

The impact of oleanolic and ursolic acid on corneal epithelial cells *in vitro*

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ABSTRACT

INTRODUCTION. Oleanolic (OA) and ursolic (UA) acids belong to the triterpene group widely present in plants. These compounds are recognised to have anti-inflammatory properties and thus are considered to be used in therapies as well as in cosmetic, natural health, or diet products.

AIM. The scientific hypothesis of our study was to show that OA and UA influence corneal epithelial cells cultured *in vitro*.

METHODS. Toxicity tests, based on MTT and Neutral Red (NR) uptake, measurement of nitric oxide (NO_x) level, as well as analysis of metalloproteinases (MMP-2 and MMP-9) amount and activity were performed.

RESULTS. UA expressed significantly higher toxicity on cells than OA. At the lowest concentration applied (5 μM), UA limited cellular metabolism and viability on average by 22% as compared to untreated control, while 25 μM resulted in values lower than 10%. On the other hand, OA at the highest (100 μM) concentration limited cellular metabolism and viability by about 20%. NO_x level significantly increased when OA and UA were applied at concentrations of 25 and 100 μM, respectively. OA and UA had a stronger impact on the level of MMP-2 than MMP-9. OA and UA reduced MMP-2 and MMP-9 in the whole range of concentrations. Tested triterpenoids had no significant impact on MMP activity.

CONCLUSIONS. OA and UA have a different impact on human corneal epithelial cells. UA is toxic for corneal epithelial cells, while OA exhibits milder activity, which may be useful for further analysis in ocular pharmacology.

KEY WORDS: corneal epithelial cells, Episkin, metalloproteinases, nitric oxide, oleanolic acid, toxicity model, ursolic acid

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INTRODUCTION

Oleanolic acid (OA) and ursolic acid (UA) belong to the group of ubiquitous pentacyclic triterpenes, which are widely found in herbs, other medicinal plants, and food. These compounds exert many biological activities such as anti-inflammatory, anticancer, antioxidant, and antiallergic [1, 2]. In view of a wide variety of activities, they were also

considered as suitable in ophthalmology in order to protect the eye surface against adverse effects of particular inflammations and allergies [1, 3]. Some of the factors acting adversely may include matrix metalloproteinases (MMPs) or high levels of nitric oxide (NO).

MMPs are a family of proteinases extracellular matrix (ECM) both in physiological processes ena-

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bling tissue remodelling or its normal functioning, and in pathological processes leading to inflammation, tissue destruction or, in the case of cancer, to tumour invasion and metastasis. Specifically, MMP-2 and MMP-9 are gelatinases that degrade type IV collagen, the major structural component of the ECM [4]. These proteases were found to take part in many ocular disorders like retinopathy [5], cataract [6], uveitis [7], or generally atopic ocular diseases [8]. In turn, local micromolar NO concentrations may exert similar effects that are connected with inflammation and local tissue destruction. On the other hand, nanomolar quantities of NO take part in maintaining physiological homeostasis on the ocular surface. Generally, NO regulate numerous vascular functions in eye, which in turn have a significant impact on proper functioning of other ocular tissues [9].

The cornea surface is created by non-keratinised epithelial cells whose arrangement and stability are essential for proper vision. These cells are subjected to continuous renewal and any manipulation of this process as well as of homeostasis of the corneal layer may lead to substantial changes in visual acuity [10]. Any substances which are administered to the surface of the eye should be analyzed in terms of their impact not only on corneal epithelial cells viability but simultaneously on the induction of soluble factors release which may exert an adverse effect on corneal epithelial layer. Therefore, the influence of triterpenes on local alteration in MMPs or NO levels in the corneal epithelium was analysed due to their potential important impact on the functionality of this tissue, and consequently on proper vision.

In our study we hypothesised that OA or UA are compounds that might modify the viability of corneal epithelium and secretion of MMPs and NO by these cells *in vitro*. For this reason, we introduced an HCE three-dimensional model (SkinEthic™ from Episkin, Lyon, France) to test NO_x and MMPs quantity and activity, as well as classic two-dimensional cultures of corneal epithelial cells in order to demonstrate changes in cell viability after their incubation with OA and UA.

