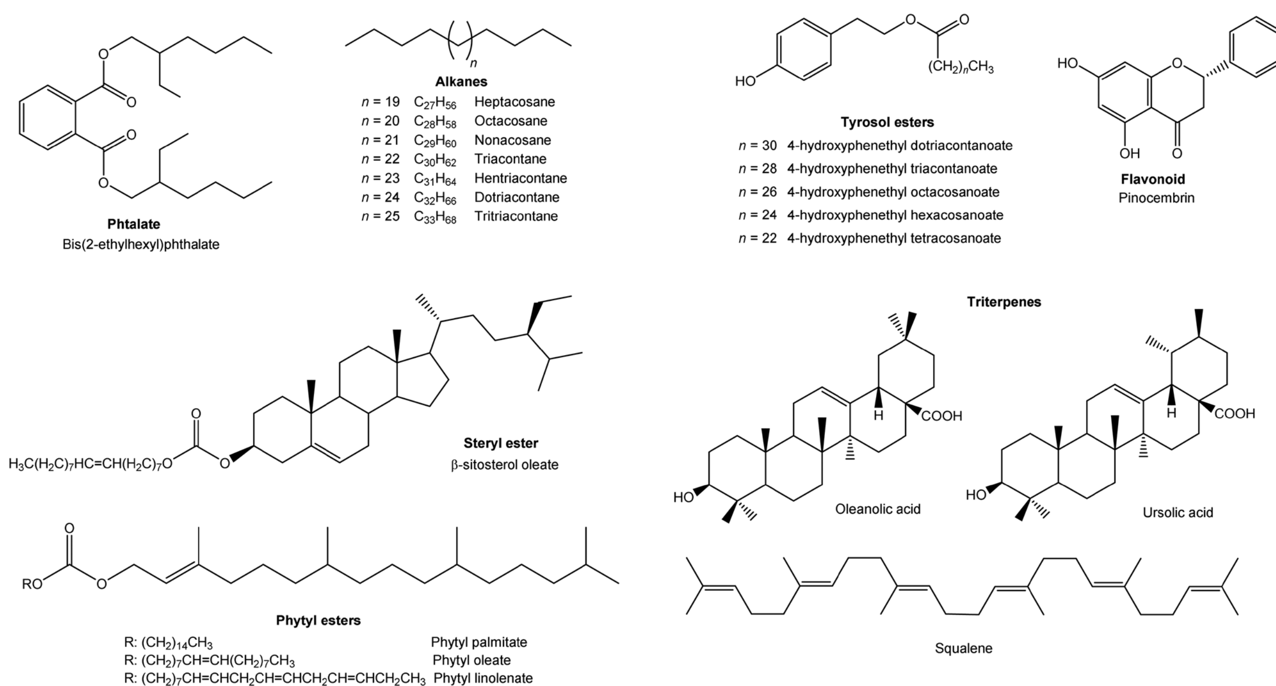

Figure 1 Classes of constituents isolated from *Fraxinus excelsior* leaves.

extracts/constituents in dimethylsulfoxide (DMSO) (50 mg/ml) were serially diluted in the medium to achieve a range of concentrations (15.6–250 µg/ml) with a maximum concentration of DMSO ≤ 0.5%. Blank control wells containing samples in culture medium only, negative control wells containing DMSO (0 and 0.5%) and culture medium; as well as positive control wells containing Triton X-100 (0.5%) and culture medium were also added. After incubation at 37°C with 5% CO_2 and 100% humidity for 22 h, the cells were treated with 12 µl of MTT in PBS (5 mg/ml). After 2–3 h of re-incubation, the purple formazan crystals were solubilized in 10% SDS (150 µl) and the plates were re-incubated for 24 h. Absorbance values were measured at λ 570 nm. At least three independent experiments were conducted and all measurements were carried out in triplicate. The percentage of cell viability was calculated as;

$$\%\ \text{Cell viability} = \frac{\text{OD treated cells} - \text{OD blank}}{\text{OD untreated cells} - \text{OD blank}} \times 100$$

The concentrations required for 50% inhibition of cell viability with respect to untreated cells (IC_{50} values) were determined using nonlinear regression analysis. All concentrations < IC_{50} which maintained cell viability above 70% compared to untreated cells were considered non-toxic and used for the immunomodulatory assays

Immunomodulatory assays

Effect of LPS on TNF-α and IL-12 production from THP-1 cells

THP-1 cells were incubated with RPMI1640 medium without LPS and with LPS (0.1–50 µg/ml). The supernatants were collected and TNF-α and IL-12 levels were measured using the Human TNF-α CytoSet (CHC1753) and Human IL-12p40 CytoSet (CHC1563) ELISA kits from Invitrogen, Thermo Fisher Scientific (USA), respectively.

Effect of FE extracts/constituents on TNF-α and IL-12 production from LPS-stimulated THP-1 cells

THP-1 cells were seeded at a density of 10^6 cells/ml in 24-well culture plates in RPMI-1640 medium with LPS (10 µg/ml) (LPS-stimulated control) and non-toxic concentrations (i.e. concentrations < IC_{50} values and with cell viability ≥ 70% compared to untreated cells) of FEH/FEE (31.3 µg/ml), FEM (31.3 and 62.5 µg/ml), pinocembrin (61, 122 µM), oleanolic acid (34.2,68.4, 136.8 µM), ursolic acid (34.2 µM), or (5) (31.25-250 µg/ml). An unstimulated control (cells in medium only), unstimulated treated control (cells and samples) and vehicle-stimulated control (LPS-stimulated cells and 0.125% DMSO) were also added. Following incubation at 37°C with 5% CO_2 and 100% humidity for 22 h, supernatants were collected and the levels of pro-inflammatory cytokines (TNF-α and IL-12) produced by LPS-stimulated THP-1 cells were measured using a sandwich ELISA assay according to the manufacturer's instructions. At least three independent experiments were performed.

