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IN VITRO CYTOPROTECTIVE STUDY OF SUNSCREEN CREAM X ON HACAT CELLS AGAINST POLLUTION MODELS

Study Sponsor : PT. Paragon Innovation and Technology
Laboratory : Skinovation Centre, Indonesia International Institute for Life Sciences
Principal Investigator : apt. Pietradewi Hartrianti, Ph.D

The investigators, data collector, author and everyone involved in this study hereby certify the validity of the data presented in the report and has come to an agreement to the conclusions written at the end of the report.

The Head of Laboratory and Laboratory Coordinator also certifies that a quality control has been performed at each stage of the method described in a specific protocol. This control leads to accurate investigations to the tested product and to guarantee the reliability of analyzed data in accordance with this standard procedure. The present report constitutes a precise description of the performing of experimentation, processing of data and detailed procedure that have been used.

Any information concerning products to study such as patents, formulae, raw materials, manufacturing procedures etc. directly or indirectly provided by the sponsor of the study, will be regarded as confidential, will remain his property and will not be disclosed without his express consent.

Date : 06 July 2022

<table>
<tr><th>Head of Laboratory</th><th>Principal Investigator</th><th>Laboratory Coordinator</th></tr>
<tr><td rowspan="3"> 
Sanjaya Mulya Wanii</td><td> 
Pietradewi Hartrianti, Ph.D</td><td> 
Priscilla Nathaniel Tandiman</td></tr>
<tr><td>Member of Investigator Team</td><td>Technician</td></tr>
<tr><td> 
Richard Sutedjo
 
Marsia Gustiananda</td><td> 
Lavisiony Gracius</td></tr>
</table>

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1. INTRODUCTION

The skin barrier function is constantly subject to external aggressions such as air pollution which induce important cell damages.

Most environmental pollutants may undergo transformation through certain processes, mainly oxidation, into free radical species capable of inducing numerous toxic effects. Upon reaction between biomolecules in the skin and free radicals, in particular Reactive Oxygen Species (ROS), peroxides are formed. The process of oxidation and peroxides resulting from the reaction will induce oxidative stress that causes damage to cell structures and connective tissues which may ultimately lead to skin aging and skin diseases (Audina, 2021).

In this study, the epidermal protective effect of sunscreen cream X against pollution was assessed on HaCaT cells by using H_2O_2 and CSE pollution models.

More specifically, the cell viability of HaCaT cells exposed to the pollution models and treated with the sunscreen was measured using MTS assay and image analysis.

Pollution stands as one of the major problems around the world that involve the introduction of harmful substances towards the environment and may negatively affect the quality of land, water, and also air. Among the other types of pollution, air pollution is reported to be the most significant cause of various health issues and even death (Dwi sutanto, 2020). Based on the World Health Organization (WHO), six major categories of air pollutants are particle pollution, ground-level ozone, carbon monoxide, nitrogen oxides, sulfur oxides, and heavy metals. Most environmental pollutants may undergo transformation through certain processes, mainly oxidation, into free radical species capable of inducing numerous toxic effects. The human skin, notably the upper epidermis layer, serves a crucial role as a barrier from the outside environment to fight off chemical and physical assaults and prevent pathogens invasion as well as excessive loss of solutes and water. However, it is also among the earliest and most vital targets of air pollutants (Drakaki, Dessinioti, & Antoniou, 2014). Upon reaction between biomolecules in the skin and free radicals, in particular Reactive Oxygen Species (ROS), peroxides are formed. The process of oxidation and peroxides resulting from the reaction will induce oxidative stress, the imbalance state between generation of ROS and antioxidant-mediated defense mechanisms. This causes damage to cell structures and connective tissues which ultimately may lead to skin aging and skin diseases like urticaria, psoriasis, and atopic dermatitis (Audina, 2021).

