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The effect of ursolic and oleanolic acids on human skin fibroblast cells

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Abstract: In this article, we look at how ursolic and oleanolic acids can be used for the purpose of quality control of natural products used in dermatocosmetology as well as of various other therapeutic preparations. Ursolic acid (UA) and oleanolic acid (OA) are pentacyclic triterpenes and they are constituents of many medicinal herbs. In this study, we analyzed the cytotoxic and anti-proliferative activity of OA and UA against normal human skin fibroblasts (HSF). Additionally, the scavenging activity of free radicals of both acids was analyzed. The sensitivity of cells to OA and UA activity was determined using a standard spectrophotometric (MTT) assay. The free radical scavenging activity of OA and UA was measured using the DPPH$^{\bullet}$ test. The F-actin cytoskeletal proteins organization was analyzed using TRITC-phalloidine fluorescent staining. The cytotoxic activity of the analyzed acids was determined using Neutral Red (NR) uptake assay. Of the two isomeric compounds, UA showed a higher cytotoxic activity against HSF cells than did OA. Our investigations showed that OA, in view of its non-toxic nature, may be used as a supplementary factor for dermal preparations. (*Folia Histochemica et Cytobiologica 2011; Vol. 49, No. 4, pp. 664–669*)

Key words: oleanolic acid, ursolic acid, HSF cell

Introduction

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) and its structural isomer oleanolic acid (3β-hydroxy-olea-12-en-28-oic acid) are natural compounds which are found in various plants, fruits and herbs.

There are many traditional uses of plants containing UA and OA in folk medicine. Herbs containing these acids have been used in the formulation of mainly anti-inflammatory preparations but also as a remedy against hepatotoxic poisons.

In recent years, both compounds have become the subject of widespread interest due to their various pharmacological activities combined with a low toxicity [1]. These triterpenic acids have also been reported as displaying various biological, pharmacological and medical activities such as anti-inflammatory [2], hepatoprotective, gastroprotective [3], anti-HIV [4], cardiovascular, hypolipidemic [5] or immunoregulatory [6]. They have also been found to be active in various stages of tumor development, including inhibition of tumor promotion, invasion and metastasis [7], and have been found to be effective inhibitors of tumor angiogenesis.

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The characteristics of UA and OA have been tested on different types of cells, e.g. leukemic cells (L1210), (K562), (HL-60) [8], macrophages [9], normal human epidermal keratinocytes (NHEK) [10], or human colon carcinoma cell line (HCT15) [11].

In the present work, we analyzed the activity of UA and OA on normal human skin fibroblasts in terms of their potential usefulness in wound healing therapies or as supplementary substances in cosmetics. Our paper describes the toxic and anti-proliferative effects of OA and UA on HSF cells and their free radical scavenging activities.

Material and methods

HSF cell culture. Freshly excised fragments of human skin were washed twice using RPMI (1640) medium (Gibco™, Paisley, UK) supplemented with antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B) (Gibco) and then placed into wells of 24-well plate. The explants were then overlaid with a warm 1:1 (v/v) mixture of 1% agarose and RPMI 1640 medium. The culture was performed by adding culture medium RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Gibco) on top of the agarose gel and incubating at 37°C in a humidified 5% CO_2/95% air incubator. Outgrowths of skin fibroblasts were separated and cultured. For the experiments, HSF cells obtained from two donors were used.

The HSF cells were cultured in 25 cm^2 culture flasks (Nunc, Roskilde, Denmark) in RPMI 1640 medium supplemented with 10% FBS (v/v) and antibiotics at 37°C in a humidified atmosphere with 5% CO_2. The total number of cells was estimated using a hemocytometer. A 100 μL cell suspension (2×10^4 cells/mL for cells proliferation activity tests or 1×10^5 cells/mL for toxicity tests) was added to appropriate wells of 96-well flat-bottomed microtiter plates (MTT and NR methods). After 24 h of incubation, the medium was discarded and a new one added which contained 2% FBS and appropriate concentrations of oleanolic and ursolic acids in the 5–100 μM range. As a control, we used HSF cells suspended in 100 μL of culture medium with 2% FBS without the addition of acids. The total cell number was equivalent to that in the sample wells. Additional controls without cells, but containing appropriate concentrations of acids in 2% FBS culture medium, were prepared to exclude non-specific dye reduction (the MTT method). As a blank control, culture medium with 2% FBS was used.