MATERIALS AND METHODS

CELL CULTURES

Human normal corneal epithelial cell line 10.014 pRSV-T (ATCC No. CRL-11515) was used. The cells were cultured as monolayers in 25-cm²

culture flasks (Nunc, Roskilde, Denmark) coated with PureCol™ ultrapure collagen (INAMED Biomaterials, Fremont, CA) at 3.1 mg/mL concentration. The cell line was maintained in defined K-SFM (keratinocyte serum-free medium) (Gibco) supplemented with 75 µg/mL endothelial cell growth factor (Sigma), 0.05 mg/mL bovine pituitary extract (Gibco), 500 ng/mL hydrocortisone (Sigma), 0.0005 mg/mL bovine insulin (Gibco), and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) (Sigma, St Louis, MO) at 37°C in a humidified atmosphere with 5% CO₂. This cell culture was used for MTT and NR toxicity tests and MGG staining.

HCE three-dimensional model (SkinEthic™ from Episkin, Lyon, France) commercially available was also used. This reconstructed HCE model consists of immortalised human corneal epithelial cells cultured on a chemically-defined permeable polycarbonate membrane insert at the air-liquid interface. The construct is a multi-layered, stratified epithelium similar to that of normal human corneal epithelium. This model represents 5–7 viable cell layers without significant histological abnormalities growing on a 0.5 cm² surface area. These layers consist of columnar basal cells, transitional wing cells, and flattened superficial cells.

The tissues were shipped at room temperature in agarose gel that contained medium to preserve the quality of the tissues. This agarose-gel was used only for transportation, to keep high viability of cells. After delivery, the inserts with corneal tissue were immediately transferred to a new 24-well culture plate. Thereafter, a liquid-liquid interface was performed because a new culture medium was added both inside the wells of the 24-well plates (medium volume 0.6 mL) as well as inside the inserts with cells (medium volume 0.15 mL). Appropriate concentrations (5; 10; 25; 50; 100 µM) of oleanolic acid (OA) and ursolic acid (UA) were added to the medium present in the insert. 24-well plates with inserts were incubated at 37°C, 5% CO₂ in a humidified incubator. The medium from the wells, but not inserts, was collected 24 hours later. As a culture medium we used the original fluid that was delivered together with cells by the manufacturer. This model was used for NO_x, MMP, and zymography analysis.

NEUTRAL RED (NR) UPTAKE ASSAY

NR cytotoxicity assay analyses the uptake and lysosomal accumulation of Neutral Red stain. Cells that are damaged do not accumulate the dye.

Cells were grown in 96-well multiplates in 100 μL of culture medium (K-SFM) with supplements and oleanolic acid (OA) (Sigma) or ursolic acid (UA) (Sigma) in the concentration range 5–100 μM for 24 hours. Subsequently, the medium was discarded and 0.4% NR (Sigma) solution medium was added to each well. The plate was incubated for three hours at 37°C in a humidified 5% CO_2 /95% air incubator. After incubation, the dye-containing medium was removed, cells fixed with 1% CaCl_2 in 4% paraformaldehyde, and thereafter the incorporated dye was solubilised using 1% acetic acetate in 50% ethanol solution (100 μL). The plates were gently shaken for 20 minutes at room temperature and the extracted dye absorbance was measured spectrophotometrically at 540 nm using a microplate reader (Emax; Molecular Devices Corp., Menlo Park, CA).

MTT ASSAY

MTT assay is based on the conversion of yellow tetrazolium salt by viable cells to purple formazan crystals. Mitochondrial succinate dehydrogenase catalyses this reaction.

Cells were grown in 96-well multiplates in 100 μL of culture medium with OA or UA addition at the appropriate concentration (5–100 μM). After 24 hours of incubation, MTT solution (5 mg/mL, 25 μL /well) (Sigma) was added, and incubation was conducted for a further three hours. The crystals of formazan were solubilised overnight in a 10% sodium dodecyl sulphate 0.01 M HCl mixture (100 μL). The product was quantified spectrophotometrically by absorbance measurement at 570 nm wavelength using an Emax microplate reader (Molecular Devices Corp.).

NO MEASUREMENT

Nitrate, a stable end product of NO, was determined in culture supernatants by a spectrophotometric method based on the Griess reaction.

