IN VITRO EFFICACY STUDY

EVALUATION OF THE PHOTOPROTECTIVE AND ANTIOXIDANT CAPACITY OF TWO COSMETIC PRODUCTS

TORSTONE SA

RIVOLI

LES MAINS SOIN TOTAL ANTI AGE LAB 01160.13

SUN CREAM SPF20 00540.07

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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
0	08/08/2019	First report release
1	28/08/2019	First revision - product name change (addition SPF20)

1. STUDY DESIGN

1.1 Title

In vitro efficacy study – evaluation of the photoprotective and antioxidant capacity of two cosmetic products.

1.2 Study aim

The study described below is aimed to evaluate the antioxidant and photoprotective capacity of the tested products in an in vitro reconstructed human epidermis model.
This evaluation was carried out by determining cell viability, histological analysis and glutathione and protein carbonyl dosage after tissue treatment with the products and subsequent UV irradiation.

1.3 Test items

Cosmetic products with the following composition

TORSTONE SA

RIVOLI
LES MAINS SOIN TOTAL ANTI AGE LAB 01160.13
SUN CREAM 00540.07

INCI: LES MAINS SOIN TOTAL ANTI AGE LAB

AQUA, ETHYL MACADAMIATE, BUTYROSPERMUM PARKII BUTTER, CAPRYLIC/CAPRIC TRIGLYCERIDE, PANTHENYL TRIACETATE, CETEARYL ALCOHOL, PROPANEDIOL, HYDROGENATED PHOSPHATIDYLCHOLINE, COCOGLYCERIDES, PENTYLENE GLYCOL, GLYCERIN, PASSIFLORA EDULIS SEED OIL, ETHYL LINOLEATE, TAPIOCA STARCH, PARFUM, CELLULOSE GUM, CETEARYL GLUCOSIDE, C20-22 ALKYL PHOSPHATE, HYDROGENATED COCO-GLYCERIDES, TOCOPHERYL ACETATE, OLEYL ALCOHOL, C20-22 ALCOHOLS, CAPRYLYL GLYCOL, ETHYLHEXYLGLYCERIN, XANTHAN GUM, PHENYLPROPANOL, SODIUM STEAROYL GLUTAMATE, SQUALANE, TAMARINDUS INDICA SEED GUM, SODIUM PHYTATE, TOCOPHEROL, ACETYL RHEUM RHAPONTICUM ROOT EXTRACT, CERAMIDE NP, SODIUM HYDROXIDE, MALIC ACID

INCI: SUN CREAM

AQUA, C12-15 ALKYL BENZOATE, DIBUTYL ADIPATE, CAPRYLIC/CAPRIC TRIGLYCERIDE, BUTYL METHOXYDIBENZOYLMETHANE, PHENYLBENZIMIDAZOLE SULFONIC ACID, POLYSILICONE-15, OCTOCRYLENE, GLYCERIN, CETEARYL GLUCOSIDE, PHENOXYETHANOL, ISOPROPYL PALMITATE, CARBOMER, SODIUM HYDROXIDE, CYCLOPENTASILOXANE, GLYCERYL STEARATE, BIS-ETHYLHEXYLOXYPHENOL METHOXYPHENYL TRIAZINE, BUTYROSPERMUM PARKII BUTTER, TRIACONTANYL PVP, GLYCERYL STEARATE CITRATE, CYCLOHEXASILOXANE, ETHYLHEXYLGLYCERIN, PARFUM, XANTHAN GUM, ACETYL TYROSINE, BUTYLENE GLYCOL, PENTYLENE GLYCOL, O-CYMEN-5-OL, DISODIUM EDTA, PROLINE, TOCOPHEROL, TAMARINDUS INDICA SEED GUM, CETYL ALCOHOL, STEARYL ALCOHOL, HELIANTHUS ANNUUS SEED OIL, ADENOSINE TRIPHOSPHATE, HYDROLYZED VEGETABLE PROTEIN, PEG-12 GLYCERYL LAURATE, PEG-35 CASTOR OIL, PVP

