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IN VITRO EFFICACY STUDY
EVALUATION OF CELL PROLIFERATION AND PROTECTION FROM ROS AFTER TREATMENT WITH COSMETIC PRODUCT ON CELL CULTURE

RIVOLI COSMETIQUES SA

L'EAU DE NUIT
Lab-01564.17
18.01.2023

COMPLIFE Italia S.r.l.
info@complifegroup.com
complifeitalia@legalmail.it
complifegroup.com

Sedi principali:
GARBAGNATE MILANESE (MI), Via Guido Rossa 1, 20024 | +39.02.990.25138
SAN MARTINO SICCOMARIO (PV), Via Monsignor Angelini 21, 27028 | +39.0382.25504

Sede legale: Via Guido Rossa, 1 20024 Garbagnate M.se (MI) Italy, | P.I/C.F. 11093320155
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KEY PERSONNEL

Customer

RIVOLI COSMÉTIQUES SA
14 Rue de L'arquebuse
1204 Genève
Switzerland

Experimenter

Dott. Andrea POGGI
Biologist
Complife Italia S.r.l

Study Director

Dott.ssa Silvana GIARDINA
Biologist
Complife Italia S.r.l

Complife Italia srl

Location
Via Angelini, 21
27028 San Martino Siccomario (PV)
Italy
tel. +39-0382 25504 - fax +39-0382 536006
Mail: info@complifegroup.com

REPORT CHANGE RECORD

The table here below reports the change log of all approved changes made to the document that make up the course after initial approval.

Rev. no	Date	Description
00	31/03/2023	Draft report release
00	25/07/2023	Final report release

STUDY DESIGN

1.1 Title

In Vitro efficacy study – evaluation of cell proliferation and ROS after treatment with a cosmetic product on cell culture

1.2 Aim the study

Evaluation of cell proliferation and ROS analysis after treatment with 3 different concentrations of a product.
The considered cell system is represented by:

- Human skin keratinocytes

1.3 Tested product

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Sample description
The tested product is a cosmetic with the following INCI composition declared by the Sponsor

Name (INCI)	GLOB

AQUA (WATER), PROPANEDIOL, 1,2-HEXANEDIOL, PANTHENYL TRIACETATE, GLYCERIN, BETA VULGARIS (BEET) ROOT EXTRACT, POLYGLYCERYL-4 CAPRATE, STEVIOSIDE, PENTYLENE GLYCOL, PARFUM (FRAGRANCE), TAMARINDUS INDICA SEED GUM, SODIUM CITRATE, CITRIC ACID, HABERLEA RHODOPENSIS LEAF EXTRACT, YEAST EXTRACT, EPIGALLOCATECHIN GALLATYL GLUCOSIDE, CI 42090 (BLUE 1), CI 60730 (VIOLET 2)

1.4 Dates

Experimental session: From 20/03/2023 to 30/03/2023
Data analysis: 31/03/2023

2.MATERIALS AND METHODS

2.1 Monitored experimental conditions

The evaluation of the modulation of cell proliferation was carried out by using cell cultures in the G0 phase of cell cycle in which the sample was added at three different concentrations (1%, 0,5% and 0,25% based on a preliminary cytotoxicity test) to study the proliferation activity.
The in vitro experimental protocol for the assessment of antioxidant efficacy of tested product was carried out by ROS analysis on cell culture pre-treated with H2O2 (10 mM) for 24 hours.

The cell cultures were analyzed after 12 hours, 36 hours and 60 hours after the beginning of treatment with tested product tested at three non-cytotoxic concentrations (according to preliminary cytotoxicity results).

2.2 Test procedure for cell proliferation assessment

The proliferation test was carried out by using:

- Human Skin keratinocytes cell culture at G0 phase not at confluence in the wells
- Test item concentration: 1%, 0.5% and 0,25% in culture medium.
- Human Skin keratinocytes without any treatment as negative control reference (CTR -).

The cells were seeded in a 12 well-plate with incomplete culture medium (missing bovine fetal serum). This induces the phase of cellular quiescence. After 72 hours, the concentrations of the product to be tested were prepared in complete medium, in order to restart the cells from the G0 phase of the cell cycle. The negative control (CTR-) is set up with only complete medium.

Culture medium containing the product at tested concentrations was added to the wells containing cells in the G0 phase of the cell cycle. Cells were exposed to the product for 12, 36 and 60 hours. At the end of incubation period, MTT coloration was performed in order to evaluate cell viability and proliferation index compared to untreated control (CTR-, 100% viability for definition).
Human skin keratinocytes were seeded at three different times (12, 36 and 60 hours) and used as references. Again, MTT coloration was performed to evaluate cell viability and proliferation index compared to untreated control (CTR-, 100% viability for definition).

2.3 MTT assay

MTT-medium is prepared at concentration of 1 mg/ml in culture medium. After exposure of cells to the test items, they were washed with PBS. After removal of the washing solution, MTT-medium solution are added to each culture well and then incubated for 3 hours at 37°C and 5% CO_2. At the end of the incubation period, the MTT-medium is removed and isopropanol are added to the wells.
The plate is shaken on a rotatory plate for 30 minutes, in order to ensure that all the crystals have dissolved from the cells and have formed a homogeneous solution. The absorbance is measured at 570 nm on a microplate reader. The results are expressed as % cell viability compared to an untreated control cell culture.

