

Triterpenoid α -amyrin stimulates proliferation of human keratinocytes but does not protect them against UVB damage

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***Rhaponticum carthamoides* plants ("maral root") are widely used in Siberian folk medicine. The present study reports for the first time the presence of pentacyclic terpenoid, α -amyrin, in methanol extract from leaves of this plant. α -Amyrin induced proliferation of human keratinocytes (HaCaT) by about 18% while other extract components were ineffective. A panel of biochemical and cell-based assays testing the antioxidative and cytoprotective activities of α -amyrin indicated no antioxidative activity of this compound. α -Amyrin did not protect HaCaT cells against the damage caused by UVB radiation.**

Key words: α -amyrin, *Rhaponticum carthamoides*, BrdU incorporation, antioxidant activity, UVB

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INTRODUCTION

Plants are a rich source of biologically active compounds used in the cosmetic and pharmaceutical industry. Introducing a plant-based product to the market, however, has to be preceded by a thorough analysis of its activity and potential toxicity. The traditional use of a specific plant often suggests the direction of research. The starting point is usually the screening of activities of a crude plant extract, followed by the identification of active constituents.

Many plant-derived compounds modulate cell proliferation, acting as cytotoxic agents (i.e., potential antineoplastic drugs) or by stimulating cell growth (i.e., potential application in wound healing). Herbal remedies have long been reported as biologically active agents promoting wound healing and used for treatment of skin disorders. Their ability to stimulate cell proliferation (Brunold *et al.*, 2004), differentiation of keratinocytes (Lee *et al.*, 2007), collagen synthesis (Aslam *et al.*, 2006) and to alleviate psoriasis symptoms (Reuter *et al.*, 2010) has been described.

Another interesting aspect is the antioxidative potential of plant-derived preparations and compounds. Reactive oxygen species (ROS) play a role in physiological processes such as cell signalling (Thannickal and Fanburg, 2000) or in the microbicidal activity of phagocytes (Babior 1984). However, if present in excess, ROS may cause cell damage by reacting with nucleic acids, membrane lipids and proteins (Muller & Gurster, 1993; Leeuwenburgh *et al.*, 1998), and in the longer run might be associated with initiation or promotion of diseases like cancer or atherosclerosis (Kehrer 1993). Hence, plant-

originated antioxidants may be valuable as potential cytoprotective agents.

Rhaponticum carthamoides (Willd) Ilijin, a perennial plant belonging to the *Compositae* (*Asteraceae*) family, has been widely used in Siberian folk medicine, mostly to treat overstrain and common weakness after illnesses. Recently it gained attention as a rich source of ecdysteroids, compounds with potentially adaptogenic and anabolic activity (reviewed by Yancey, 2004). In addition to ecdysteroids, several other biologically active compounds have been isolated from *R. carthamoides*, including flavonoid glycosides, polyphenolic acids (Sharaf *et al.*, 2001; Miliauskas *et al.*, 2005), *N*-feruloserotonin isomers (Pavlik *et al.*, 2002) and thiophene polyacetylenes (Chobot *et al.*, 2003). A number of preparations based on *R. carthamoides*-derived compounds, mainly designed for sportsmen, are commercially available. An *R. carthamoides* plantation has recently been established in Lubiewice, Poland by FITOSTAR® (Biskup *et al.*, 2007).

Since *R. carthamoides* is highly interesting from the pharmacognostic point of view, we analyzed biological activities of crude *R. carthamoides* extracts, extract fractions and pure compounds present in *R. carthamoides* leaf tissue (Biskup & Łojkowska, 2009). The present study documents for the first time the presence of a triterpenoid α -amyrin in *R. carthamoides* leaf tissue extract.

α -Amyrin is a pentacyclic terpenoid synthesized by a number of plants, i.e. *Callicarpa acuminata* H. B. K. (*Verbenaceae*) from Mexico, *Protium heptaphyllum* (*Burseraceae*) from the Amazon, *Carmona retusa* (*Boraginaceae*) found in Philippines, and many more (Anaya *et al.*, 2003; Villaseñor *et al.*, 2004; Oliveira *et al.*, 2005). This compound is usually found in plant extracts together with its isomer β -amyrin, differing in the placement of one methylene group (C-19 and C-20, respectively).