Corneal epithelial cells (EpiSkin HCE model) were incubated for 24 hours with OA and UA concentrations (5; 10; 25; 50; 100 μM). Thereafter, culture supernatants were collected. The samples were frozen at -80°C and stored for no longer than one month. 100 μL of supernatant was plated in 96-well flat-bottomed plates in triplicate and incubated with 100 μL of Griess reagent (1% sulphanimide/0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride) (Sigma) in 3% H_3PO_4 (POCH Gliwice, Poland) at room temperature for 10 minutes. The optical density was measured at 570 nm

using a microplate reader (Emax; Molecular Devices Corp.). A standard curve was achieved using 0.5–25 μM sodium nitrite (NaNO_2) for calibration and quantitative analysis.

MAY-GRÜNWARD-GIEMSA (MGG) STAINING

Briefly, cells were fixed with methanol for 10 minutes and stained with May-Grünwald stain for three minutes, and diluted in an equal volume of distilled water for a further three minutes. The stain was removed and Giemsa stain diluted (1:20) in water was added for 20 minutes. After that time the dye was removed and slides were rinsed three times with distilled water, air dried, and observed under a light microscope (Olympus BX51).

ZYMOGRAPHY

MMP activity was measured using SDS-PAGE zymography.

Gelatine (300 $\mu\text{g}/\text{mL}$) (Sigma) was incorporated into a 7.5% polyacrylamide gel. Culture supernatant samples were mixed with equal volumes of 2 \times sample buffer (0.25 M Tris-HCl pH 6.8, 10% (v/v) glycerol, 0.05% (w/v) bromophenol blue, and 5% (w/v) SDS) and separated by electrophoresis. The gel was washed in distilled water, incubated for one hour at room temperature with 2.5% (v/v) Triton X-100, washed again in distilled water, and incubated for 24 hours at 37°C with developing buffer (0.05 M Tris-HCl, pH 7.4, 0.01 M CaCl_2 and 0.02% (w/v) NaN_3). After washing, the gel was stained for one hour with 0.2% Coomassie blue in 50% (v/v) methanol and 10% (v/v) acetic acid. Thereafter, the gel was de-stained with 20% (v/v) methanol and 10% (v/v) acetic acid. Bands of digested substrate indicated metalloproteinase activity.

MMP-2 AND MMP-9 ELISA ASSAY

The levels of MMP-2 and IL-6 were tested immunoenzymatically (ELISA) using commercially available kits (R&D, Minneapolis, MN, USA), according to the manufacturers' instructions.

Supernatant samples were added to 96-well microplates coated with monoclonal antibodies against human MMP-2 or MMP-9 and incubated for two hours at room temperature. After three washes in the appropriate buffer, polyclonal secondary antibodies conjugated to horseradish peroxidase were added and incubated for two hours at room temperature. The optical density was measured at 450 nm using a microplate reader (Molecular Devices Corp.,