Sunscreen cream X claims that it has the ability to protect the skin against free radicals by ingredients which act as antioxidants. In this research project, Sunscreen cream X will be tested on HaCaT (Immortalized human keratinocyte line) cells that compose around 95% of the skin epidermis, to determine whether it truly possesses the ability to fight free radicals or not. Several pollution models that are used in this project to test the product cover the hydrogen peroxide (H_2O_2) model and the cigarette smoke extract (CSE) model. The H_2O_2 model is used because it is easily accessible, cheap, and can be used at a wide range of pH and temperature (Simonenko, Gomonov, Rolle, & Molodkina, 2015). Uncontrolled exposure to ROS including H_2O_2 may cause oxidative stress which has the potential to damage biomolecules (Imlay, 2013). In addition, H_2O_2 exposure has been correlated to apoptosis and DNA damage via a mitochondrial pathway (Xiang et al., 2016). This model pathway serves as a representation of free radicals' effect on skin cells. Whereas, the CSE model is utilized because it is quite simple, and the materials needed to construct the model are inexpensive. Cigarette smoke is known to contain numerous chemicals such as nicotine, pesticide residues, metal-carbon monoxide, hydrogen cyanide, acrolein, ROS (superoxide, nitric oxide, H_2O_2), and many more which are released into the atmosphere as air pollutants (Bernard et al., 2019). Several components of cigarette smoke including ROS and acrolein may also induce oxidative stress that further damages the cells. This model employs CSE exposure against HaCaT cells in order to represent the effect of burning and smoke from air pollution on skin cells (Benedikter et al., 2017). Moreover, it allows the observation of the effect of direct H_2O_2 and cigarette smoke against HaCaT cells and whether both models will yield comparable results. The aim of

the study is to measure cytoprotective capability of sunscreen cream X against pollution models such as cigarette smoke extract and H_2O_2 using HaCaT cells

2. MATERIALS AND METHODS

a. In vitro model/Subject

- Model: HaCaT (Immortalized human keratinocytes) cell
- Culture conditions: 37°C, 5% CO2
- Culture medium: DMEM supplemented with
  L-glutamine 2 mM
  Penicillin 1% - Streptomycin 1%
  Fetal bovine serum (FBS) 10%

b. Test Compound (Groups of study or experimental design)
It has to be reminded that the sponsor of the study is responsible for the initial identification, composition, and purity of the product, as well as all characteristics allowing to define and identify tested products before the study is started.

So as to ensure that results are still meaningful after prolonged storage, stability tests should have been preceded by the sponsor.

Receipt of product are reported below:

Sponsor	Product Reference	Product name	Aspect	Product Storage	Study Date
PT Paragon Technology and Innovation	Sunscreen -0921-K	Sunscreen product	Liquid cream	In a jar, protected from light	October 2021 – December 2021
	Sunscreen -0921-L	Sunscreen base	Liquid cream	In a jar, protected from light	October 2021 – December 2021
	Raw Mat-0921-F	Bifida Ferment Lysate	Liquid	In a transparent bottle, protected from light	October 2021 – December 2021
	Raw Mat-0921-G	Bisabolol	Liquid	In a transparent bottle, protected from light	October 2021 – December 2021
	Raw Mat-0921-M	*Pongamia Glabra*	Powder	In a plastic clip, protected from light	October 2021 – December 2021
	Raw Mat-0921-N	Propanediol, Water, *Artemisia Capillaris* Flower Extract	Liquid	In a transparent bottle, protected from light	October 2021 – December 2021
	Raw Mat-0921-O	Saccharide Isomerate, Aqua, Citric Acid, Sodium Citrate	Liquid	In a transparent bottle, protected from light	October 2021 – December 2021
	Raw Mat-0921-Q	Titanium Dioxide, Dimethicone, Silica	Powder	In a plastic clip, protected from light	October 2021 – December 2021

	Raw Mat-0921-V	Zinc Oxide, Triethoxycaprylylsilane	Powder	In a plastic clip, protected from light	October 2021 – December 2021

c. Methods

c.1 Method origin

Determination of the pollution protection performance by means of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay and spectrophotometer to evaluate the skin protection against H_2O_2 and CSE brought by the sunscreen product in a reliable manner. This method is initially described by Song *et al.* (2018), then modified appropriately to assess the anti-pollution protection activity based on the *in vitro* standard approach.

c.2 Method Principle

The present method consists in evaluating the protection brought by a sunscreen product against the pollution models such as H_2O_2 and CSE, by means of MTS assay and spectrophotometric method, using HaCaT cell model on which the product and pollution inducers have been added homogeneously.

The test is based on the assessment of MTS assay and absorbance of the cell read on a UV-Vis plate reader after being treated with sunscreen samples and exposure to a controlled dose of H_2O_2 and CSE as inducers. MTS assay is used to assess cell viability and is indicative of the mitochondrial metabolic rate (Lü et al., 2012). Only cells with active metabolism are able to reduce the tetrazolium compounds in the presence of intermediate electron acceptor reagents such as phenazine methosulfate (PMS) or phenazine ethyl sulfate (PES) into soluble formazan products, mainly by NADPH-dependent dehydrogenases located in the mitochondria (Berridge et al., 2005; Kuete et al., 2017). Spectrophotometer can be used to measure the reduction, where the amount of colored formazan is linearly dependent on the number of viable cells (Riss et al., 2019). It is cost-effective, rapid, more accurate, sensitive, and does not need specialized equipment or skills making it a preferred method for cell metabolic viability and proliferation analysis (Aslantürk, 2018).

c.3 Description of the method

Step 1: Sample Preparation

The sample solutions (product, base, API, ZnO + TiO_2 Only, API without ZnO + TiO_2, product without ZnO + TiO_2) were made into 1% w/v solution in DMEM and sterilized with a 10 watt at wavelength of 352 nm for 15 minutes in a 96-well plate. Sample solutions were serially diluted into a series of concentrations of 0.5%, 0.25%, 0.125, 0.0625, and 0.03125% w/v.