It has been demonstrated that a mixture of both isomers produces pronounced antinociception in the capsaicin test (decreased face rubbing behavior in rats), (Holanda Pinto *et al.*, 2008). The analgesic effect was observed only in the case of second phase reaction, corresponding to inflammatory pain rather than to acute neurogenic pain (Holanda Pinto *et al.*, 2008). On the other hand, the anti-inflammatory activity of these compounds

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Abbreviations: BrdU, bromodeoxyuridine; c-H₂DCF-DA, carboxy-2',7'-dichlorodihydrofluorescein diacetate; CPDs, cyclopyrimidine dimmers; DMEM, Dulbecco's Minimal Essential Medium; DPPH, diphenyl 1-picrylhydrazyl free radical; FCS, fetal calf serum; FRAP, ferric reducing antioxidant power; GC-MS, gas chromatography-mass spectrometry; ROS, reactive oxygen species.

measured in carrageenan-induced mouse paw edema test seemed to be very low (Villaseñor *et al.*, 2004). A mixture of α - and β -amyrin isomers protects liver against acetaminophen-induced injuries (Oliveira *et al.*, 2005) and ameliorates L-arginine-induced acute pancreatitis (Melo *et al.*, 2010). α -Amyrin esters were also reported to exhibit significant biological activity, namely cytotoxic (Barros *et al.*, 2011) and anti-inflammatory (Akihisa *et al.*, 2010).

Having confirmed the presence of α -amyrin in *R. carthamoides* leaf tissue, we focused on analyzing its biological activities.

MATERIALS AND METHODS

Plant material and extraction procedure. *Raponticum carthamoides* leaves were obtained from the FITO-STAR[®] plantation in Lubiewice (Kujawsko-pomorskie vojvodship), Poland. Voucher specimens are deposited at the Intercollegiate Faculty of Biotechnology, UG and MUG. Plant secondary metabolites were extracted by 30-min. sonication (PolSonic) in methanol (1g dry leaves powder in 20 ml). Plant residue was removed by filtration through double Whatman filter paper (no. 1) and methanol was evaporated, using a vacuum rotary evaporator. Extract was weighed and dissolved in methanol again (4 ml).

Detection of amyirin by gas chromatography combined with mass spectrometry (GS-MS). Samples were dried under nitrogen and silylized with 100 μ l of a mixture of bis(trimethylsilyl)acetamide and chlorotrimethylsilane (99:1; Sigma Aldrich) for 24 h at 25°C. The analyses were carried out on a Clarus 500 (Perkin Elmer) gas chromatograph equipped with a split ratio of 1:30 for the injection port and direct connection to a FID. An RTX 5 column (30 m \times 0.25 mm i.d., film thickness 0.1 μ m) was used. The carrier gas was argon. For reliable analysis, the injector and detector temperatures were 310°C. The following temperature program was applied: 100–310°C, rate 4°C/min. Mass spectra (70 eV) were recorded on a SSQ 710 mass spectrometer (Finnigan). The samples were introduced through a Hewlett-Packard 5890 gas chromatograph equipped with the same columns and under the same chromatographic conditions as for the GC analysis but with helium as carrier gas. The ion source was maintained at 220°C. The identification α -amyirin was based on the mass spectrum and retention time.

Cell culture conditions. HaCaT cells (spontaneously immortalized human keratinocytes, originally provided by Dr. M.R. Pittelkow; Mayo Clinic, Rochester, MI, USA) were grown in DMEM GlutaMAX (Gibco) medium, supplemented with 10% fetal calf serum (FCS; Gibco). After reaching about 70–80% confluence cells were harvested by trypsinization and seeded at a density 10⁵ cells per flask. The medium was exchanged twice a week.

Methylene blue assay (Faurischou *et al.*, 2008). Cells were seeded in 24-well plates at 10⁵ cells/well and let to attach overnight. α -amyirin (Sigma), dissolved in 2-propanol, was added to the final concentration of 10, 20 or 40 μ g/ml and cells were incubated for 24 or 48 h. Final 2-propanol concentration was 0.8% v/v in all assays, and it did not affect cell viability. After time periods indicated cells were fixed in 0.4% formaldehyde and stained with methylene blue (Merck) for 20 min. Cells were then washed with distilled water and lysed in 0.1 M HCl and relative protein content was determined spectrophotometrically at the 595 nm.

Clonogenic assay (Faurischou *et al.*, 2008, modified). Cells were seeded in 6-cm diameter Petri dishes at a density of 200 cells/dish and let to attach overnight. α -Amyrin (Sigma), dissolved in 2-propanol, was added to the final concentration of 5, 10 or 20 μ g/ml and cells were incubated for 10 days. After time periods indicated cells were fixed in 0.4% formaldehyde and stained with methylene blue (Merck) for 20 min. Cells were then washed with distilled water and colonies were counted.