Statistical analysis

Measurements were performed in triplicate or quadruplicate, with the data presented as means ± standard deviation (SD) of at least three independent experiments. Statistical significance was analysed with one-way analysis of variance (followed by *Fisher's Protected Least Significant Difference* test), with $P < 0.05$ considered significantly different when comparing each treatment concentration versus untreated cells. GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, CA) was employed to generate curves, analyse all data, and perform the statistical analyses.

Results

Characterisation of constituents

Various phytochemiclas were isolated from *F. excelsior* (Figure 1). In FEH, these were identified as as a mixture of squalene and nonacosane (**1**),[43–46] and as bis(2-ethylhexyl)phthalate (**2**).[47,48] FEE contained a mixture of heptacosane, octacosane, nonacosane, triacontane, hentriacontane, dotriacontane and tritriacontane (**3**),[45] β-sitosteryl oleate, phytyl palmitate, phytyl oleate, and phytyl linolenate (**4**),[49–54] tyrosol esters identified as 4-hydroxyphenethyl dotriacontanoate, 4-hydroxyphenethyl triacontanoate, 4-hydroxyphenethyl octacosanoate, 4-hydroxyphenethyl hexacosanoate, and 4-hydroxyphenethyl tetracosanoate (**5**),[55–58] pinocembrin (**6**),[59] a mixture of oleanolic acid and ursolic acid (**7**)[60, 61] and ursolic acid (**8**).[61,62] NMR and MS data for all compounds are reported in Supplementary Figure S1–S23.

Cell viability assays

Viability of THP-1 cells at various concentrations

Preliminary experiments were performed to determine the optimal THP-1 cell concentration (0.75 < optical density <1.25) to be used for subsequent assays. This was found to be in the range of 7.0–8.0 × 10^5 cell/ml (equivalent to 5.0 × 10^5 cell/ml as the final concentration per well) (Supplementary Figure S24).

Effect of FEH, FEE and FEM on THP-1 cell viability

FEH, FEE, FEM led to a concentration-dependent decrease in THP-1 cell viability. FEH, FEE significantly reduced cell viability compared to the control at concentrations ≥ 31.25 µg/ml. FEH, at 31.25 µg/ml, maintained 87% cell viability compared to untreated cells, whereas FEE at the same concentration significantly lowered cell viability (ca. 35% compared to untreated cells). The IC_{50} values for FEH and FEE were 56.9 and 39.9 µg/ml, respectively. FEM significantly reduced THP-1 cell viability at concentrations ≥ 125 µg/ml with an IC_{50} value of 124.7 µg/ml (Figure 2).

Effect of FEE constituents on THP-1 cell viability

Pinocembrin significantly lowered the number of viable THP-1 cells compared to the control at concentrations > 122.0 µM (IC_{50} value of 178.6 µM). Pinocembrin at 122.0 µM maintained approximately 86% of THP-1 cell viability, but reduced cell viability to approximately 20% of the control level at 243.9 µM (Figure 3). Cell viability in the presence of ursolic acid exceeded 94% at concentrations ≤ 34.2 µM.