MTT assay. The sensitivity of cells to the activity of OA and UA was determined using a standard spectrophotometric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells grown in 96-well multiplates in 100 μl of culture medium supplemented with 2% FBS were incubated for 3 h with MTT solution (5 mg/mL, 25 μL/well) (Sigma, St. Louis, MO, USA). The yellow tetrazolium salt was metabolized by viable cells to purple crystals of formazan. The crystals were solubilized overnight in a mixture consisting of 10% sodium dodecyl sulfate (SDS) (Sigma) in 0.01 M HCl. The product was quantified spectrophotometrically by absorbance measurement at a 570 nm wavelength using an E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

Neutral Red (NR) uptake assay. Cells were grown in 96--well multiplates in 100 μL of culture medium (RPMI 1640) supplemented with 2% FBS and various concentrations of oleanolic and ursolic acids (5–100 μM). Subsequently, the medium was discarded and 0.4% NR (Sigma) solution in 2% FBS medium was added to each well. The plate was incubated for 3 h at 37°C in a humidified 5% CO_2/95% air incubator. After incubation, the dye-containing medium was removed, the cells fixed with 1% $CaCl_2$ in 4% paraformaldehyde, and thereafter the incorporated dye was solubilized using 1% acetic acetate in a 50% ethanol solution (100 μL). The plates were gently shaken for 20 min at room temperature and the extracted dye absorbance was spectrophotometrically measured at 540 nm.

Labeling of cytoskeleton F-actin. Cells were incubated in 4-well Lab-Tek chamber slides in 1 mL of culture medium supplemented with 2% FBS and oleanolic or ursolic acids (20 μM). After incubation, the cells were rinsed with RPMI 1640 medium and exposed to paraformaldehyde (10%, v/v) solution for 20 min, rinsed three times with PBS, exposed to Triton X-100 (0.2%, v/v) solution for 5 min and rinsed three times with PBS. 0.5 mL PBS containing tetra-methylrhodamine-isothiocyanate-phalloidin (TRITC-phalloidin, 1 μg/mL) (Sigma) was added to each well; incubation in the dark at 37°C/5% CO_2 for 30 min was accomplished. Cells were observed under a fluorescence microscope (Olympus, BX51). Quantitative analysis of fluorescent images was performed using an AnalySIS imaging software system.

1,1-diphenyl-2-picrylhydrazyl (DPPH•) free radical scavenging test. The free radical scavenging activity of oleanolic and ursolic acids was measured using a DPPH• assay. This method is based on the ability of antioxidants to reduce the stable dark violet radical DPPH• (Sigma) to the yellow-colored diphenyl-picrylhydrazine. Briefly, 100 μl of DPPH• solution (0.2 mg/mL in ethanol) was added to 100 μL of different oleanolic and ursolic acids concentrations (5–100 μM) and standards. Trolox (Sigma) at increasing concentrations (1–50 μg/mL) was used as a reference for the free radical scavenging activity. After 15 min of incubation at room temperature, the absorbance of the solution was measured at 515 nm. The lower the absorbance, the higher the free radical scavenging activity of the analyzed acids. The activity of oleanolic and ursolic acids was determined by comparing their absorbance to that of a blank solution (reagents without acids addition) and a standard.

The capacity to scavenge DPPH• radical was calculated using the following formula:

DPPH• scavenging effect (%) = $[(X_C - X_A/X_C) \times 100]$, where X_C is the absorbance of the control, and X_A is the absorbance in the presence of OA or UA [12, 13].

Results

The cytotoxic and anti-proliferative activities of OA and UA were measured using NR uptake and MTT tests. The experiments with HSF cells seeded at a density of 2×10^4 cells/ml were continued for 72 h (with results read every 24 h) (anti-proliferative activity analysis). The experiments with HSF cells seeded at a density of 1×10^5 cells/ml were conducted for only 24 h (cytotoxicity analysis).