Step 2: HaCaT cell culture

The HaCaT cell line was obtained from the American Type Culture Collection. It was maintained at 37°C with 5% CO_2 and passaged regularly along with cell counting to keep track of cell growth.

Step 3: Cigarette Smoke Extraction

The apparatus was made with a falcon tube, pasteur pipette, and nylon tubing constructed together with epoxy glue as illustrated in Figure 3.1. The cigarette was lit inside the fume hood and the vacuum pump was employed for the extraction process, where the cigarette smoke passed through and bubbled into the PBS. 10 mL of 1x phosphate buffered saline (PBS) solution was utilized for

every one cigarette (Dji Sam Soe, Indonesia) to make 100% CSE solution and it was sterilized by vacuum membrane filtration using Whatman filter paper no. 1 and sterile syringe filtration through a 0.22 µm-pore filter.

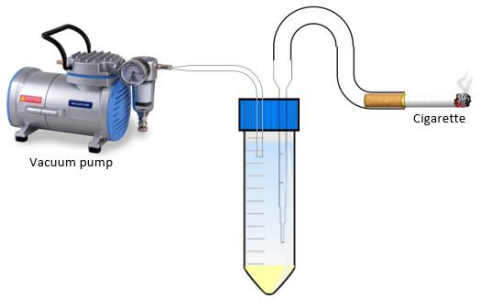

Figure 3.1 *Cigarette smoke extraction apparatus*

Step 4: Cytotoxicity Study (MTS assay)

Sunscreen cream X and ascorbic acid underwent cytotoxicity study (at least 3 replicates) to determine the safe concentrations for HaCaT cells, while H_2O_2 and CSE were tested and optimized to investigate the concentration that significantly reduces HaCaT cell viability but not until complete death. HaCaT cells were seeded in 96-well plates and exposed to different concentrations of sunscreen samples (1, 0.5, 0.25, 0.125, 0.0625, and 0.03125%), ascorbic acid (200, 100, 50, 25, 12.5, and 6.25 ppm), H_2O_2 (200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 µM), and CSE (16, 8, 4, 2, 1, 0.5, 0.25, and 0.125%) for 24 hours. MTS reagent was added afterwards and the absorbance was measured with UV-Vis plate reader to determine cell viability. Image analysis was also done using Primo Vert inverted microscope (Carl Zeiss, Germany).

HaCaT cells were seeded in 96-well plates and exposed to different concentrations of sunscreen samples (1, 0.5, 0.25, 0.125, 0.0625, and 0.03125%), ascorbic acid (200, 100, 50, 25, 12.5, and 6.25 ppm), H_2O_2 (200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 µM), and CSE (16, 8, 4, 2, 1, 0.5, 0.25, and 0.125%) for 24 hours.

MTS reagent was added afterwards and the absorbance was measured with UV-Vis plate reader to determine cell viability. Image analysis was also done using Primo Vert inverted microscope (Carl Zeiss, Germany).

Step 5: *In Vitro* Cytoprotective Evaluation against Pollution Models

Cells were seeded in 96-well plates (1.0×10^4 density) and pretreated with sunscreen samples (product, base, API, API without ZnO + TiO_2, Product without ZnO + TiO_2) and ascorbic acid as positive control for 1 hour, then co-treated with 6.25 uM H_2O_2 or 0.5%nCSE insult for another 24 hours. MTS assay was performed to determine the protection percentage.

c.4 Materials and Equipment used

The materials and equipment used are listed on Table.