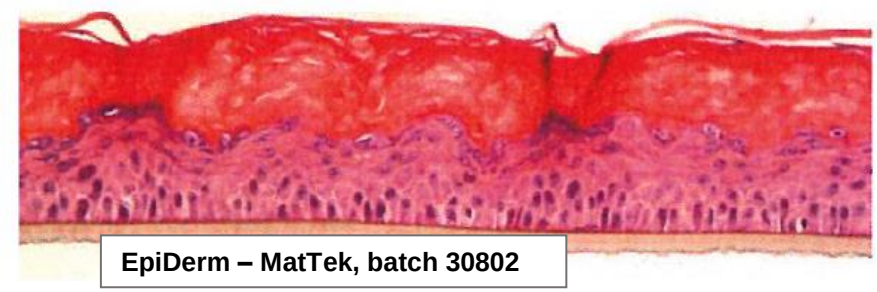
1.4 Date of test execution

Test: 19/06/2019– 21/06/2019
Biochemical dosages: 27/06/2019 – 11/07/2019
Data Analysis: 12/07/2019

2. MATERIALS AND METHOD

2.1 Experimental model

The biological model used in the test consists of three-dimensional reconstructed human epidermis, built from primary cultures of keratinocytes (EpiDerm – MatTek, batch 30802). Particularly, this is 0.6 cm^2 reconstructed epidermis by airlifted culture of keratinocytes for 17 days in chemically defined medium on inert polycarbonate filter at the air/liquid interface. The test system has an ultra-structure (tissue morphology and thickness) very similar to the human in vivo skin. All the tissue units were subjected to quality controls to ensure the suitability of the biological model and verify the absence of pathologies to ensure the operator safety.



EpiDerm – MatTek, batch 30802

2.2 Experimental treatments

For the test execution, the tissues were pre-treated with the test products and then subjected to two cycles of UV irradiation at 24h intervals by using a solar simulator. The sample treatment lasted 48 hours.

The epidermis was first treated with 30 µL of the test products as it is and then irradiated with Suntest CPS+ sun simulator, with 5 J/cm^2 UV dose (exposure time 10 minutes).

At the end of the treatment period the tissues were washed with PBS and cell viability by MTT assay, histological analysis with hematoxylin-eosin staining, protein carbonyl content and glutathione dosage in the tissue homogenate were determined.

The results were compared with negative control (untreated epidermis, CTR-) and positive control (tissues treated only with UV rays, CTR +).

The treatments were performed in duplicate.

In summary, the experimental protocol provided:

- untreated epidermis (negative control, CTR-);
- irradiated epidermis (positive control, CTR+);
- epidermis treated with tested products and then irradiated.

2.3 Cell viability study – MTT assay

The MTT test (3,(4,5-dimethylthiazol-2)2,5 difeniltetrazolium bromide) is a standard, simple and accurate colorimetric method for cell viability assessment. The assay is based on the intracellular reduction of the yellow tetrazolium salts by the mitochondrial enzyme succinate dehydrogenase in blue/purple formazan crystals. The reaction may therefore take place only in metabolically active cells and the value of the optical density obtained by spectrophotometric reading can be correlated to the amount of viable cells.

At the end of each treatment the tissues were rinsed with PBS, stained with MTT solution 1 mg/mL and incubated for three hours at 37°C/5% CO_2. Then the tissues were treated with isopropanol and incubated for two hours at room temperature. After incubation absorbance readings was performed at 540 nm by microplate reader (isopropanol was used as blank for reading). For each test condition the ratio of the average optical density of the treated tissues on the average optical density of negative controls determines the viability rate. The difference between the viability of the sample and the positive control is calculated, direct index of the protective capacity of the product.

2.4 Histological analysis – Hematoxylin eosin staining

Hematoxylin and eosin stain (abbreviated HE) is the principal stain in the microscopic study of animal tissues and histopathological routine analysis.