Toxic and anti-proliferative activity of OA and UA, NR and MTT analysis

Figure 1 shows the cytotoxicity of OA and UA on HSF cells. OA revealed no toxic effects on fibroblasts, while UA at concentrations exceeding 20 μM significantly influenced cell viability. IC_{50} values for UA measured by MTT and NR methods were 47.5 μM and 33.7 μM, respectively. The results of the NR method showed that OA in concentration- and time-dependent manner slightly decreased cell viability. The lowest value (53.3% of viability compared to non-treated control) was reached after 72 h of incubation with 100 μM of OA concentration. UA expressed toxic characteristics in concentrations < 5 μM. IC_{50} values after 24 h, 48 h and 72 h of incubation were 18.2 μM; 9.1 μM and 6 μM, respectively (Figure 2). Analy-

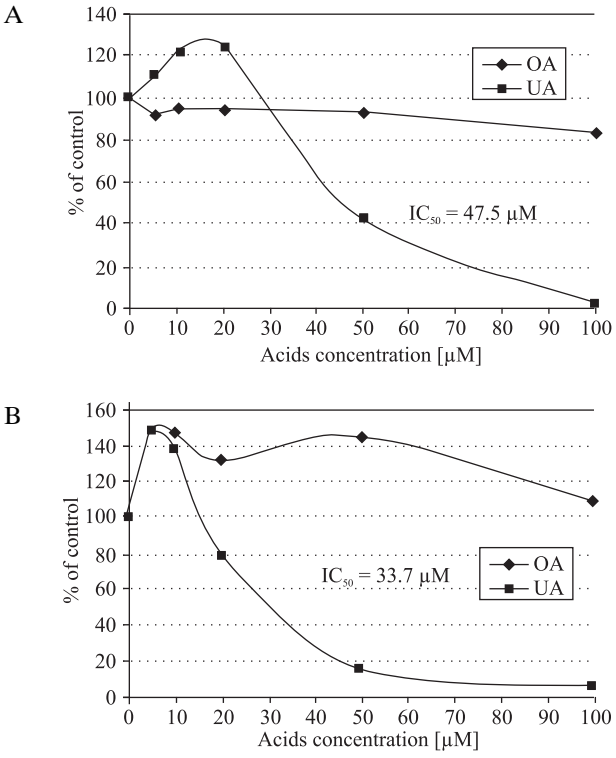

Figure 1. The effect of 24 h treatment of HSF cells (1×10^5 cells/ml) with OA and UA. The MTT assay (**A**) and Neutral Red (NR) uptake assay (**B**). The results of viability are expressed as a percentage of the control arbitrarily set to 100%. UA at concentrations lower than 20 μM was not toxic to HSF cells but at higher concentrations decreased viability of fibroblasts. OA expressed no toxic activities against normal HSF cells