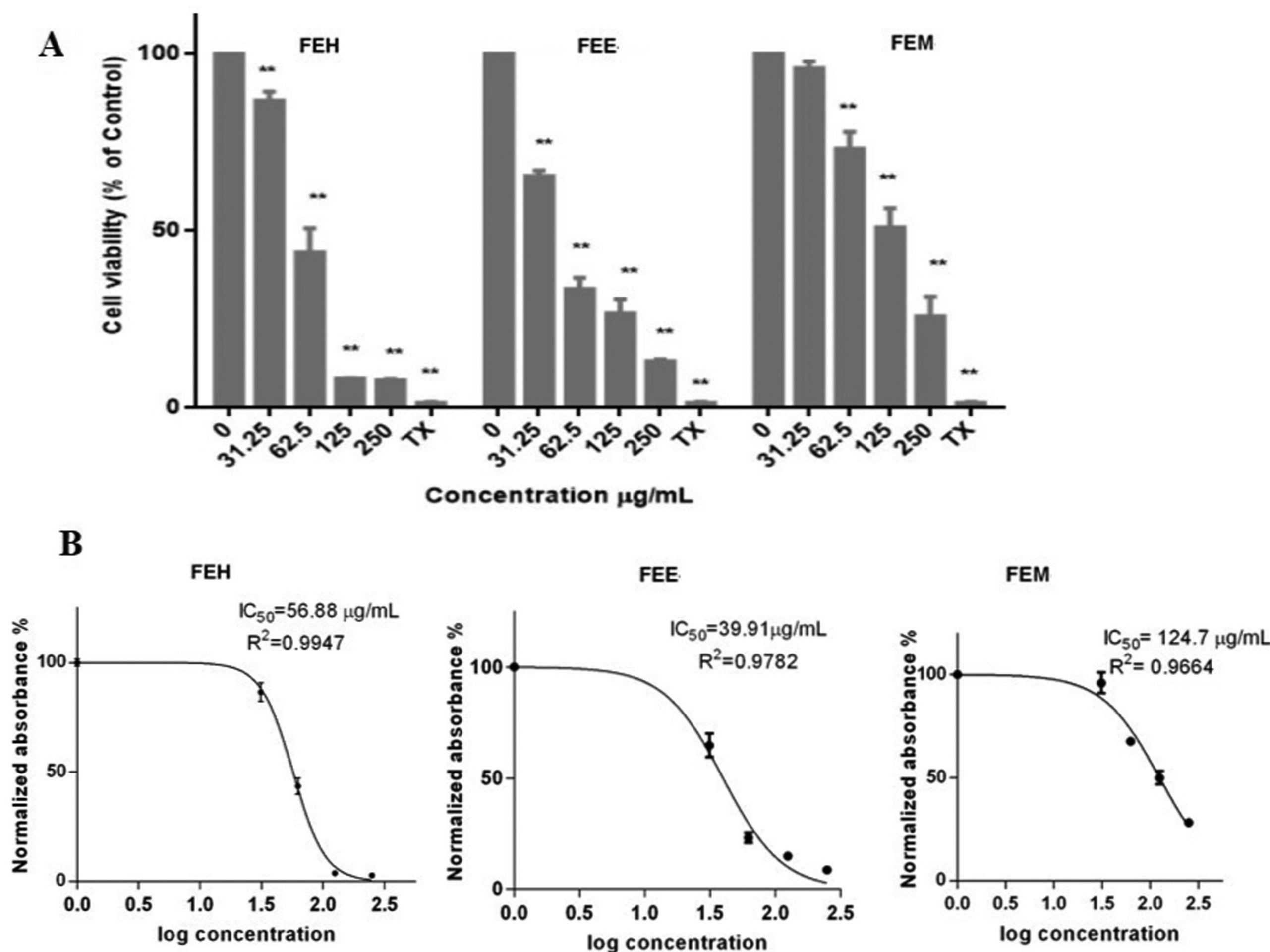

Figure 2 (A) Effect of FEH, FEE and FEM on THP-1 cell viability using an MTT assay. THP-1 cells (5 × 10^5 cells/ml) were transferred to 96-well plates and incubated in the presence of FEH, FEE, FEM at 37°C in 5% CO_2 and 100% humidity for 22 h. Untreated cells (0) and cells treated with Triton X-100 (0.5%) were used as negative and positive controls, respectively. The absorbance was measured at 570 nm. The values are presented as the means of $n = 3$ ± SD from three independent experiments. (**B**) Calculation of the IC_{50} values of FEH, FEE, FEM using non-linear regression analysis. The values are presented as the means of $n = 4$ ± SD from three independent experiments.

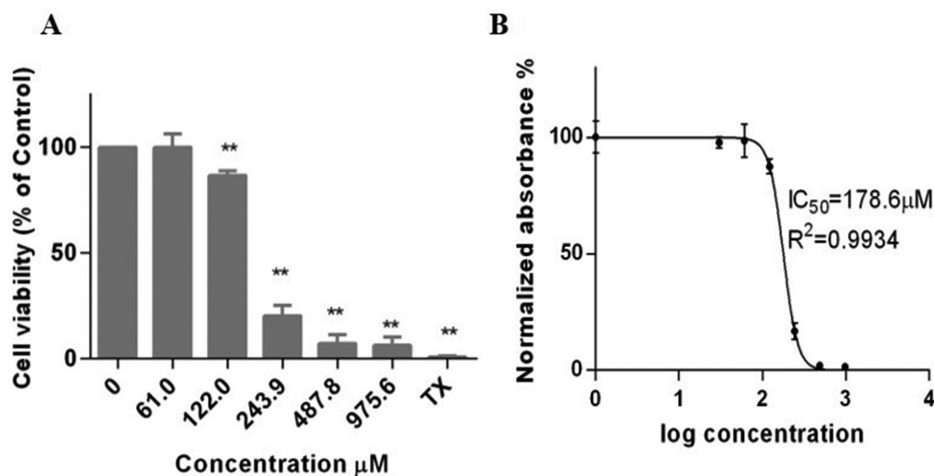

Figure 3 (A) Effect of various concentrations of pinocembrin on THP-1 cell viability using an MTT assay. THP-1 cells (5 × 10^5 cells/ml) were transferred to 96-well plates and incubated in the presence of various concentrations of pinocembrin at 37°C in 5% CO_2 and 100% humidity for 22 h. Untreated cells (0) and cells treated with Triton X-100 (0.5%) were used as negative and positive controls, respectively. The absorbance was measured at 570 nm. The values are presented as the means of n = 3 ± SD from three independent experiments. (B) Calculation of the IC_{50} value of pinocembrin using nonlinear regression analysis. The values are presented as the means of n = 4 ± SD and are representative of three independent experiments.

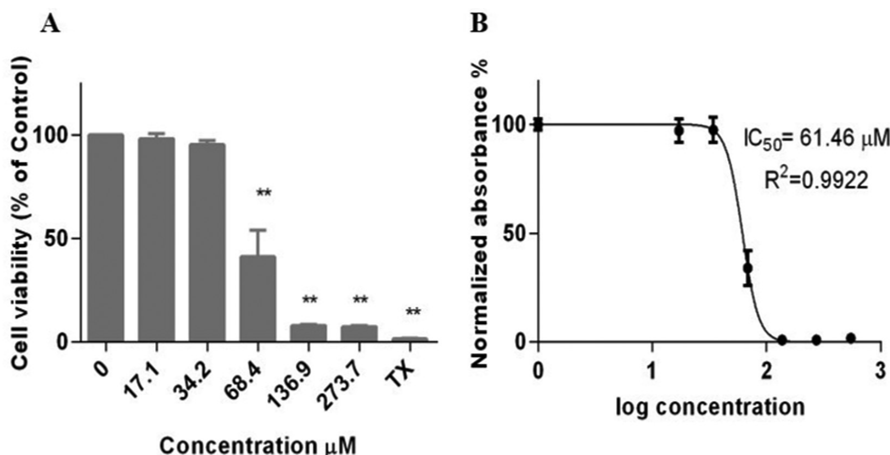

Figure 4 (A) Effect of various concentrations of ursolic acid (UA) on THP-1 cell viability using an MTT assay. THP-1 cells (5 × 10^5 cells/ml) were transferred to 96-well plates and incubated in the presence of various concentrations of ursolic acid at 37°C in 5% CO_2 and 100% humidity for 22 h. Untreated cells (0) and cells treated with Triton X-100 (0.5%) were used as negative and positive controls, respectively. The absorbance was measured at 570 nm. The values are presented as the means of n = 3 ± SD from three independent experiments. (B) Calculation of the IC_{50} value of ursolic acid (UA) using nonlinear regression analysis. The values are presented as the means of n = 4 ± SD and are representative of three independent experiments.