Bromodeoxyuridine (BrdU) incorporation assay. Cells were seeded in 6-well plates at 4 \times 10⁵ cells/well and let to attach overnight. After 24 h of incubation with α -amyirin (10, 20, 40 μ g/ml) cells were pulsed for 20 min with 10 μ M BrdU and fixed in ice-cold 70% ethanol for at least 20 hr. Then, cells were washed with PBS, incubated in 2 M HCl for 30 min, washed in neutralizing buffer (0.2 M Na₂B₄O₇, pH 8.5) and resuspended in dilution buffer (0.5% Tween 20 and 0.5% bovine serum albumin in PBS) prior to the addition of anti-BrdU mouse antibodies (Becton Dickinson). Goat anti-mouse antibodies labeled with Alexa-Fluor 488 (1:1000; Invitrogen) were applied as secondary antibodies. DNA was stained with 7-amino-actinomycin D (7AAD; Beckman Coulter). The percentage of BrdU-positive cells was determined by flow cytometry analysis (Beckman Coulter). BrdU incorporation rate (FL1) was plotted versus cellular DNA content (FL3) using Cell Lab QuantaTM software.

Antioxidant activity

DPPH assay. The ability of α -amyirin to scavenge the diphenyl 1-picrylhydrazyl (DPPH) free radical was quantified as described by Konczak-Islam *et al.* (2003). Results are expressed as IC₅₀, which is the concentration of the analyte able to scavenge 50% of the DPPH free radical. Ascorbic acid (Sigma) and α -tocopherol (MP Biochemicals, Inc.) were used as positive controls (hydrophilic and hydrophobic, respectively).

FRAP assay. Total antioxidant potential of α -amyirin was evaluated using the FRAP assay (ferric reducing antioxidant power), according to the protocol of Benzie and Strain (1996). In this assay ability of a compound to reduce ferric (Fe³⁺) to ferrous (Fe²⁺) ions is estimated, with the use of an indicator dye (2,4,6-tripyridyl-*S*-triazine). Results are expressed as IC₅₀, which is the concentration of the analyte that reduces 50% of the ferric ions. Ascorbic acid and α -tocopherol were used as positive controls

Scavenging of intracellular reactive oxygen species (ROS). Cells were seeded in 24-well plates at 10⁵ cells/well and let to attach overnight. After 2-hr incubation with α -amyirin (final concentration 20 μ g/ml) cells were washed with PBS and loaded with 50 mM carboxy-2',7'-dichlorodihydrofluorescein diacetate (c-H₂DCF-DA; Sigma) dissolved in DMSO (final DMSO conc. 0.5% v/v) for 30 min. Then cells were washed and ROS generation was induced by exposition to UVA radiation (5 kJ/m² for 15 min.). ROS generation was measured indirectly as the fluorescence of oxidized derivative of c-H₂DCF (485/538 nm Ex/Em), by a microplate reader (Wallace Victor 1420) and expressed as a fold increase calculated versus the control (vehicle-treated non-irradiated cells).

Protection against UVB irradiation

Cells were seeded in 60-mm diameter Petri dishes at the density 10⁵ cells/plate (for cyclopuridine dimers – CPDs — measurement) or in 24-well plates at 10⁴

Table 1. Influence of α -amyrin on proliferation of HaCaT cells, measured as changes in total protein content (methylene blue assay), ability to form cell colonies following treatment (clonogenic assay) and to incorporate BrdU (measured by means of flow cytometry, with the use of specific antibodies).

Concentration of α -amyrin ($\mu\text{g/ml}$)	Total protein content (% of control)		Colony forming ability (% of control)	BrdU incorporation	
				(% total)	(% of control)
	Incubation time				
	24 h	48 h	10 days	24 h	
0	100 \pm 9.0	100 \pm 6.0	100 \pm 8.6	48.8 \pm 3.2	100.0 \pm 6.6
5	NA	NA	99.4 \pm 7.0	52.9 \pm 0.8	108.4 \pm 1.6
10	110.9 \pm 14.3	107.4 \pm 7.9*	110.3 \pm 7.4	56.3 \pm 3.6*	115.3 \pm 7.3*
20	111.0 \pm 13.8*	116.9 \pm 9.1*	100.1 \pm 5.2	57.8 \pm 1.1*	118.4 \pm 2.2*
40	104.1 \pm 5.5	117.4 \pm 7.5*	NA	NA	

Asterisk (*) indicates a value statistically different from the control ($p < 0.05$); NA, not analyzed

cells/well (for caspase 3/7 activation measurement) and let to attach overnight.