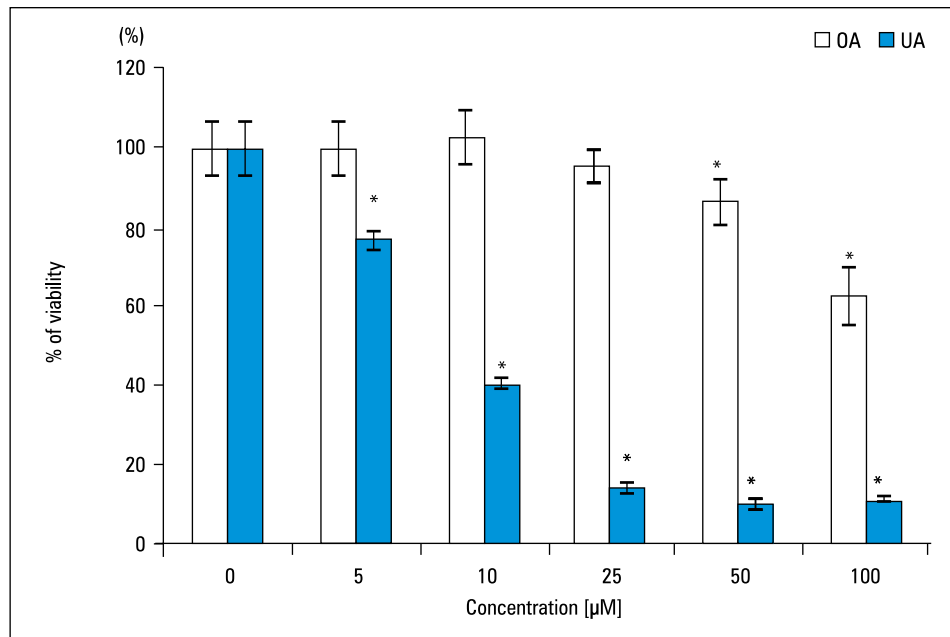


FIGURE 1. The effect of 24-hour treatment of human corneal epithelial cells with oleanolic acid (OA) and ursolic acid (UA). MTT assay. The results are presented as a percentage of the controls, arbitrarily set to 100%. The figure shows an average of three independent experiments. * $p \leq 0.05$ — compared with the control

Emax, Menlo Park, CA, USA), and MMP-2 or MMP-9 concentrations were calculated from the standard curves prepared from recommended substrates. The sensitivity of tests was: 0.082 ng/mL for human MMP-2 and 0.156 ng/mL for human MMP-9.

STATISTICAL ANALYSIS

Results are presented as means \pm SD from three experiments. Data were analysed using one-way ANOVA analysis. Differences of $p \leq 0.05$ were considered significant.

RESULTS

Cytotoxic and metabolism changing activity (MTT and NR tests).

Oleanolic acid (OA) at concentrations higher than 50 μ M decreased metabolic activity of corneal epithelial cells. At 100 μ M of OA applied, it was $37\% \pm 7\%$ lower than control. Ursolic acid (UA) expressed stronger activity than OA and significantly limited metabolism of cells even at the lowest concentration (5 μ M) ($23\% \pm 2\%$) applied as compared to untreated controls. Concentrations of UA higher than 25 μ M decreased metabolism of corneal epithelial cells to the level lower than 10% (Fig. 1).

Similar results were obtained when the cytotoxic activity of both compounds was tested using

NR uptake assay. However, OA significantly decreased cell viability only at the highest concentration (100 μ M) ($18\% \pm 3.5\%$) applied, as compared to controls. UA activity coincided with the results obtained in MTT test (Fig. 2).

Staining of cells with May-Grünwald-Giemsa (MGG) dye confirmed corneal epithelial cell sensitivity to UA and the low impact of OA on these cells (Fig. 3).

NITRIC OXIDE (NOX) LEVEL

Control level of NO_x produced by corneal epithelial cells was $0.51 \pm 0.01 \mu$ M. Both OA and UA at concentrations lower than 10 μ M had no impact on NO_x production by corneal epithelial cells as compared to controls. Starting from 25 μ M concentration, UA gradually increased the NO_x level in culture medium. At the highest concentration used (100 μ M) it was $1.93 \pm 0.12 \mu$ M. On the other hand, OA only at the concentration of 100 μ M increased the NO_x level to the value of $0.67 \pm 0.01 \mu$ M (Fig. 4).

OA AND UA IMPACT ON PRODUCTION OF METALLOPROTEINASES (MMP-2 AND MMP-9)

Metalloproteinase (MMP-2 and MMP-9) levels were tested quantitatively by ELISA, and qualitative activity of enzymes was tested by zymography. The levels of both enzymes were very low.