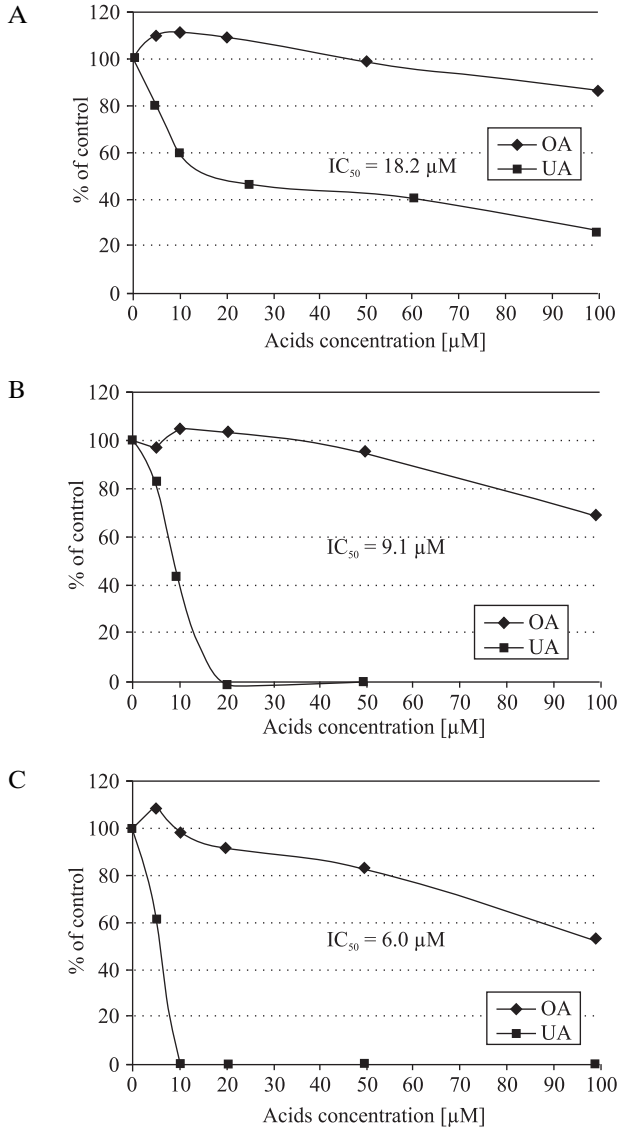

Figure 2. Antiproliferative, dose-dependent effect of OA and UA on HSF cells (2×10^4 cells/ml) after 24 h (**A**), 48 h (**B**) and 72 h (**C**) of culture. The Neutral Red (NR) uptake assay. The results of viability are expressed as a percentage of the control arbitrarily set to 100%. UA in a time-dependent manner decreased viability of HSF cells. OA only at a high concentration (100 μM) influenced on cell membrane permeability

sis performed using the MTT method confirmed these results, with IC_{50} values of 14.1 μM, 11.1 μM and 8.6 μM after 24 h, 48 h and 72 h of incubation, respectively (Figure 3).

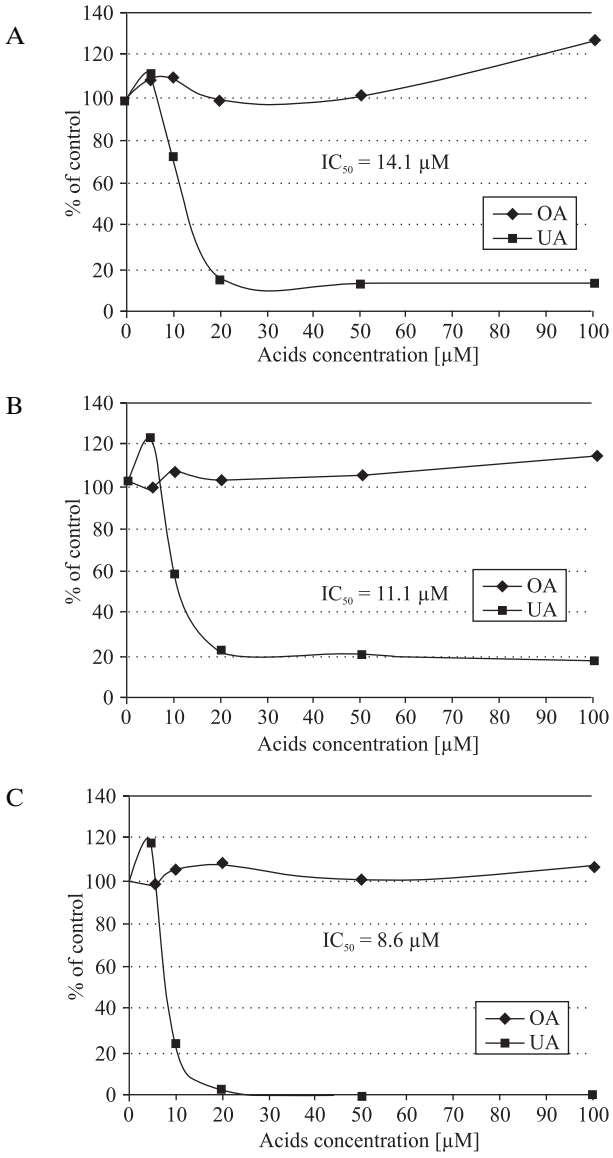

Figure 3. Antiproliferative, dose-dependent effect of OA and UA on HSF cells (2×10^4 cells/ml) after 24 h (**A**), 48 h (**B**) and 72 h (**C**) of culture. The MTT assay. The results of viability are expressed as a percentage of the control arbitrarily set to 100%. UA expressed cytotoxic activities at concentrations exceeding 5 μM while OA was not toxic to HSF cells