However, at 68.4 µM, this compound significantly decreased the number of viable THP-1 cells to ~40% compared to the control with an IC_{50} value of 61.5 µM (Figure 4). Cell viability exceeded 84% with oleanolic acid concentrations ≤ 68.4 µM, and only at a concentration of 273.7 µM did the reduction of cell viability exceed 50% compared to the control with an IC_{50} value of 199.8 µM (Figure 5). The mixture of tyrosol esters (**5**) showed no significant effect on THP-1 cell viability at all tested concentrations compared to the control (untreated cells). The cell viability exceeded 87% at the highest concentration used (250 µg/ml) (Figure 6).

Immunomodulation assays

Effect of LPS on TNF-α and IL-12 production from THP-1 cells

Incubation of THP-1 cells with increasing LPS concentrations (0–50 µg/ml) led to a significant and concentration-dependent increase in TNF-α and IL-12 levels when compared to cells in RPMI-1640 medium alone. The maximal TNF-α and IL-12 concentrations were seen with LPS used in the range of 10–50 µg/ml. Based on these results, the optimum concentration of LPS used for subsequent experiments was determined to be 10 µg/ml (Supplementary Figure S25).

Effect of FEH, FEE, FEM on TNF-α production from LPS-stimulated THP-1 cells

THP-1 cells incubated with LPS produced increased TNF-α levels compared to the unstimulated control. Treatment with various concentrations of FEH, FEE, FEM in the absence of LPS had no effect on the TNF-α levels produced. In the presence of LPS, however, FE extracts at all tested concentrations significantly reduced TNF-α levels compared to the LPS-stimulated control. The most noticeable reduction was

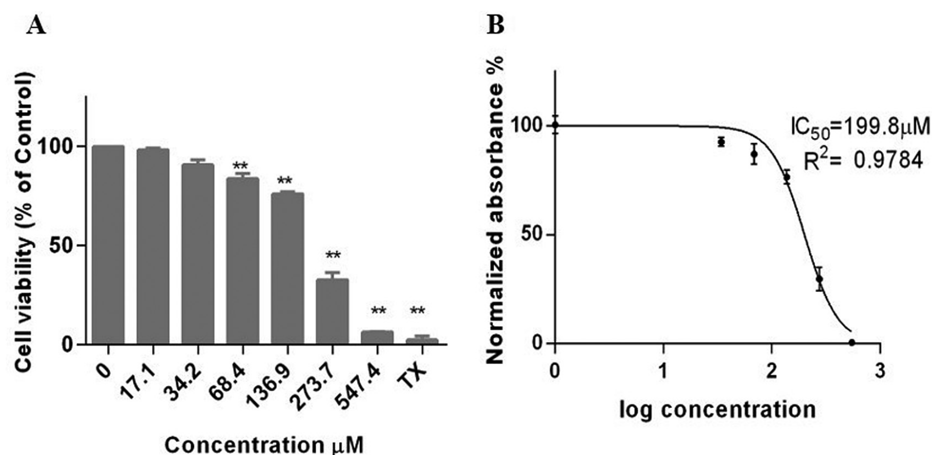

Figure 5 (A) Effect of various concentrations of oleanolic acid on THP-1 cells viability using an MTT assay. THP-1 cells (5 × 10^5 cells/ml) were transferred to 96-well plates and incubated in the presence of various concentrations of oleanolic acid at 37°C in 5% CO_2 and 100% humidity for 22 h. Untreated cells (0) and cells treated with Triton X-100 (0.5%) were used as negative and positive controls, respectively. The absorbance was measured at 570 nm. The values are presented as the means of n = 3 ± SD from three independent experiments. (B) Calculation of the IC_{50} value of oleanolic acid using nonlinear regression analysis. The values are presented as the means of n = 4 ± SD and are representative of three independent experiments.

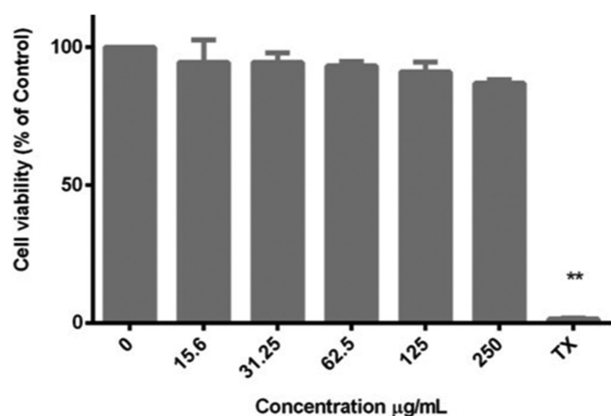

Figure 6 Effect of various concentrations of the mixture of tyrosol esters (**5**) on THP-1 cell viability using an MTT assay. THP-1 cells (5 × 10^5 cells/ml) were transferred to 96-well plates and incubated in the presence of various concentrations of (5) at 37°C in 5% CO_2 and 100% humidity for 22 h. Untreated cells (0) and cells treated with Triton X-100 (0.5%) were used as negative and positive controls, respectively. The absorbance was measured at 570 nm. The values are presented as the means of n = 3 ± SD from three independent experiments.

observed for FEH (tested at 31.3 μg/ml). The vehicle-LPS-stimulated control showed no statistical difference compared to the LPS-stimulated control (Figure 7).