No	Materials	No	Equipment

1	96-well plate	1	HaCaT cells
2	T-25 flask	2	DMEM
3	20 mL Falcon tubes	3	Penicillin-streptomycin
4	50 mL Falcon tubes	4	Fetal Bovine Serum
5	10 mL syringe	5	Products
6	0.22 µm PES syringe filter	6	Ascorbic acid
7	Cigarette smoke extraction apparatus	7	Cigarette
8	Vacuum pump	8	Trypsin
9	Inverted light microscope	9	MTS reagent
10	Analytical balance	10	Pipette tips 1000, 200, 10 µL
11	Pipette 1000, 200, 10 µL	11	Microcentrifuge tubes
12	Biological Safety Cabinet	12	Hydrogen peroxide 30%
13	UV-Vis Plate-reader	13	Parafilm seal
14	Microcentrifuge		
15	Marker		
16	Autoclave		
17	UV-LED lamp Box (Sankyo Denki, Japan)		

d. Data Management, Calculation and Statistical Analysis

Raw data were conducted and collected in triplicate, and analyzed using Microsoft Excel software.

Statistical analysis was performed in Prism version 8.0.1 (GraphPad Software, USA). Collected data were tested for Shapiro-Wilk normality test, and analyzed using one-way ANOVA followed by post-hoc using Dunnett's method with significance level (α) set at 0.05.

Formulas used in this report:

Standard error of the mean: sem = Sd/n

The standard error of the mean (sem) is a measure of how far the sample mean is likely to be from the true population mean. The sem is calculated as the sd divided by the square root of sample size.

Percentage of cell viability:

$$ell\ viability(\%) = \frac{Absorbance\ (sample)\ -\ Absorbance\ (blank)}{Absorbance\ (negative\ control)\ -\ Absorbance\ (blank)} \times 100$$

Percentage of protection:

$$Protection(\%) = \frac{(Absorbance\ (sample) - Absorbance\ (blank)) - (Absorbance\ (internal\ control) - Absorbance\ (blank}{Absorbance\ (negative\ control) - Absorbance\ (blank)} \times 100$$

3. RESULT

3.1 Cytotoxicity Study against Sunscreen Cream X

MTS assays were performed to determine the working concentration of the sunscreen samples that are not cytotoxic to the HaCaT cells. The cells were incubated in various concentrations (1, 0.5, 0.25, 0.125, 0.0625, and 0.03125%) of product, base, API, API without ZnO + TiO_2, product without ZnO + TiO_2, and ZnO + TiO_2 only. Moreover, one-way ANOVA was conducted to analyze means between groups and determine its significance. The statistical analysis signified a significant difference ($p < 0.05$) of cell viability in all concentrations tested.