Free radical scavenging activity. DPPH method

The results are presented in Table 1. UA at applied concentrations (5; 10; 20; 50 and 100 μM) had a very weak scavenging activity which was 1–2% higher than the control (pure methanol). It was equivalent to the scavenging activity of Trolox at a 0.5 μM concentration.

OA at concentrations up to 50 μM also had a weak scavenging activity which amounted to 2–3% higher than the control, equivalent to 0.9 μM of Trolox activity. However, 100 μM of OA increased the scavenging action by 9.22% above the control, which was equal to 4.45 μM of Trolox activity.

Cytoskeleton organization

F-actin cytoskeletal proteins organization was analyzed using TRITC-phalloidine fluorescent staining. The control cells are presented in Figure 4. OA did not influence cytoskeleton structure (Figure 5), while UA (35 μM) changed the shape of cells and the amount of microfilaments compared to the control (Figure 6).

Discussion and conclusion

Oleanolic acid (OA) and ursolic acid (UA) are widely distributed in the plant kingdom. They occur in

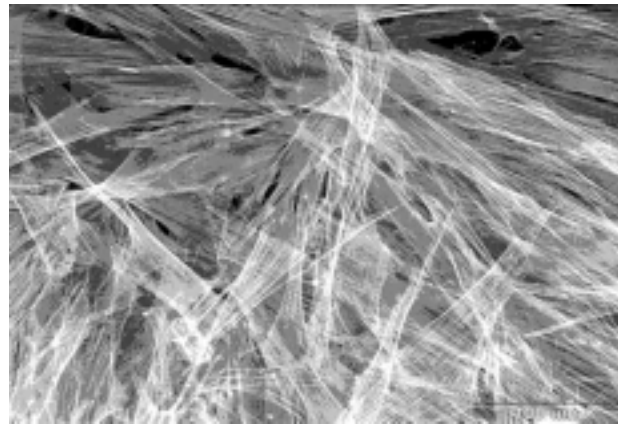

Figure 4. Cytoskeleton organization in HSF cells. The control sample. TRITC-phalloidin fluorescent staining. Bar 100 μm. Magnification × 200

Table 1. DPPH scavenging effect (%). The % of reduced DPPH$^{\cdot}$ radical by oleanolic and ursolic acids is compared to the control (0% of reduction)

Concentration [mM]	5	10	20	50	100
Oleanolic acid	2.68 ± 0.2	2.6 ± 0.2	2.13 ± 0.2	2.76 ± 0.1	9.22 ± 0.5
Ursolic acid	1.65 ± 0.2	1.42 ± 0.2	2.29 ± 0.3	3.23 ± 0.2	2.68 ± 0.3

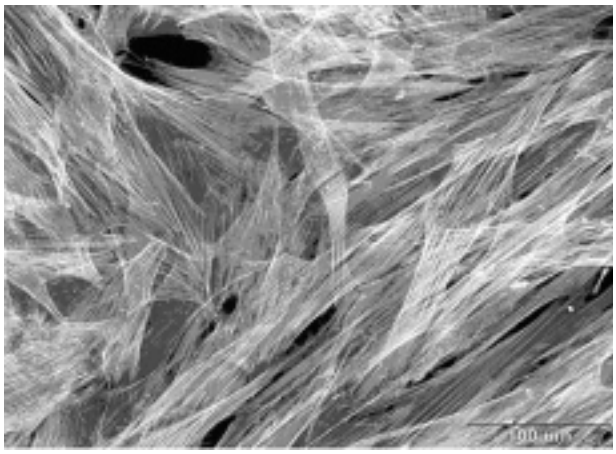

Figure 5. Cytoskeleton organization in HSF cells after 24 h incubation with oleanolic acid at a 35 μM concentration. Bar 100 μm. Magnification × 200