Effect of FEE constituents on TNF-α production from LPS-stimulated THP-1 cells

THP-1 cells incubated with LPS produced increased TNF-α levels compared to the unstimulated control. Pinocembrin (61 and 122 μM) and ursolic acid (34.2 μM) in the absence of LPS did not change the TNF-α produced. In the presence of LPS, however, ursolic acid (34.2 μM) significantly decreased TNF-α production by approximately 25% compared to the LPS-stimulated control. Pinocembrin also led to a potent, significant, and concentration-dependent decrease in TNF-α levels in LPS-stimulated cells compared to the stimulated control. Pinocembrin, at 61.0 and 122.0 μM, reduced TNF-α levels by approximately 40% and 50%, respectively compared to the stimulated control (Figure 8). Treatment with oleanolic acid (34.2, 68.4, 136.8 μM) in the absence of LPS had no effect on the TNF-α levels. In the presence of LPS, oleanolic acid led to a significant, and concentration-dependent, decrease in TNF-α levels (ca. 10–15%) compared to the stimulated control (Figure 9). Treatment with various concentrations of the mixture of tyrosol esters (5) in the absence of LPS had no effect on TNF-α levels. In the presence of LPS, (5) at all the tested concentrations showed no statistically significant differences in TNF-α levels compared to the stimulated control (Figure 10).

Effect of FEH, FEE, FEM on IL-12 production from LPS-stimulated THP-1 cells

THP-1 cells incubated with LPS produced a significant increase in IL-12 levels *versus* the unstimulated control. Treatment with various concentrations of FEH, FEE, FEM in the absence of LPS had no effect on IL-12 production. In the presence of LPS, FE extracts at all tested concentrations significantly reduced IL-12 levels compared to the LPS-stimulated control. The most noticeable and significant reduction in IL-12 production (almost 90% reduction compared to the stimulated control) was observed for FEH and FEE (both at 31.3 μg/ml). IL-12 production was also significantly lowered by FEM in a concentration dependent manner. When tested at 31.3 and 62.5 μg/ml, FEM generated a 60% and 85% reduction in IL-12 levels versus the stimulated control, respectively. The vehicle-stimulated control showed no statistical difference in comparison with the LPS-stimulated control (Figure 11).

Effect of FEE constituents on IL-12 production from LPS-stimulated THP-1 cells

THP-1 cells incubated with LPS significantly increased IL-12 levels compared to the unstimulated control. Treatment with pinocembrin (61 and 122 μM) and ursolic acid (34.2 μM) in the absence of LPS had no effect on IL-12 levels. In the presence of LPS, ursolic acid (34.2 μM) significantly decreased IL-12 production by approximately 50% compared to the

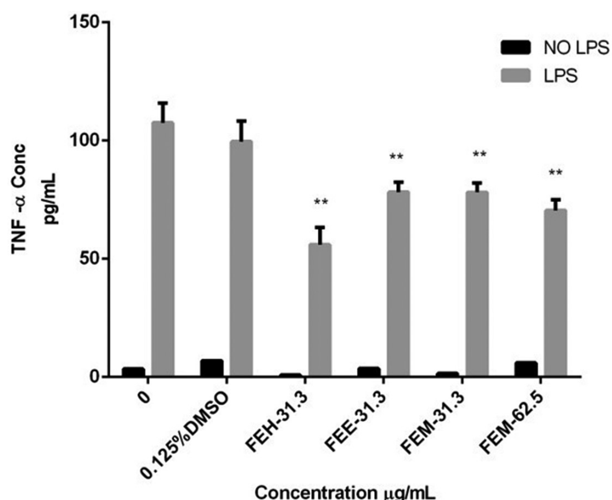

Figure 7 Effect of FEH, FEE, FEM on LPS-stimulated TNF-α production from THP-1 cells. THP-1 cells (1 × 10^6 cells/ml) were incubated with FEH (31.3 µg/ml), FEE (31.3 µg/ml), FEM (31.3 and 62.5 µg/ml) in the presence of LPS (10 µg/ml) (grey colour columns). Incubations without LPS are shown as black colour columns. Incubations were carried out at 37°C, 5% CO_2 and 100% humidity for 22 h. After that, the supernatant was collected and TNF-α levels were measured by ELISA. Values are expressed as the means of $n = 3 \pm$ SD. *$P < 0.05$ and **$P < 0.01$ for treated groups *versus* stimulated control (THP-1 cells incubated with LPS alone).

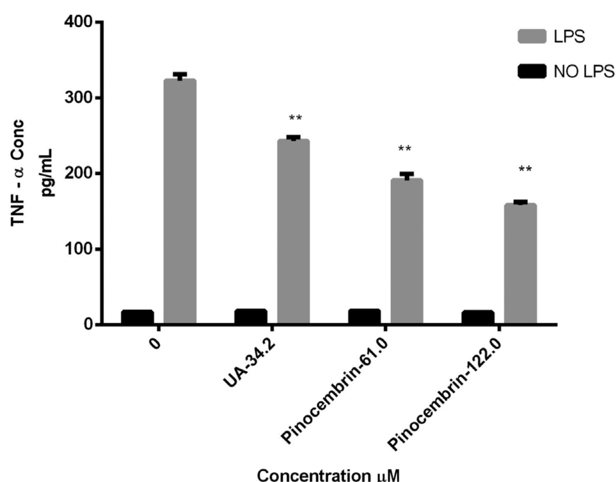

Figure 8 Effect of different concentrations of ursolic acid (UA) and pinocembrin on LPS-stimulated TNF-α production from THP-1 cells. THP-1 cells (1 × 10^6 cells/ml) were incubated with ursolic acid (34.2 µM) or pinocembrin (61 and 122 µM) in the presence of LPS (10 µg/ml) (grey colour columns). Incubations without LPS are shown as black colour columns. Incubations were carried out at 37°C, 5% CO_2 and 100% humidity for 22 h. After that, the supernatant was collected and TNF-α levels were measured by ELISA. Values are expressed as the means of $n = 3 \pm$ SD. **$P < 0.01$ for treated groups versus stimulated control (THP-1 cells incubated with LPS alone).