The hematoxylin or Mayer's hemalum is a basic dye which colors in blue violet negatively-charged cellular components such as nucleic acids, membrane proteins and cellular membranes, elastin. These components are called basophils and are found predominantly in the nucleus, which thus assumes the blue color. Eosin is instead acid and colors in pink the positively charged components as many cellular proteins, mitochondrial proteins, collagen fibers. These components are called eosinophils or acidophilus and determine a pinkish color of all remaining cellular areas, that is cytoplasm and extracellular substances.

After each treatment one tissue/condition is fixed in 10% formalin solution for histological analysis.

The tissues are cut in sections and adhered to a glass slide. After this preparation they are first immersed in the hematoxylin and biting solution, then after washing are immersed in the eosin solution. The slide is examined by light microscopy (10X magnification) and a specific damage score is assigned.

2.5 GLUTATHIONE synthesis study – GSH dosage

Glutathione (GSH) is an important and universal antioxidant, it plays a key role in resisting and preventing oxidative damage. The determination of total glutathione (GSSG + GSH) is carried out by means of colorimetric method. Commercial kits were used for this purpose. The biological sample is first deproteinized with the 5% 5-Sulfosalicylic Acid Solution, centrifuged to remove the precipitated protein, and then assayed for glutathione.

The measurement of GSH uses a kinetic assay in which catalytic amounts of GSH cause a continuous reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to TNB and the GSSG formed is recycled by glutathione reductase and NADPH. The GSSG present will also react to give a positive value in this reaction.

The reaction rate is proportional to the concentration of glutathione up to 2 μM. The yellow product, 5-thio2-nitrobenzoic acid (TNB) is measured with microplate reader at 450 nm. The assay uses a standard curve of reduced glutathione to determine the amount of glutathione in the biological sample.

The results are expressed as nmoles GSH per ml of of cell homogenate. The % variation in GSH content between positive control/negative control and samples is calculated and the protection index too. The protection efficacy is reported as the inverted ratio between the GSH difference between sample and CTR- respect to the GSH difference between CTR+ and CTR- (%).

2.6 Protein damage study – Protein carbonyl dosage

The carbonylated proteins are considered as a universal marker of oxidative damage at the protein level. Therefore the dosage of these compounds is used as an index of oxidative stress related to the protein component. During the oxidation process, the protein amino acids are modified or degraded; they thus form new functional groups such as carbonyls and hydroxyls, with the consequent loss of protein functional activity. The usage of protein CO groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins.

The determination of the carbonylated protein content was done through a colorimetric method. In particular, the carbonyl content was determined by the derivatization of protein carbonyl groups with 2,4-dinitrophenylhydrazine (2,4-DNPH) to form a yellow precipitate, the 2,4-dinitrophenylhydrazone (2,4-DNP) derivate, that can be detected spectofotometrically at 405 nm, proportional to the present carbonyl groups.

The samples were pretreated with 10% streptozocin solution in order to remove nucleic acid interference. DNPH solution and then trichloroacetic acid (TCA) solution was added for the protein precipitation. The samples were separed by centrifugation and the precipitate was washed with acetone. Then the protein precipitate was dissolved in 6M guanidine solution until the complete solubilization and the absorbance of the solutions was measured at 405 nm (absorption wavelength of 2,4-dinitrophenylhydrazone).

The results are expressed as carbonyl concentration (nmoles) in 100 μL of cell homogenate. The % variation in carbonyl content between positive control/negative control and samples is calculated and the protection index too. The protection efficacy is reported as the inverted ratio between the carbonyl difference between sample and CTR- respect to the carbonyl difference between CTR+ and CTR- (%).

2.7 Statistical analysis

Obtained results were subjected to statistical analysis by means of Student test. The variations are considered significant for *p<0,05.