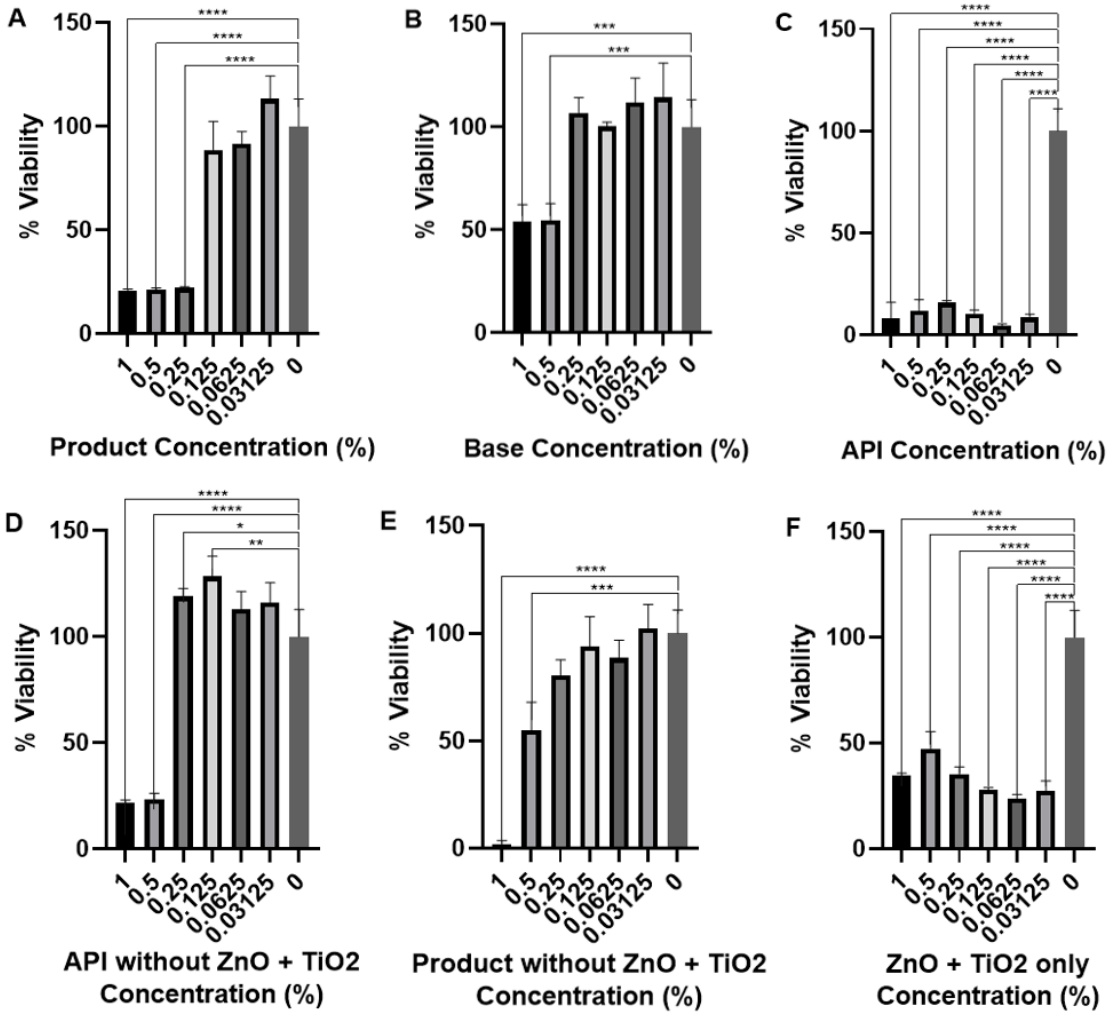

Figure 1. *Effect of Sunscreen Samples on HaCaT cell viability (%). Graphs represent average HaCaT viability against several tested concentrations of (A) product, (B) Base, (C) API, (D) API without ZnO + TiO_2, (E) product without ZnO + TiO_2, and (F) ZnO + TiO_2 only. Results are expressed as means (n = 3). Statistical significance was determined by one-way ANOVA with Dunnett post hoc test, comparing the viability of untreated wells vs treated wells.* Asterisk *(*) represents significance in which (*)* means $p < 0.05$, *(**)* means $p < 0.005$, *(***) means $p < 0.0005$,* and *(****) means $p < 0.0001$.*

From the results, it is found that the product, base, API without ZnO + TiO_2, and product without ZnO + TiO_2 groups, are safe starting from the concentration 0.125% (88.61%), 0.25% (106.80%), 0.25%

(118.76%), and 0.25% (80.33%) respectively when compared to the untreated wells. On the other hand, all concentrations of API and ZnO + TiO_2 groups were toxic and reduced the cell viability to < 50%.

3.2 Cytotoxicity Study against Ascorbic Acid

The safe concentration of ascorbic acid to be used as a positive control was also determined with MTS assay by incubation of HaCaT cells in varying concentrations (200, 100, 50, 25, 12.5, and 6.25 ppm) of ascorbic acid. Statistical analysis was carried out using one-way ANOVA and showed significant difference ($p < 0.05$) between concentrations.

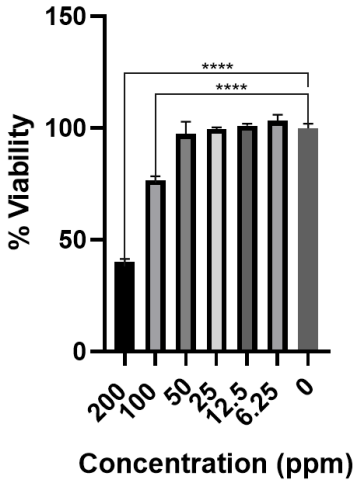

Figure 2. *Effect of Ascorbic Acid on HaCaT cell viability (%). Graphs represent average HaCaT viability against several tested concentrations of ascorbic acid. Results are expressed as means (n = 3). Statistical significance was determined by one-way ANOVA with Dunnett post hoc test, comparing the viability of untreated wells vs treated wells.* Asterisk *(*)* represents significance in which *(****) means p < 0.0001.*

As shown in **Figure 2**, the viability of the HaCaT cells are influenced in a concentration-dependent pattern where ascorbic acid is found to be completely safe starting from concentration 50 ppm (97.32%) in comparison to the untreated wells. The viability is markedly reduced in concentration 100 ppm (76.65%) and experienced further reduction in 200 ppm (40.03%).

3.3 Determination of H_2O_2 and CSE Working Concentration

To determine the working concentration of H_2O_2 and CSE that effectively reduces cell viability by around 50%, MTS assays were conducted. HaCaT cells were incubated in several different concentrations of H_2O_2-containing medium (200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 µM) and CSE-containing medium (16, 8, 4, 2, 1, 0.5, 0.25, and 0.125%) for 24 hours. One-way ANOVA was performed and the statistical analysis showed that in both models the cell viability has significant difference ($p < 0.05$) in all concentrations tested.