LPS-stimulated control. Pinocembrin led to a potent, significant, and concentration-dependent decrease in IL-12 compared to the LPS-stimulated control. When tested at 61.0 and 122.0 µM, it reduced IL-12 levels by approximately 75% and 90%, respectively (Figure 12). Treatment with oleanolic acid (34.2, 68.4, 136.8 µM) in the absence of LPS had no effect

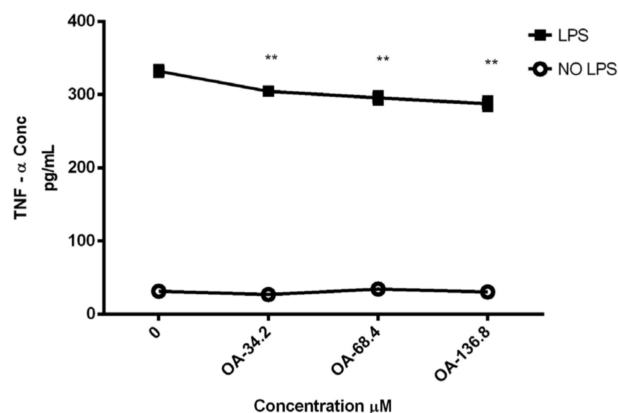

Figure 9 Effect of different concentrations of oleanolic acid (OA) on LPS-stimulated TNF-α production from THP-1 cells. THP-1 cells (1 × 10^6 cells/ml) were incubated with oleanolic acid (34.2, 68.4 and 136.8 µM) in the presence of LPS (10 µg/ml) (square filled symbols). Incubations without LPS are shown as open circles. Incubations were carried out at 37 °C, 5% CO_2 and 100% humidity for 22 h. After that, the supernatant was collected and TNF-α levels were measured by ELISA. Values are expressed as the means of $n = 3 \pm$ SD. *$P < 0.05$ and **$P < 0.01$ for treated groups *versus* stimulated control (THP-1 cells incubated with LPS alone).

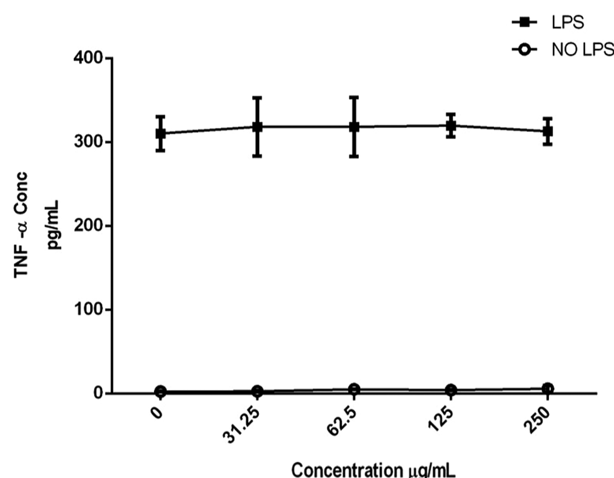

Figure 10 Effect of different concentrations of the mixture of tyrosol esters (**5**) on LPS-stimulated TNF-α production from THP-1 cells. THP-1 cells (1 × 10^6 cells/ml) were incubated with different concentrations of (**5**) (0–250 µg/ml) in the presence of LPS (10 µg/ml) (square filled symbols). Incubations without LPS are shown as open circles. Incubations were carried out at 37°C, 5% CO_2 and 100% humidity for 22 h. After that, the supernatant was collected and TNF-α levels were measured by ELISA. Values are expressed as the means of $n = 3 \pm$ SD. *$P < 0.05$ for treated groups *versus* stimulated control (THP-1 cells incubated with LPS alone).

on IL-12 levels. Oleanolic acid decreased IL-12 production in LPS-stimulated cells in a manner that was concentration-dependent compared to the LPS-stimulated control. When tested at 34.2, 68.4 and 136.8 µM, it reduced IL-12 levels by approximately 20%, 40% and 70%, respectively (Figure 13). Treatment with various concentrations of (5) in the absence of LPS had no effect on IL-12 levels. In the presence of LPS, (5) at all the tested concentrations showed no significant difference in IL-12 levels compared to the LPS-stimulated control (Figure 14).

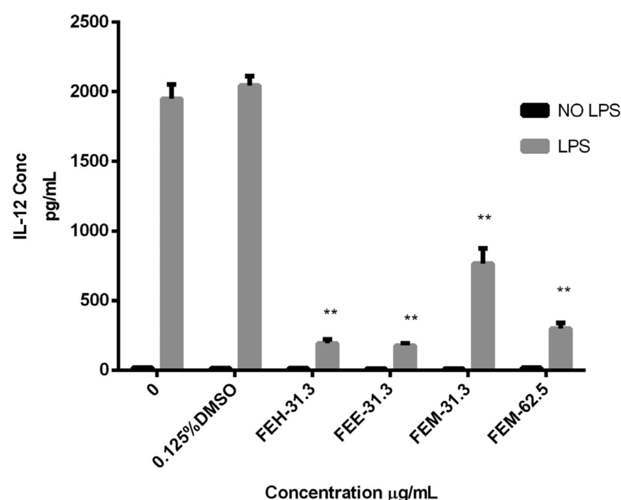

Figure 11 Effect of FEH, FEE, FEM on LPS-stimulated IL-12 production from THP-1 cells. THP-1 cells (1×10^6 cells/ml) were incubated with FEH (31.3 µg/ml), FEE (31.3 µg/ml), FEM (31.3 and 62.5 µg/ml) in the presence of LPS (10 µg/ml) (grey colour columns). Incubations without LPS are shown as black colour columns. Incubations were carried out at 37°C, 5% CO_2 and 100% humidity for 22 h. After that, the supernatant was collected and IL-12 levels were measured by ELISA. Values are expressed as the means of $n = 4 \pm$ SD. $^{**}P < 0.01$ for treated groups *versus* stimulated control (THP-1 cells incubated with LPS alone).