2.8 Bibliography

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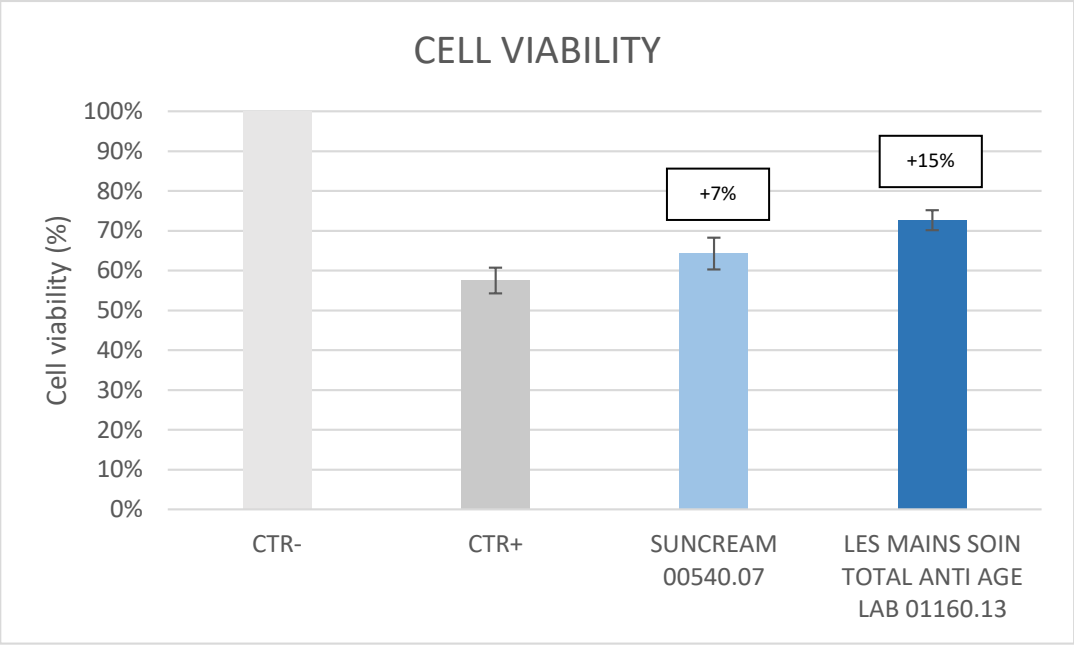
3. RESULTS

In the tables and graphs below the obtained data are reported.

3.1 CELL VIABILITY

Table and graph 1
Cell viability in CTR-, CTR+ and epidermis treated with SUNCREAM 00540.07 and LES MAINS SOIN TOTAL ANTI AGE LAB 01160.13.The results are expressed as mean ± st. dev. (expressed in %) and as protection efficacy (%).

	Cell Viability (%)	Protection vs CTR+ (%)
CTR-	**100%**	-
CTR+	**57.5% ± 3.2%**	-
SUNCREAM 00540.07 (+UV irradiation)	**64.3% ± 4.0% ***	**6.8%**
LES MAINS SOIN TOTAL ANTI AGE LAB 01160.13 (+UV irradiation)	**72.7% ± 2.5% ***	**15.1%**



3.2 HYSTOLOGICAL ANALYSIS

Table 2
Histological analysis in CTR-, CTR+ and epidermis treated with SUNCREAM 00540.07 and LES MAINS SOIN TOTAL ANTI AGE LAB 01160.13. Scores for the recorded alterations are reported.

Experimental conditions	Judgment
CTR-	No alteration -
CTR+	Necrosis +++
SUNCREAM 00540.07 (+UV irradiation)	Necrosis ++
LES MAINS SOIN TOTAL ANTI AGE LAB 01160.13 (+UV irradiation)	Necrosis ++