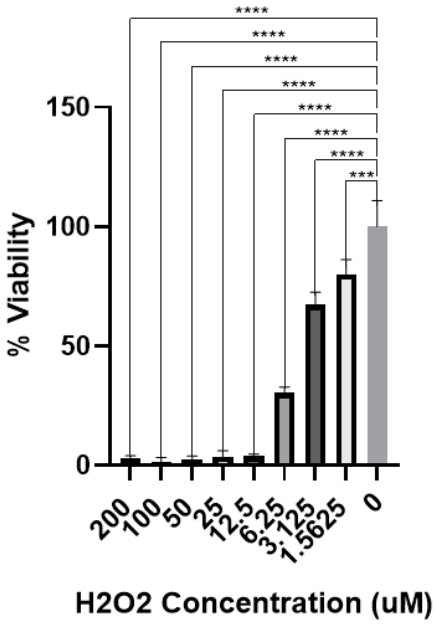

Figure 3. *Effect of CSE on HaCaT cell viability (%). Graphs represent average HaCaT viability against several tested concentrations of CSE. Results are expressed as means (n = 3). Statistical significance was determined by one-way ANOVA with Dunnett post hoc test, comparing the viability of untreated wells vs treated wells.* Asterisk *(*) represents significance in which (***) means p < 0.0005* and *(****) means p < 0.0001.*

The viability results of HaCaT cells treated with H_2O_2 showed that cell viability decreased in concentrations-dependent manner (Figure 3). Cell viability is quite affected in concentration 1.5626 µM (79.84%) and 3.125 µM (67.20%), where the viability is reduced compared to that of the untreated wells. Reduction in cell viability can be seen more drastically in 6.25 µM (30.23%), while most of the cells can be categorized dead in concentration 12.5 until 200 µM as the viability is only up to 3.79%.

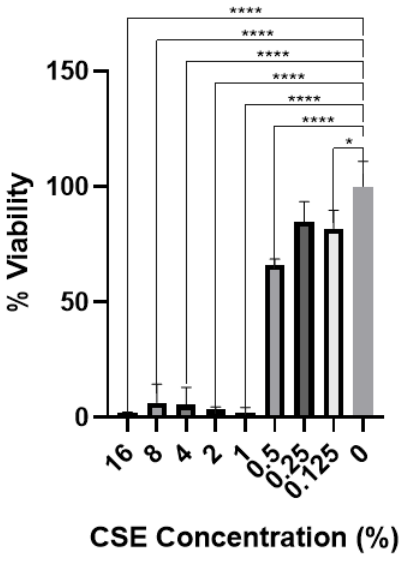

Figure 4. *Effect of H_2O_2 on HaCaT cell viability (%). Graphs represent average HaCaT viability against several tested concentrations of H_2O_2. Results are expressed as means (n = 3). Statistical significance was determined by one-way ANOVA with Dunnett post hoc test, comparing the viability of untreated wells vs treated wells.* Asterisk *(*) represents significance in which (*)* means *p < 0.05* and *(****) means p < 0.0001.*

The results displayed that the viability of HaCaT cells treated with CSE decreased proportionally along with increasing CSE concentration (Figure 4). The viability is not much affected in concentration 0.125% (81.85%) and 0.25% (84.76%), when compared to the untreated wells. More significant reduction in viability

can be seen in 0.5% (66.11%), and the cells are considered dead in concentration 1 until 16% with viability of only up to 6.47%.

3.4 *In Vitro* Cytoprotective Evaluation against Pollution Models

Oxidative protection assay was carried out to investigate the cytoprotective ability of Sunscreen samples. HaCaT cells were grown, seeded, and subsequently pretreated with 0.125% Sunscreen samples for 1 hour then treated with Sunscreen samples together with 6.25 µM of H_2O_2 or 0.5% CSE for another 24 hours. MTS reagent was added afterwards and the absorbance measurement of cells was done. Cells pretreated with different concentrations (50, 25, 12.5, and 6.25 ppm) of ascorbic acid and co-treated with H_2O_2 or CSE were used as positive control, while cells treated only with H_2O_2 or CSE were used as internal control. The % viability of cells was calculated by comparing treated cells with untreated cells.