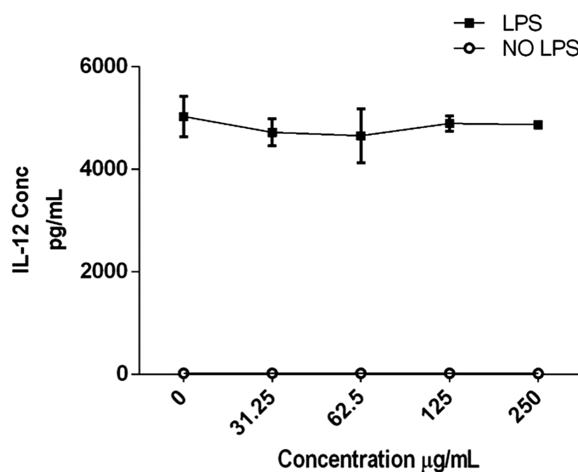

Figure 14 Effect of different concentrations of the mixture of tyrosol esters (**5**) on LPS-stimulated IL-12 production from THP-1 cells. THP-1 cells (1×10^6 cells/ml) were incubated with different concentrations of (**5**) (0–250 µg/ml) in the presence of LPS (10 µg/ml) (square filled symbols). Incubations without LPS are shown as open circles. Incubations were carried out at 37°C, 5% CO_2 and 100% humidity for 22 h. After that, the supernatant was collected and IL-12 levels were measured by ELISA. Values are expressed as the means of $n = 3 \pm$ SD. $^{*}P < 0.05$ for treated groups *versus* stimulated control (THP-1 cells incubated with LPS alone).

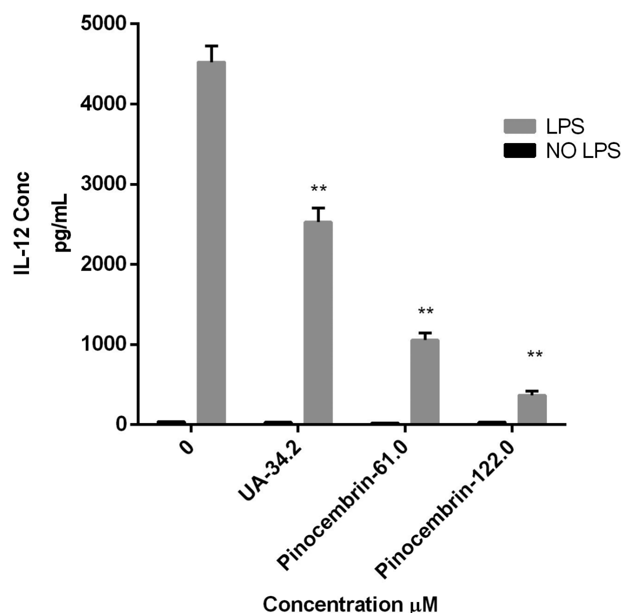

Figure 12 Effect of different concentrations of ursolic acid (UA) and pinocembrin on LPS-stimulated IL-12 production from THP-1 cells. THP-1 cells (1×10^6 cells/ml) were incubated with ursolic acid (34.2 µM) or pinocembrin (61 and 122 µM) in the presence of LPS (10 µg/ml) (grey colour columns). Incubations without LPS are shown as black colour columns. Incubations were carried out at 37°C, 5% CO_2 and 100% humidity for 22 h. After that, the supernatant was collected and IL-12 levels were measured by ELISA. Values are expressed as the means of $n = 3 \pm$ SD. $^{**}P < 0.01$ for treated groups *versus* stimulated control (THP-1 cells incubated with LPS alone).

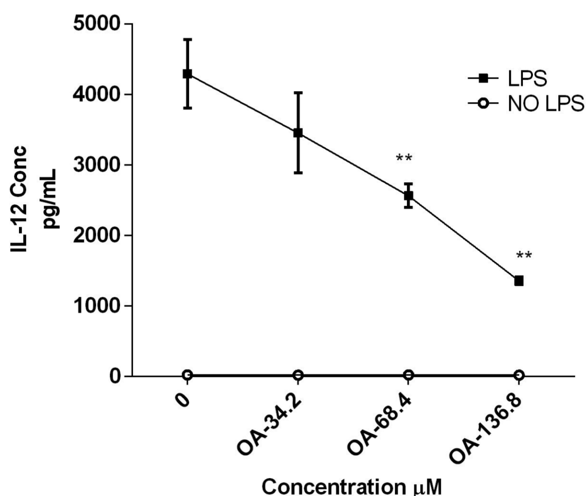

Figure 13 Effect of different concentrations of oleanolic acid (OA) on LPS-stimulated IL-12 production from THP-1 cells. THP-1 cells (1×10^6 cells/ml) were incubated with oleanolic acid (34.2, 68.4 and 136.8 µM) in the presence of LPS (10 µg/ml) (square filled symbols). Incubations without LPS are shown as open circles. Incubations were carried out at 37°C, 5% CO_2 and 100% humidity for 22 h. After that, the supernatant was collected and IL-12 levels were measured by ELISA. Values are expressed as the means of $n = 3 \pm$ SD. $^{*}P < 0.05$ and $^{**}P \leq 0.01$ for treated groups *versus* stimulated control (THP-1 cells incubated with LPS alone).