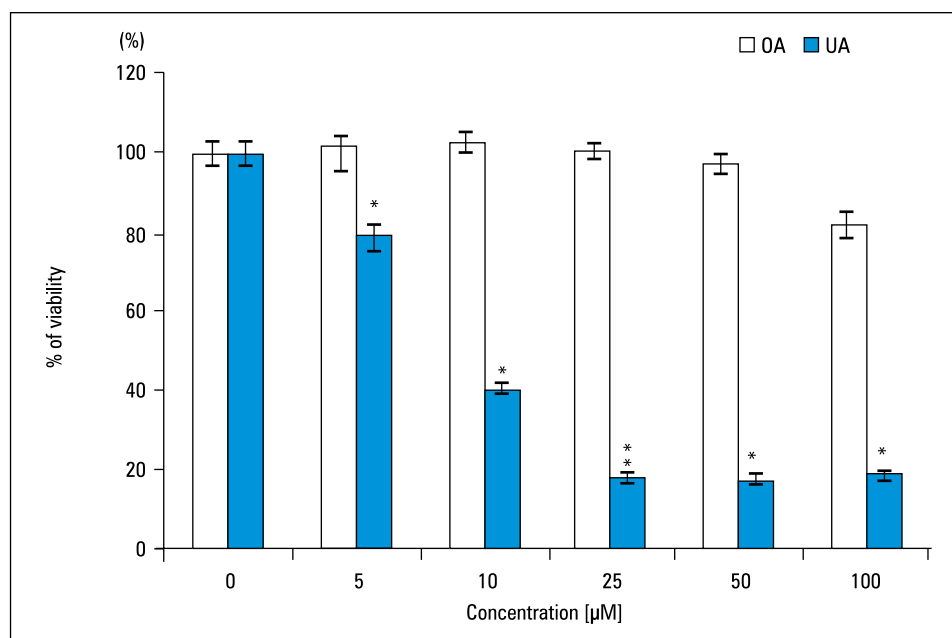


FIGURE 2. The effect of 24-hour treatment of human corneal epithelial cells with oleanolic acid (OA) and ursolic acid (UA). Neutral Red uptake assay. The results are presented as a percentage of the controls, arbitrarily set to 100%. The figure shows an average of three independent experiments. * $p \leq 0.05$ — compared with the control

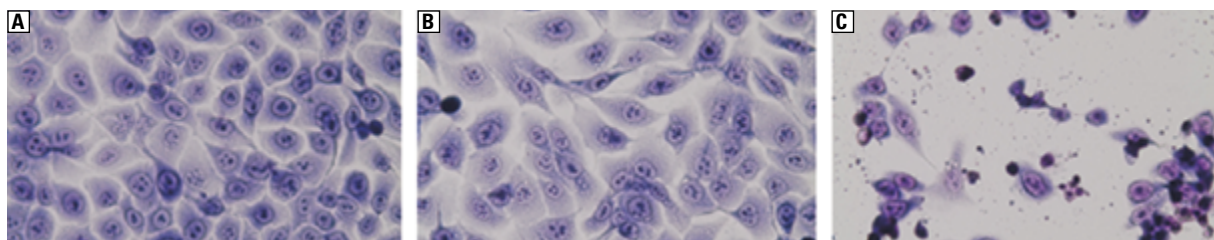


FIGURE 3. May-Grünwald-Giemsa (MGG) staining of corneal (pRSV-T) cells. Control cells (A), cells treated with oleanolic acid (OA) (100 µM) (B), and treated with ursolic acid (UA) (100 µM) (C) for 24 hours. Magnification 200 ×. Bar = 20 µm

The control amount of MMP-2 protein was 0.424 ng/mL. The protein level was decreased when the whole range of OA concentrations was used. The most significant reductions, about three-times as compared to control, were observed when low concentrations of terpenes (5–10 µM) were applied. UA similarly to OA decreased MMP-2 level in a concentration-dependent manner. At the highest concentrations of UA applied (50 µM and 100 µM) the decreased protein level reached values about 30% lower than those observed in controls (Fig. 5).

MMP-9 control amount was 0.572 ng/ml. Application of OA and UA reduced the level of this protein. At the lowest concentration (5 µM) OA reduced the MMP-2 level by half, while at its highest concentration (100 µM) UA reduced this reading to about 40%, as compared to controls (Fig. 6).

Zymography analysis revealed that enzymatic activity could be found only when the highest concentration (100 µM) of both OA and UA was used. However, it was not possible to find separate bands of proteases or their pre-forms, but the whole slots after electrophoresis revealed low gelatine proteolytic activity (Fig. 7).