3.4.1 Hydrogen Peroxide (H_2O_2)

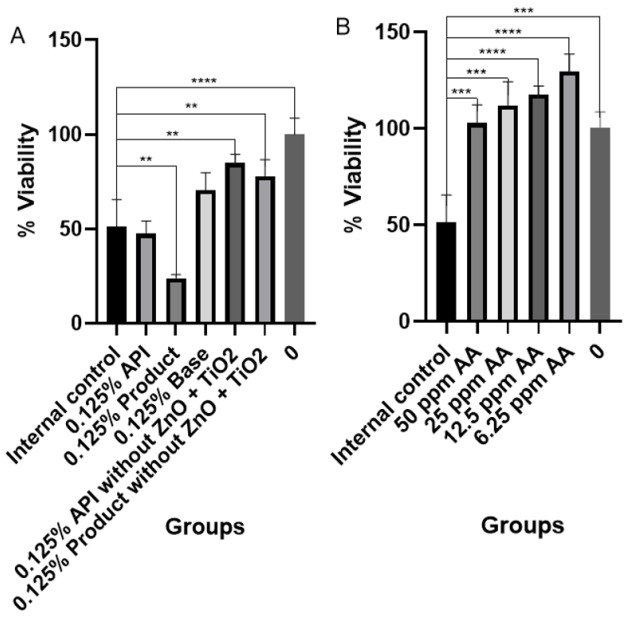

Figure 5. *Protective effect of Sunscreen samples and Ascorbic Acid on H_2O_2 induced HaCaT cell viability (%). Graphs represent average HaCaT cells viability treated with H_2O_2 and (A) 0.125% Sunscreen samples, and (B) several concentrations of Ascorbic acid (AA). Results are expressed as means of the 2nd trial (n = 3 replications in 1 experiment). Statistical significance was determined by one-way ANOVA with Tukey post hoc test, comparing the viability of untreated wells vs treated wells. Internal control is treated with 6.25 µM H_2O_2 only. Asterisk (*) represents significance in which (**)* means *$p < 0.005$, (***) means $p < 0.0005$,* and *(****) means $p < 0.0001$.*

The viability of HaCaT cells treated with sunscreen samples can be observed in **Figure 5**. Cell viability reductions are observed in the API, product, base, API without ZnO + TiO_2, and product without ZnO + TiO_2 groups compared to that of the untreated cells. However, when compared against the internal control, API without ZnO + TiO_2 and product without ZnO + TiO_2 groups significantly increase the cell viability by 65% (1.7x) and 52% (1.5x) accordingly, that signifies the protective effects exerted by the samples against H_2O_2. For the ascorbic acid utilized as positive control, all concentrations showed higher viability than the untreated cells and displayed significant increase in the viability by 100% (2.0x), 118% (2.2x), 129% (2.3x), and 152% (2.5x) at concentrations of 50 ppm, 25 ppm, 12.5 ppm, and 6.25 ppm respectively, compared to the internal control indicating its cytoprotective action.

3.4.2 Cigarette Smoke Extract (CSE)

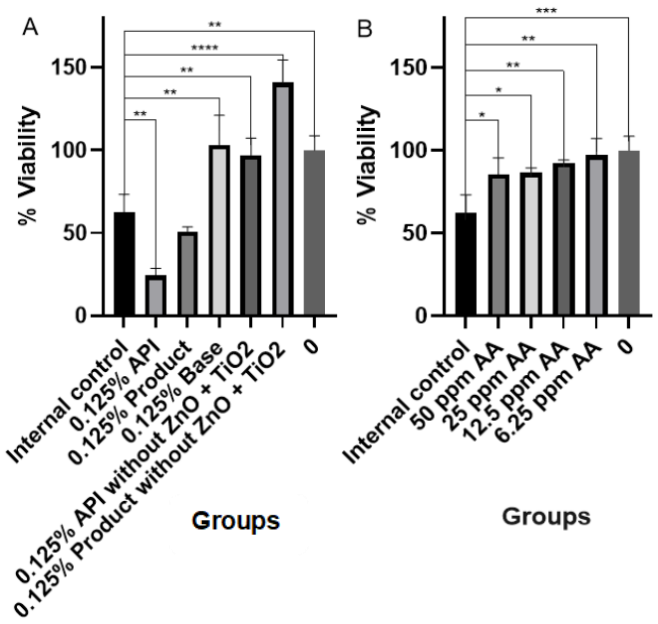

Figure 6. *Protective effect of Sunscreen samples and Ascorbic Acid on CSE induced HaCaT cell viability (%). Graphs represent average HaCaT cells viability treated with CSE and (A) 0.125% Sunscreen samples, and (B) several concentrations of Ascorbic acid (AA). Results are expressed as means (n = 3). Statistical significance was determined by one-way ANOVA with Dunnett post hoc test, comparing the viability of untreated wells vs treated wells. Internal control is treated with 0.5% CSE only. Asterisk (*) represents significance in which (*)* means $p < 0.05$, *(**)* means $p < 0.005$, *(***) means $p < 0.0005$,* and *(****) means $p < 0.0001$.*