Discussion

The phytochemical analysis of *F. excelsior* afforded triterpenes, tyrosol esters, alkanes, phytyl and steryl esters, a flavonoid and a phtalate derivative. The occurrence of squalene – a natural constituent of wheat germ, shark liver oil, amaranth and olive oils[44, 63] – is reported for the first time in this species. Ursolic acid, oleanolic acid, and tyrosol esters have

previously been isolated from oleaceous plants.[57, 64–66] Ursolic acid has been previously isolated from *F. excelsior* leaves.[29] However, oleanolic acid and tyrosol esters are reported for the first time in the species and the genus, respectively. Alkanes previously isolated from *F. excelsior* include nonacosane, hentriacontane, and tetratriacontsane.[29] The presence of heptacosane, octacosane, triacontane, dotriacontane, and tritriacontane is reported here for the first time. Wihtin the Oleaceae, phytyl esters have been reported in unripe green olives[67] and in olive oil.[68] Phytyl linolenate, phytyl stearate and phytyl docosanoate have been reported in *F. excelsior leaves*.[69] The presence of phytyl palmitate and phytyl oleate, along with the steryl ester, β-sitosteryl oleate, is reported for the first time in this species. The occurrence of pinocembrin is reported for the first time in *Fraxinus*. Although minimal contact with plasticware was ensured throughout our study, it cannot be confirmed if bis(2-ethylhexyl)phthalate, previously reported as a plant-derived natural product,[47, 48] is a constituent of *F.excelsior* or if it was isolated as a result of experimental contamination.[70]

THP-1 cells are commonly used to assess the antiproliferative activity of natural products.[71, 72] The effect of FEH and FEE on THP-1 cell viability was similar to the activity previously reported for *F. excelsior* on SW742 (colorectal adenocarcinoma), SKLC6 (lung carcinoma), MCF-7 (breast ductal carcinoma), PLC/PRF/5 (liver hepatoma), AGS (Caucasian gastric adenocarcinoma) and A375 (melanoma cancer) cells.[34] Among the tested constituents, the effect of oleanolic acid on THP-1 cell viability was in agreement with the literature.[72] No significant effect was observed for the mixture of tyrosol esters (5) at all tested concentrations, in agreement with a previous report showing that tyrosol esters were non-cytotoxic towards human breast, lung and nervous cancer cells.[57] Pinocembrin and ursolic acid displayed less and more antiproliferative activity on THP-1 cells than previous reports, respectively.[72, 73]

LPS-stimulated THP-1 cells are commonly used to evaluate the immunomodulatory activity of natural products.[74–76] LPS is present in the outer membrane of Gram-negative bacteria and is a key pathogen-associated molecular pattern that interacts with TLR4 receptors to trigger the release of cytokines by monocytes and macrophages.[77–79] We observed that LPS-stimulated THP-1 cells produced TNF-α and IL-12, and that FE extracts at all tested concentrations significantly reduced the levels of these cytokines. The observation that treatment with the highest concentrations of extracts in the absence of added LPS had no effect on TNF-α and IL-12 levels clearly indicated the absence of any contaminating LPS/endotoxin in our extraction process. Previous studies have revealed that *F. excelsior* reduced TNF-α in LPS-stimulated neutrophils[18] and in LPS-stimulated monocytes via inhibiting NF-κB activation.[80] Another *Fraxinus* species, *Fraxinus rhynchophylla*, has been reported to reduce the expression of IL-12 mRNA *in vivo*.[81] This is the first report of the effect of FE on TNF-α and IL-12 production by LPS-stimulated THP-1 cells. The effect of FEH on TNF-α levels may be linked to the presence of squalene in this extract as this compound is known to downregulate TNF-α gene expression.[82] Among the constituents isolated from FEE, both ursolic and oleanolic acid reduced TNF-α levels in THP-1 cells, with ursolic acid showing a more potent suppression in agreement with the literature.[72] Ursolic acid is known to reduce TNF-α levels by blocking TLR4 receptors and suppressing NF-κB and MAPKs activation.[83–88] The effect of ursolic acid on IL-12 was in agreement with the literature.[89] Oleanolic acid can also reduce TNF-α and IL-12 levels by blocking TLR4 receptors and NF-kB activation.[90–93] Previous reports have indicated that pinocembrin reduced TNF-α and IL-12 expression *in vivo*,[94] and reduced TNF-α levels in LPS-stimulated macrophages and murine microglial cells by blocking TLR4 receptors and inhibiting the p38/MAPK, PI3K/Akt and NF-κB signalling pathways.[95–100] Tyrosol has been reported to inhibit the LPS-induced TNF-α release from human monocytes[101] while tyrosol esters with chain lengths of C12:0 and C14:0 can significantly reduce TNF-α mRNA expression in methylglyoxal-treated RAW264.7 cells.[102] The effects of pinocembrin and the tyrosol esters (5) on TNF-α and IL-12, and of ursolic acid on IL-12 by LPS-stimulated THP-1 cells are reported here for the first time.

Conclusion

We demonstrated for the first time that *F. excelsior* leaves, as well as some of its constituents, could significantly reduce the production of TNF-α and IL-12 in LPS-stimulated THP-1 cells. *F. excelsior* leaves are used traditionally to treat arthritis, gout, rheumatism, minor articular pain, neuralgia where the production of these major pro-inflammatory cytokines mediate the effects.[11, 12, 14–16] This study validates, to some extent, the traditional use of *F. excelsior* in inflammatory and pain disorders. Further investigations are warranted to unravel the exact mode of action of the tested constituents and discover other potentially active compounds in the tested extracts.

Supplementary Material

Supplementary data are available at *Journal of Pharmacy and Pharmacology* online.

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Author contributions

R.M.Q. performed all experiments. R.M.Q. and V.S. designed the phytochemical analysis. R.M.Q. and D.R. designed the pharmacological analysis. R.M.Q., V.S. and D.R. analysed the data. R.M.Q. and V.S. wrote the original draft of the manuscript. R.M.Q., V.S. and D.R. edited the manuscript. All authors have read and agreed to the submitted version of the manuscript.

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Conflict of Interest

The authors declare that they have no conflicts of interest to disclose.

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