Legend

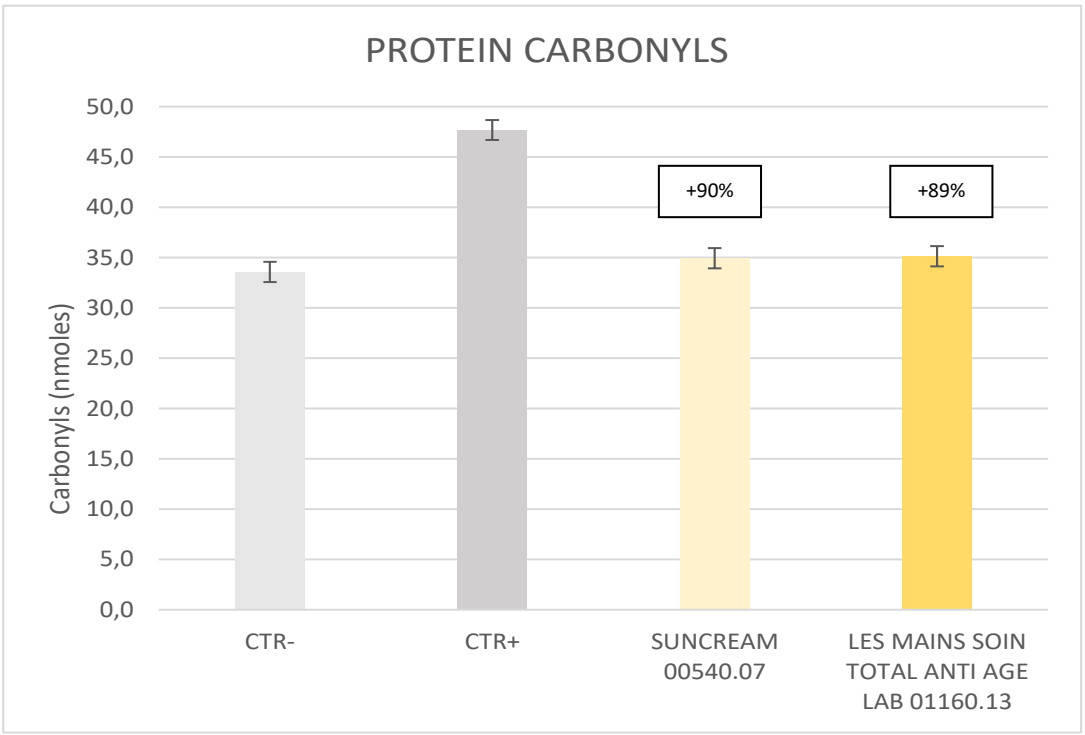
- (0)	**Absent**
+ (<10%)	**Slight**
++ (≥10-<40%)	**Moderate**
+++ (≥40%)	**High**

3.3 PROTEIN DAMAGE STUDY – PROTEIN CARBONYL DOSAGE

Table and graph 3

Carbonyl protein content in CTR-, CTR+ and epidermis treated with SUNCREAM 00540.07 and LES MAINS SOIN TOTAL ANTI AGE LAB 01160.13. The results are expressed as mean ± st. dev. (expressed in nmoles), variation vs CTR+/CTR- and protection efficacy (%).

	Carbonyl proteins (nmoles)	Var vs CTR- (%)	Var vs CTR+ (%)	Protection (%)
CTR-	**33.6 ± 0.9**	-	-	-
CTR+	**47.7 ± 1.2**	**42.0%**	-	-
SUNCREAM 00540.07 (+UV irradiation)	**34.9 ± 0.9**	**4.1%**	**-26.7% ***	**90.3%**
LES MAINS SOIN TOTAL ANTI AGE LAB 01160.13 (+UV irradiation)	**35.1 ± 0.4**	**4.7%**	**-26.3% ***	**88.9%**