DISCUSSION

Natural compounds have been widely used to treat different human ailments. Among natural substances showing activity as health reinforcing stimuli, terpenes make up the most important group. Oleanolic (OA) and ursolic (UA) acids are members of pentacyclic triterpenes and are currently considered to have possible beneficial effects on ocular tissues. These compounds have already been shown

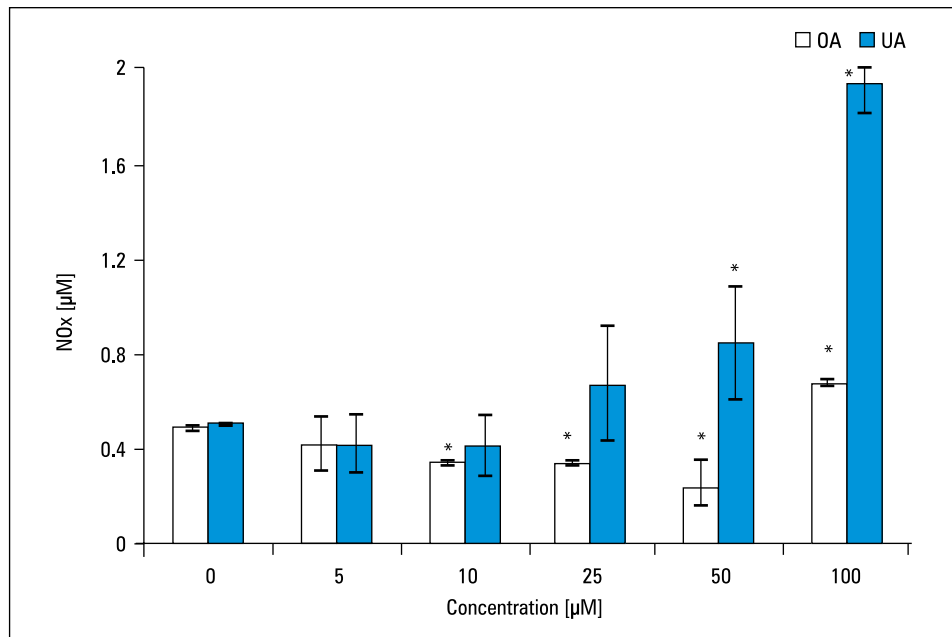


FIGURE 4. The effect of oleanolic (OA) and ursolic (UA) acid exerted on nitric oxide (NO) (nitrite and nitrate — NOx) secretion from human corneal epithelium *in vitro*. Incubation time — 24 hours. The results are presented as mean ± SD of three independent experiments. * $p \leq 0.05$ — compared with the respective control

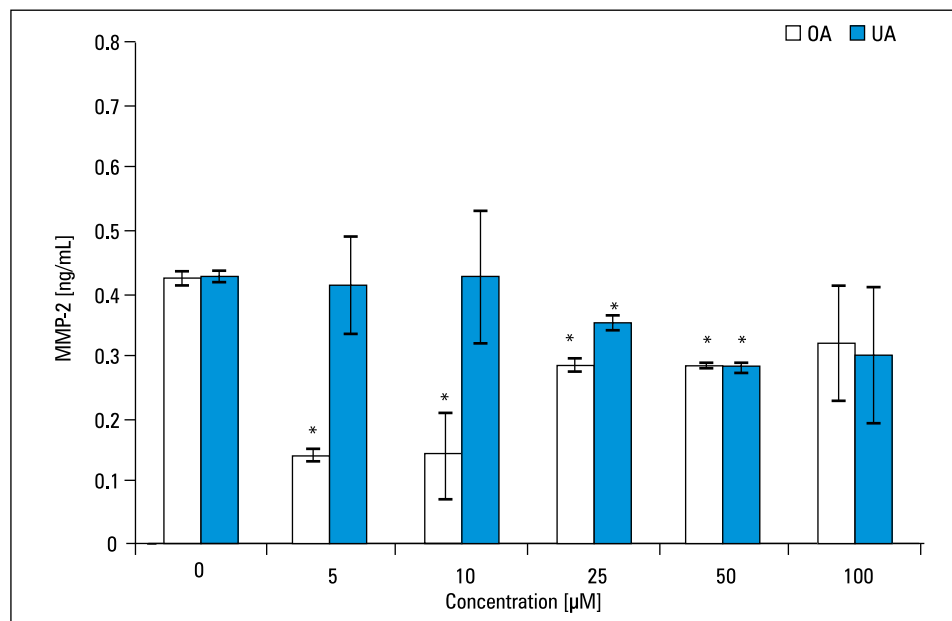


FIGURE 5. The effect of 24-hour treatment of human corneal epithelial cells with oleanolic acid (OA) and ursolic acid (UA). ELISA assay of MMP-2 level. The figure shows an average of three independent experiments. * $p \leq 0.05$ — compared with the control