α -Amyrin (20 $\mu\text{g/ml}$) was added to the culture medium 2 h before the UVB irradiating or (in the case of apoptosis assay) also immediately after treatment. Cells were irradiated with UVB light at an intensity of 10 mJ/cm^2 (for CPDs measurement) or 20 mJ/cm^2 (for caspase 3/7 activation measurement).

CPDs quantification by ELISA. Genomic DNA was isolated either immediately after irradiation or 24 h later, using Invisorb Spin Cell Mini Kit (Invitek). DNA concentration and purity was estimated spectrophotometrically (NanoDrop® ND-1000). Subsequently CPDs were detected by ELISA with the use of specific antibody, as described elsewhere (Faurichou *et al.*, 2008). Briefly, DNA samples were denaturated at 100°C for 10 min and then rapidly chilled on ice. Denaturated DNA solution (1 μg) was transferred into a 96-well plate precoated with 0.003% protamine sulphate. Plates were incubated in 37°C until completely dry, rinsed with distilled water thrice and dried again.

Unspecific antibody-binding sites were blocked with 2% FCS solution and anti-CPDs antibody was added (MBL International). After washing, the plates were incubated with biotin-F(ab)² fragment of anti-mouse IgG (H₁L) (Invitrogen) followed by treatment with peroxidase-streptavidin (Invitrogen) and incubation with the α -phenylene diamine substrate solution. Reaction was stopped by the addition of 2 M H₂SO₄. The absorbance was determined at 492 nm (Wallace Victor 1420).

Quantification of apoptosis. After UVB treatment cells were incubated for 4 hr in the DMEM medium without phenol red (Gibco). UVB-incuded cell death was quantified with the Caspase-Glo 3/7 (Promega Corporation) kit according to manufacture's instructions. Luminescence was measured using a microplate reader (Wallace Victor 1420).

Statistical analysis. All experiments were performed in triplicates. One-way analysis of variance was performed with the use of GraphPad Prism 4 program, and $p < 0.05$ value was considered as statistically significant.

RESULTS AND DISCUSSION

In the present work α -amyrin production in *R. carthamoides* plant tissue is documented for the first time. The mass spectrum of the trimethylsilyl derivative showed characteristic ions at m/z 189 and 203 in addition to the base peak at m/z 218 and a molecular ion at m/z 498

(Fig. 1). The amount of α -amyrin detected by means of GC-MS analysis was 597.55 \pm 38.2 $\mu\text{g/g}$ dry weight.

The aim of the study was to elucidate if the biological activity of *R. carthamoides* extracts may be attributed to the presence of α -amyrin. According to the literature, α -amyrin can be considered cytotoxic (Chaturvedula *et al.*, 2004; Chung *et al.*, 2009). However, contrary to expectations, our preliminary investigations indicate its ability to actually stimulate cell proliferation.

Subsequently we investigated the effect of α -amyrin on survival and proliferation of eukaryotic cells, as well as its antioxidant potential and ability to protect against cell damage by UVB radiation.

HaCaT cells, non-tumorigenic, spontaneously immortalized human keratinocytes were used as a model. They are both easy to maintain and closely resemble normal keratinocytes. Even after multiple passages HaCaT cells retain the ability to proliferate and differentiate (Boukamp *et al.*, 1988). These characteristics make them a valuable model for studying the ability of the tested compounds to induce wound-healing processes.

In the first part of our studies, testing the direct α -amyrin effect on cell growth, three methods were applied: methylene blue assay (in order to screen for potential α -amyrin cytotoxicity), clonogenic assay (to check the long-term effect on cell growth) and BrdU incorporation analyzed by flow cytometry (to estimate cell proliferation rate).