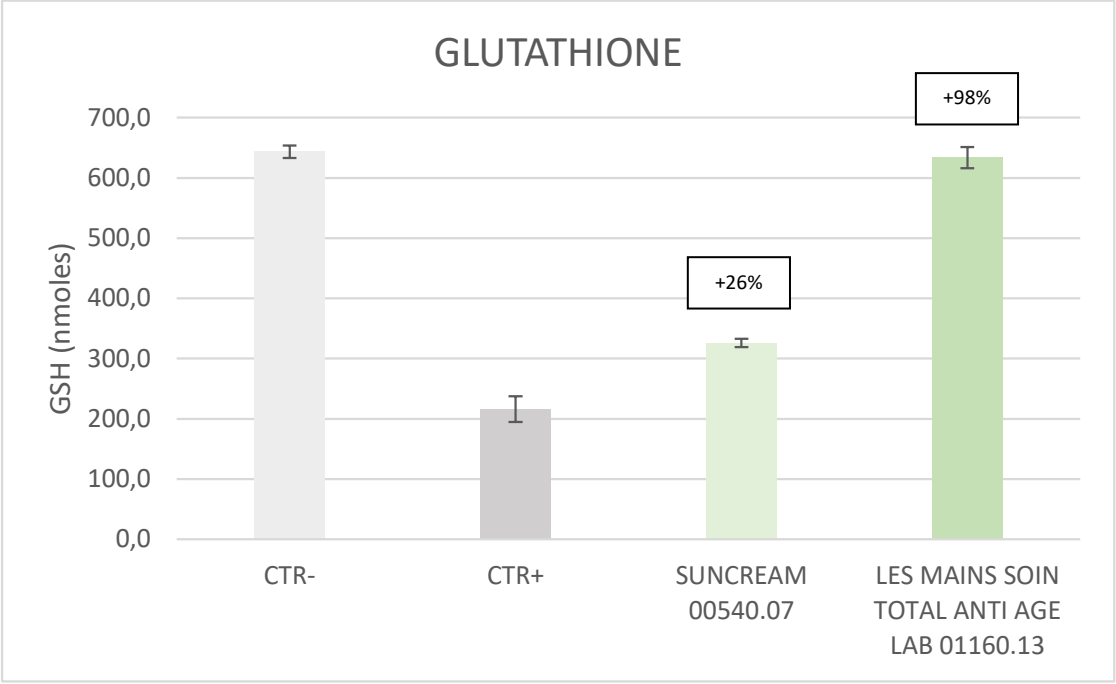


3.4 GLUTHATIONE DOSAGE

Table and graph 4

Glutathione content in CTR-, CTR+ and epidermis treated with SUNCREAM 00540.07 and LES MAINS SOIN TOTAL ANTI AGE LAB 01160.13.The results are expressed as mean ± st. dev. (expressed in nmoles), variation vs CTR+/CTR- and protection efficacy (%).

	GSH (uM)	Var vs CTR- (%)	Var vs CTR+ (%)	Protezione / Protection (%)
CTR-	**643.5 ± 10.6**	**-**	**-**	**-**
CTR+	**216.0 ± 21.2**	**-66.4%**	**-**	**-**
SUNCREAM 00540.07 (+UV irradiation)	**326.0 ± 7.1**	**-49.3% ***	**50.9%***	**26.0%**
LES MAINS SOIN TOTAL ANTI AGE LAB 01160.13 (+UV irradiation)	**633.5 ± 17.7**	**-1.6%**	**193.3%***	**97.7%**



The tissue treatment with UV rays showed a significant reduction of the cell viability, high tissue alterations, a significant increase in carbonyl proteins and a significant reduction of glutathione amount (*p<0.05)

The tissues pretreated with the products and then exposed to UV irradiation showed a significantly higher viability, a reduction of tissue damage, a significant reduction in protein carbonyl content and a significant increase in glutathione amount compared to the positive control (*p <0.05).

The tested products showed a protective efficacy in the reconstructed epidermis model against toxic effects of UV rays.

4. CONCLUSION

According to the obtained data and referring to the applied experimental protocol, we can assess that the treatment with SUNCREAM 00540.07 and LES MAINS SOIN TOTAL ANTI AGE LAB 01160.13 **has protected reconstructed epidermis against toxic effect of UV rays, by preserving cell viability, glutathione synthesis and containing the oxidative protein damage as well as tissue alterations.**

According to the exposed data

TORSTONE SA

RIVOLI
SUNCREAM 00540.07
LES MAINS SOIN TOTAL ANTI AGE LAB 01160.13

in the described experimental conditions showed
PHOTOPROTECTIV AND ANTIOXIDANT EFFICACY against UV RAYS

Experimenter

Dr. Andrea POGGI

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- A part of the study was carried out in collaboration with a partner laboratory.

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