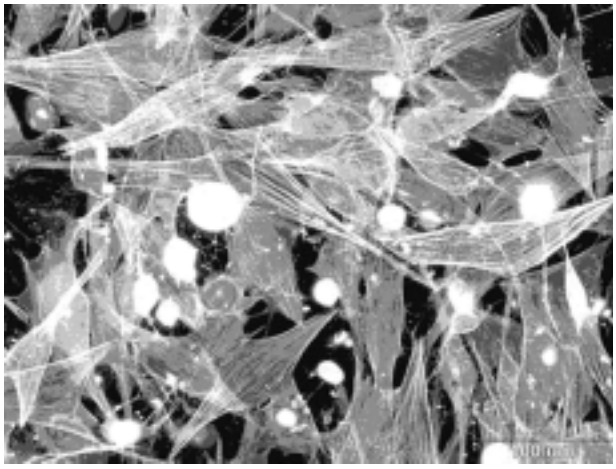

Figure 6. Cytoskeleton organization in HSF cells after 24 h incubation with ursolic acid at a 35 μM concentration. Bar 100 μm. Magnification × 200

different forms such as free acids or aglycones of triterpenoid saponins. These compounds have been shown to exert many pharmacological and medical effects, including hepatoprotective, anti-inflammatory, cardiovascular and anti-tumor [1]. The anti-tumor activities are based on cytotoxic and reactive oxygen species (ROS) scavenging actions. In our study, we analyzed whether these activities also concern, in an equal degree, normal human skin fibroblasts (HSF). We tried to answer the question as to whether UA and/or OA may be used as supporting factors in dermal preparations.

We found that UA was toxic for HSF cells in concentrations exceeding 5 μM. This effect was not observed in the case of OA. Moreover, while both acids had a slight antioxidant activity, OA at a concentration of 100 μM exerted significantly increased scavenging action. Our results confirmed the observations of Sohn et al. [14] who showed that the IC_{50} values of anti-proliferative effect against normal endothelial cells were determined to be 5 μM for UA and 20 μM for OA. We, however, were unable to establish this value for OA. The pharmacological properties of pentacyclic triterpenes have mainly been tested on tumor cells. UA and its esterified derivatives have been reported to possess strong cytotoxic activity against some tumor cell lines [15]. They inhibit the proliferation of breast, leukemia, prostate, liver, colon and skin cancer cells. This effect is associated with interaction of DNA enzyme synthesis changes in cell-cycle-related or apoptosis-associated molecular pathways [16]. We suggest that a similar effect also appears in normal cells after UA treatment.

We revealed that UA action against normal cell viability strongly depends on cell culture density, and therefore on the direct cell-cell interactions. The toxic properties of UA increased in a dose-dependent manner, and in concentrations above 20 μM, cell viability was decreased. At low culture density, cytotoxic effects appeared only at a UA concentration of 5 μM. Therefore, when considering the biological activity of UA and OA, special attention should be paid to the kinds of tested cells, their density in culture, and terpenoid acids concentrations.

Besides anti-tumor effects, pentacyclic triterpene acids may express anti-inflammatory and antioxidant activities. Oh et al. [17] indicated that UA may regulate apoptosis through scavenging of ROS. These highly reactive radicals are produced continuously in human cells and derive from endogenous and exogenous sources. We did not find ROS scavenging activity by UA at concentrations up to 100 μM. This finding confirmed the results of Ramos et al. [18] which indicated that highly lipophilic UA did not protect the DNA of HepG2 cells, a phenomenon which could be linked to its low free radical scavenging activity. In our study, only OA at a relatively high concentration (100 μM) expressed significant ROS scavenging effects.

In conclusion, we showed that for the treatment and health of normal tissues, OA, but not UA, should be used. Because of its non-toxic nature, OA may be used as a supplementary factor for dermal preparations. However, its immunomodulatory activity should be tested by using OA in skin wound healing therapies.

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