The short-term (24 h and 48 h) influence of α -amyrin on cell growth and survival was tested with the methylene blue assay. There was no decrease in the cell viabil-

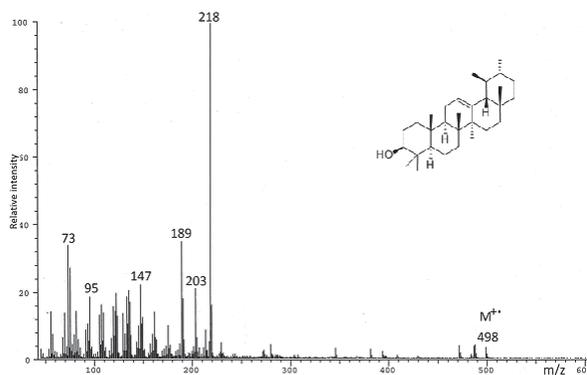


Figure 1. Mass spectrum (70 eV) of trimethylsilyl derivative of α -amyrin ($\text{C}_{30}\text{H}_{50}\text{O}$), from *R. carthamoides* leaves. Inset: structure of α -amyrin.

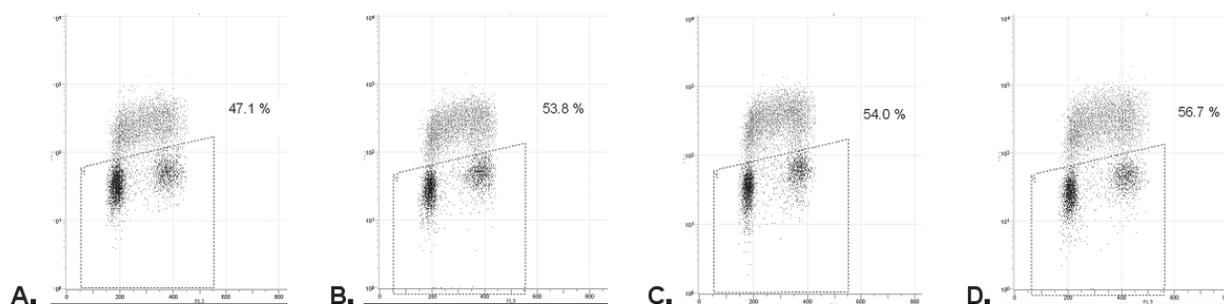


Figure 2. α -Amyrin influence on DNA synthesis measured by BrdU incorporation by HaCaT cells.

Result of one representative experiment is shown. Percentage of BrdU-positive cells in: (A) vehicle control (0.8% propanol) (B) 5 μ g/ml α -amyrin, (C) 10 μ g/ml α -amyrin, (D) 20 μ g/ml α -amyrin.

ity within the range of α -amyrin concentrations tested; from 10 to 40 μ g/ml (Table 1). On the contrary, after a 48-h incubation a statistically significant ($p < 0.05$) enhancement of cell growth was observed (Table 1).

The potential toxic/growth inhibitory effect of a long-time (10 days) α -amyrin treatment was tested using the clonogenic assay. No perturbations in colony forming were observed over the whole experiment (Table 1).

In order to confirm ability of α -amyrin to stimulate cell proliferation, observed previously in the methylene blue assay, the BrdU incorporation rate by HaCaT cells was analyzed by flow cytometry. In accordance with earlier findings HaCaT cell growth increase by ca. 15–18% was observed after 24 h of incubation with α -amyrin (Table 1 and Fig. 2). At the same time no alterations in cell cycle distribution were observed (data not shown).

An ability to stimulate growth of keratinocytes (as well as fibroblasts) by plant-derived components was observed earlier for *Phyllanthus muellerianus* (Kuntze) Exell aqueous extract and its active constituents, geraïin and furosin. The rate of BrdU incorporation by cells grown in medium with reduced serum content was almost doubled following geraïin or fucosin treatment, with cell viability being ca. 40% higher, compared to the control. The same compounds promoted keratinocyte differentiation, upregulating the synthesis of cytokeratins and involucrin (Agyare *et al.*, 2010). Promotion of keratinocytes growth was also caused by a pomegranate seed oil (Aslam *et al.*, 2006) and acidic subfractions of *Hibiscus sabdariffa* L. polysaccharides (Brunold *et al.*, 2004). The postulated mechanism was activation of membrane-associated growth receptors, eg. KGFR, EGFR InsR (Brunold *et al.*, 2004).

Various terpenoids were also reported to influence cell proliferation. The triterpenoid uvaol (at low doses,

ca. 12.5 μ M) and maslinic acid (up to 100 μ M) increased the proliferation rate of MCF-7 cells by between 20 and 40%. A marked growth stimulation was seen already after 24 h of incubation (Allouche *et al.*, 2011). Lupeol, isolated from *Leptodenia hastate*, and its ester derivatives, i.e. lupeol hemisuccinate and, to a lower extent, lupeol palmitate and acetate had a stimulatory effect on human epidermis cell proliferation (Nikiéma *et al.*, 2001).