2.4 Test procedure for the evaluation of antioxidant potential by ROS analysis

The assessment of antioxidant potential by ROS analysis was carried out by using:

- Human Skin keratinocytes Cell culture at confluence in the wells pre-treated for 24 hours with H2O2 (10 mM).
- Test item concentration: 1%, 0.5% and 0,25% in culture medium.

- Human Skin keratinocytes without any treatment was used as negative control reference (CTR -) and Human Skin keratinocytes exposed to H2O2 but not treated with tested product was used as positive control reference (CTR +).

The cells were seeded in a 12 well-plate for 24 hours and then exposed to a oxidant solution (H2O2 , 10mM) for 24 hours in order to stimulate the ROS appearance. After that, the concentrations of the product to be tested were prepared in complete medium and put in contact with stressed cell culture system. The negative control (CTR-) and positive control (CTR+) is set up in parallel.

Culture medium containing the product at tested concentrations was added to the wells containing oxidative stress. Cells were treated to the product for 12, 36 and 60 hours. At the end of incubation period, ROS analysis was performed in order to evaluate the index of oxidative stress compared to controls (CTR- and CTR+).

2.5 ROS dosage

ROS levels were analyzed by using a commercially available kit. The test was performed on cells collected by the wells at each experimental time point. The assay employs a proprietary quenched fluorogenic probe, Reactive oxygen species (ROS) are generated as a result of the reduction of oxygen during aerobic respiration and by various enzymatic systems within the cell. At physiological levels, ROS contribute to cell signaling and host defense. Increased ROS generation, above the detoxification capacity of the biological system, results in oxidative stress and cellular damage. The main damage to cells results from the ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA. .Fluorescence intensity is proportional to the total ROS levels within the sample.

2.6 References

DB-ALM Method Summary n. 17: MTT Assay – Summary

2.7 Statistical analysis

Statistical analysis by T-test was conducted between the samples and the controls (CTR – and CTR+). The variations are considered significant for $p<0,05$.

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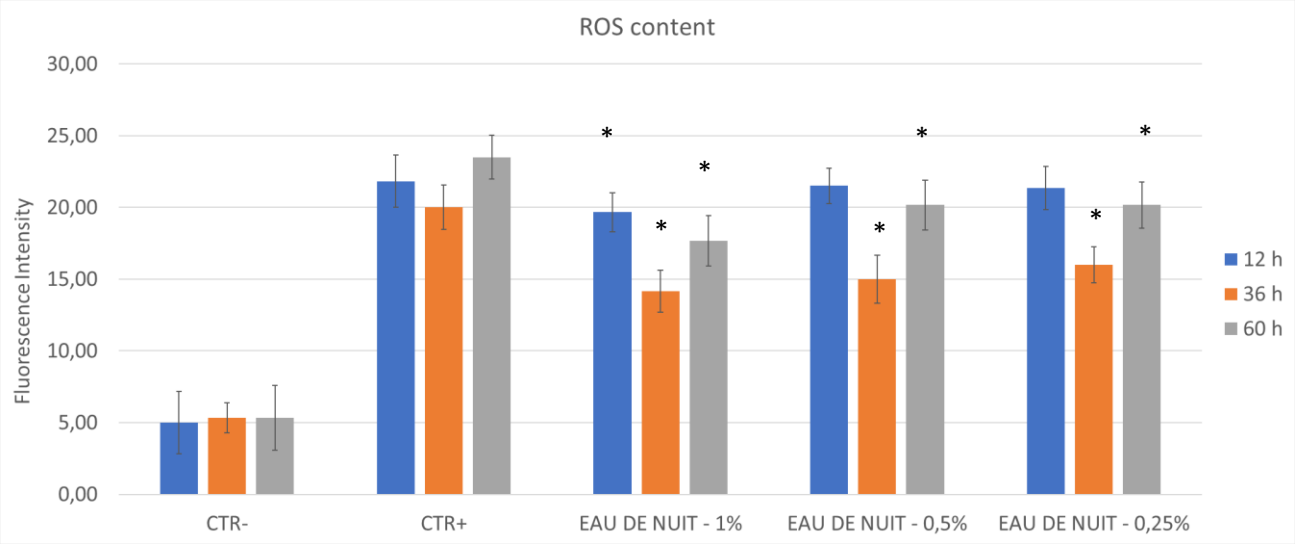
3.RESULTS

In the tables and graphs below the data collected after ROS analysis were reported.

Table 1: ROS analysis on human skin keratinocyte cell culture (12h, 36h and 60h) compared to CTR+. Significant data vs CTR+ are reported with asterisk (*).