to have antiallergic properties [3] as well as being useful when uploaded onto nanoplatfoms in anti-inflammatory applications [1]. In our study we showed that, in principle, only oleanolic acid at concentrations lower than 50 µM is not toxic for human corneal epithelial cells. On the other hand, ursolic acid significantly reduced the viability of

cells even at the lowest (5 µM) concentration used. These results are in agreement with literature data showing that UA modulates mitochondrial redox activity and decreases mitochondrial membrane potential; this results in cell cycle phase arrest and consequently apoptosis of cells [11, 12]. Moreover, UA treatment of cells may decrease the anti-apop-

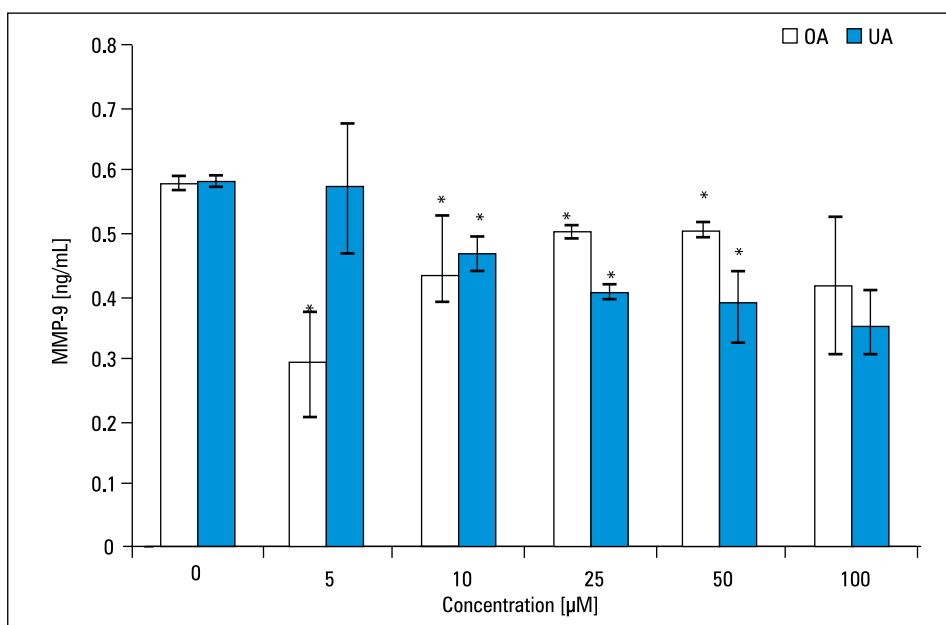


FIGURE 6. The effect of 24-hour treatment of human corneal epithelial cells with oleanolic acid (OA) and ursolic acid (UA). ELISA assay of MMP-9 level. The figure shows an average of three independent experiments. * $p \leq 0.05$ — compared with the control

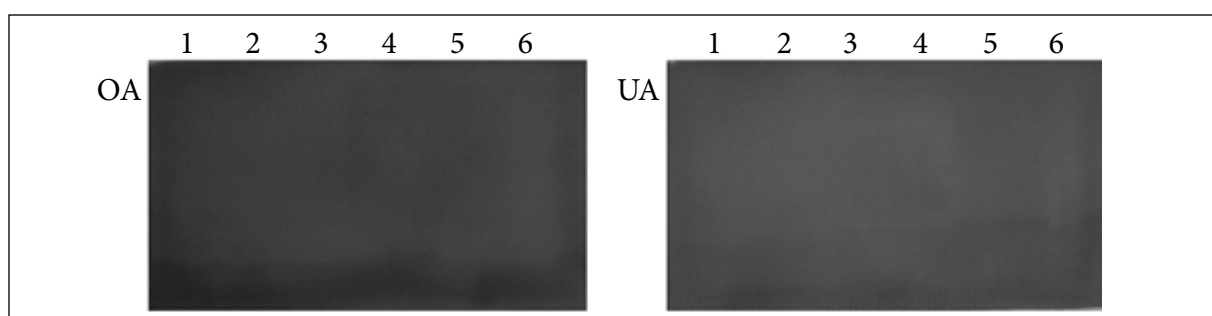


FIGURE 7. SDS-PAGE (gelatin zymography) analysis of metalloproteinases (MMPs) production. Lane 1 — control; lane 2 — 5 μM of the OA/UA; lane 3 — 10 μM of the OA/UA; lane 4 — 25 μM of the OA/UA; lane 5 — 50 μM of the OA/UA; lane 6 — 100 μM of the OA/UA