It should be mentioned that *R. carthamoides* extracts exhibit a significant antioxidative potential (Koleckar *et al.*, 2010) and hence the antioxidative activity of the triterpenoid α -amyrin was evaluated. Two biochemical assays (DPPH and FRAP) were applied and a cell model, in which the ability of α -amyrin to scavenge intracellular ROS induced by UVA irradiation was analyzed. Ascorbic acid, a well-known antioxidant, was used as a positive control. Both those biochemical assays gave negative results, indicating that α -amyrin exhibits no direct antioxidant activity (Table 2). This is not surprising, as α -amyrin lacks structures mostly related to the antioxidant activity of a compound, such as *o*-dihydroxybenzene (catechol) structures (present for example in flavonoids; Lu & Foo, 2001) or 6-hydroxychroman rings (found in tocopherols; Prieto *et al.*, 1999). At the same time the level of intracellular ROS, induced by UVA irradiation, was not decreased following α -amyrin pre-treatment. This observation confirms that α -amyrin cannot interact directly with ROS. On the other hand, some compounds not recognized as potent antioxidants (eg. sesquiterpene lactones), are able to alter the cell redox status indirectly by influencing cellular antioxidant mechanisms. The latter may be achieved for example by inducing such pathways as Nrf2/ARE (Umemura *et al.*, 2008) or modifying the activity of antioxidant enzymes like glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT) or su-

Table 2. Antioxidant activity of α -amyrin analyzed with the use of biochemical assays (DPPH and FRAP) and a cell model (scavenging of intracellular ROS induced by UVA irradiation)

Method applied	DPPH assay	FRAP assay	Intercellular ROS scavenging
Compound analyzed	IC ₅₀ (μ g/ml)	IC ₅₀ (μ g/ml)	Fold increase vs. control (non-irradiated cells)
α -amyrin	>150	>150	2.42±0.18
ascorbic acid (hydrophilic positive control)	5.6±4.7	3.9±0.5	2.09±0.15 *
α -tocopherol (hydrophobic positive control)	10.8±3.6	20.9±2.8	ND ^a
vehicle treated, irradiated cells (negative control)	—	—	2.38±0.04

Asterisk (*) indicates a value significantly different from the control ($p < 0.05$). ^adue to the low α -tocopherol solubility in cell medium, its ROS scavenging potential was not determined

peroxide dismutase (SOD) (Jodynis-Lieber *et al.*, 2000). These aspects of α -amyirin activity, however, require further examination.

A number of plant compounds, including some terpenoids (eg. certain dicyanoterpenoid derivatives or triterpene saponines), have been found to protect eukaryotic cells against damage caused by UVB radiation (Cai *et al.*, 2008; Dinkova-Kostova *et al.*, 2008). Therefore in the next set of experiments we tried to find out if α -amyirin could also exhibit such capacity. First, we assessed the ability of α -amyirin to limit the number of cyclo-pyrimidine dimers (CPDs) formed as a consequence of UVB treatment. Secondly, we analyzed apoptosis induction, measured as caspase 3/7 activation resulting from exposing cells to UVB. The obtained data indicate that α -amyirin failed to decrease CPDs formation whether the cells were collected immediately after irradiation (analysis of direct, passive protection, usually attributed to light absorption) or 24 hours later (analysis of indirect involvement in repair mechanisms; data not shown).

Similarly, α -amyirin did not prevent apoptosis induced by UVB radiation when cells were pretreated with α -amyirin before irradiation or when it was added immediately after UVB treatment. An about four-fold increase of caspase 3/7 activity was observed in cells exposed to UVB versus non-irradiated cells: respectively, 3.65 ± 0.21 , 3.67 ± 0.39 and 3.61 ± 0.22 for the vehicle control (0.8% isopropanol), cells preincubated for 2 h with 20 $\mu\text{g}/\text{ml}$ α -amyirin and cells treated with 20 $\mu\text{g}/\text{ml}$ α -amyirin directly after irradiation.

Summarizing, α -amyirin induced proliferation rate of human keratinocytes (HaCaT) by up to 18% and therefore it is an interesting candidate for use in cosmetic industry as an agent promoting wound healing and skin regeneration. However, more detailed analysis should be performed in order to further examine the mechanism of its action and its cellular targets, effect on cell differentiation markers expression, as well as the effect on other cell lines. At the same time it was demonstrated that α -amyirin has no apparent antioxidant activity nor does it protect against UVB light.

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