	12 h			36 h			60 h		
	mean value	sd	%var vs ctr+	mean value	sd	%var vs ctr+	mean value	sd	%var vs ctr+
CTR-	5,00	2,19		5,33	1,03		5,33	2,25	
CTR+	21,83	1,83		20,00	1,55		23,50	1,52	
EAU DE NUIT - 1%	19,67	1,37	**-9,9%**	14,17	1,47	**-29,2%**	17,67	1,75	**-24,8%**
EAU DE NUIT - 0,5%	21,50	1,22	**-1,5%**	15,00	1,67	**-25,0%**	20,17	1,72	**-14,2%**
EAU DE NUIT - 0,25%	21,33	1,51	**-2,3%**	16,00	1,26	**-20,0%**	20,17	1,60	**-14,2%**



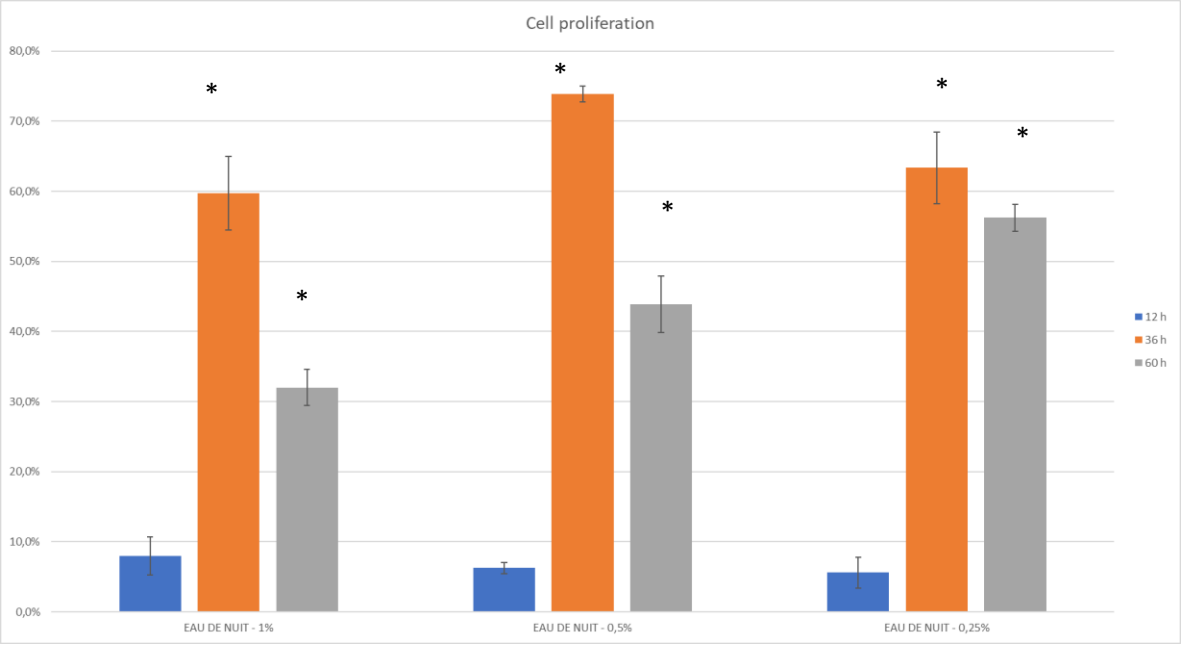
Comment to the results

The tested product resulted effective in protect cell culture to oxidative stress.
In particular, the tested product at 1%, 0,5% and 0,25% significantly enhances the reduction of ROS presence after 36 and 60 hours.
the tested product at 1% showed significant reduced data of ROS amount in stressed cell culture just after 12 hours.

In the tables and graphs below the data collected during the test of cell proliferation were reported.

Table 2: Cell proliferation study on human skin keratinocyte cell culture (12h, 36h and 60h) compared to CTR- (100%). Significant data vs CTR- are reported with asterisk (*).

	12 h		36 h		60 h	
	mean value	sd	mean value	sd	mean value	sd
EAU DE NUIT - 1%	**7,9%**	2,7%	**59,7%**	5,3%	**32,0%**	2,5%
EAU DE NUIT - 0,5%	**6,2%**	0,8%	**73,9%**	1,1%	**43,9%**	4,0%
EAU DE NUIT - 0,25%	**5,6%**	2,2%	**63,3%**	5,1%	**56,3%**	1,9%



Comment to the results

The tested product resulted effective in increasing cell proliferation on the considered *in vitro* system.
In particular, the tested product at 1%, 0,5% and 0,25% enhances significantly cell proliferation after 36 and 60 hours.

4. CONCLUSIONS

According to the data obtained and reported in this report, we can assess that the treatment of cell cultures with tested product enhances cells proliferation and reduce the ROS amount in the considered in *vitro* system.

According to tested experimental patterns,

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significantly enhances proliferation capability of cell culture in the specific in vitro model, and it highlight a potential antioxidant efficacy reducing the ROS amount

Experimenter

Dr. Andrea POGGI

Study Director

Dr. Silvana GIARDINA

Digitally signed by Silvana Giardina
Date: 2023.07.25 17:17:09 +02'00'

- The result of the study reported in this document is only referred to the tested sample and the specific experimental conditions.
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