otic Bcl-2 protein level by its phosphorylation and degradation, but it does not affect pro-apoptotic Bcl-xS or Bax protein amounts. This triterpenoid was also found to activate Fas/FasL pathway and is involved in caspase cascade activation leading directly to apoptotic death of treated cells [12, 13]. In turn, OA expressed protective effects on normal cells against artificially induced oxidative or inflammatory stress. This compound stabilised mitochondrial membranes or decreased COX-2 and consequently PGE₂ production or NADPH oxidase expression [14]. However, the activity of both UA and OA is dependent on the compound concentration. Lower concentrations of OA expressed mainly anti-proliferative effects, but with its increase pro-apoptotic properties were observed depending on the increase in its concentration [15, 16]. These

reports confirm observations made in our study showing higher activity of UA compared with OA on corneal epithelial cells, but simultaneously the impact of both factors on mitochondrial metabolism was stronger than cellular membrane stability and direct viability of cells.

In our study we found that the higher the concentration of triterpenoids, the higher the NO_x level in culture medium. It is consistent with previously published results indicating that both OA and UA stimulate NO release by normal cells. The process was mainly connected with the increased level of inducible nitric oxide synthase (iNOS) activity. The mechanism of such activity has been designated as taking place through NF-κB transactivation and calcium-independent mechanisms. Moreover, triterpenoids upregulated the NO accumulation in

a dose-dependent manner [17, 18]. These results are also closely connected and confirm those respecting apoptosis level because NO is a recognised compound that induces apoptosis through changes in the expression of pro-apoptotic proteins like P53 or Bax [18].

However, there are also reports indicating that OA derivatives or UA may inhibit NOS activity and consequently decrease NO level [19, 20]. Nevertheless, we may also suppose that the increased level of NO observed in our study may be linked with the increased apoptotic cell death, uncoupled metabolic pathways, and release of enzymes or nitrates or nitrites deposited in cells into the culture medium.

Metalloproteinases classified as gelatinases (MMP-2 and MMP-9) are very important because they are able to degrade type IV collagen being the most significant element of extracellular matrix and basement membranes [21]. They are generally produced in small amounts but exhibit high proteolytic activity. After quantitative analysis of MMP-2 and MMP-9 levels we observed that OA and UA decrease the amounts of these proteins. These results are consistent with other authors' findings indicating that these pentacyclic terpenoids significantly reduce the expression of metalloproteinases [21–23]. The mechanism of the action was defined as running through inactivation of JNK, Akt, and mTOR pathways or downregulation of NF- κ B [2, 22, 23]. When zymography was performed we found that only the highest concentration (100 μ M) of both OA and UA expressed gelatinolytic activity. Similarly to NO $_x$, it could be the result of the release of proteins from disintegrating cells undergoing apoptosis. This explanation is also supported by the lack of clearly expressed bands of MMPs activity in zymography performed in our study. Instead of this, the whole slot line showed gelatinolytic activity. Therefore, generally low quantities of MMP-2 and MMP-9 in controls and decreased amounts of these proteins after corneal epithelial cell incubation with OA and UA resulted in a very low, almost undetectable activity of these MMPs measured by zymography.

On the other hand, increased activity of MMP-9 and MMP-2 was observed in lens epithelial cells (LECs) of patients with age-related cataract or in aqueous humour (AH) and uvea in animal models [6, 7, 24]. It suggests that OA or UA activity decreasing MMP levels in eye tissues might be an interesting alternative in the treatment of ocular diseases. The choice of the compound

is also important, and our research indicates that OA may be more useful than UA due to lower concentrations needed to achieve the desired effect. However, further studies are needed to exclude the eventual toxic activities of these triterpenoids or to determine their effective doses for treatment of certain eye tissues.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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