Based on the graph, the viability of API and product groups of the sunscreen samples are lower than the untreated cells which suggested that the cells were affected by the inducer resulting in decreased viability, whereas the base, API without ZnO + TiO_2, and product without ZnO + TiO_2 groups exhibited comparable or even higher viability compared to that of the untreated cells. The sunscreen base, API without ZnO + TiO_2, and product without ZnO + TiO_2 groups expressed significant increase in the viability by 65% (1.7x), 55% (1.6x), and 125% (2.3x) accordingly, in comparison to the internal control denoting protection action against CSE. The ascorbic acid also showed significant improvement in the cell viability by 36% (1.4x), 38% (1.4x), 48% (1.5x), and 55% (1.6x) in respect to concentrations of 50 ppm, 25 ppm, 12.5 ppm, and 6.25 ppm compared to the untreated group indicating the presence of cytoprotection effects.

4. CONCLUSION

Based on the results obtained , it can be concluded that **this product can protect skin (represented by keratinocyte models) against free radicals from pollution at the recommended range of usage based on *in vitro* cytoprotective tests against pollution models (H_2O_2 and Cigarette smoke extract**).

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1. SUPPLEMENTARY DATA, TABLES AND FIGURES

Table 1. MTS protective assay viability of HaCaT cells after treatment with sunscreen samples and 6.25 µM of H_2O_2. Internal control represents cells treated with 6.25 µM of H_2O_2 only

Groups	% Viability
Internal control	51.35% ± 14.07
Untreated	100.00 ± 8.59
0.125% Product	23.63% ± 2.09
0.125% Base	70.56% ± 9.23
0.125% API	47.44% ± 6.59
0.125% API without ZnO+TiO_2	84.89% ± 4.63
0.125% product without ZnO+TiO_2	77.87% ± 8.82

Table 2. MTS protective assay viability of HaCaT cells after treatment with ascorbic acid and 6.25 µM of H_2O_2. Internal control represents cells treated with 6.25 µM of H_2O_2 only

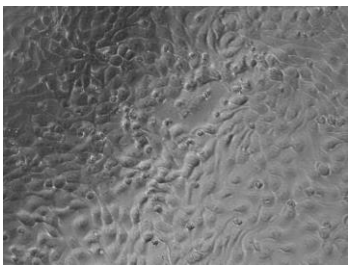
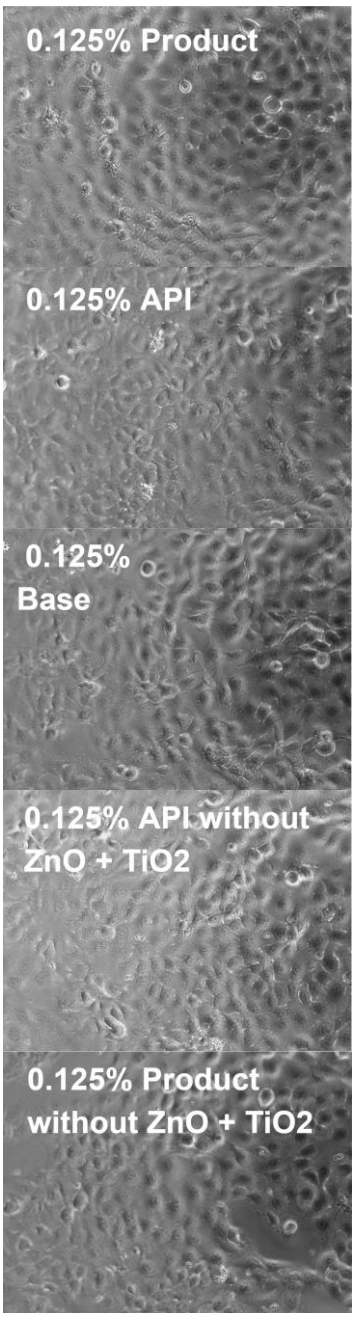
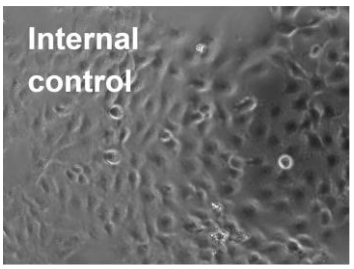
Concentration (ppm)	% Viability
Internal control	51.35% ± 14.07
Untreated	100.00 ± 8.59
50	102.83% ± 9.35
25	111.95% ± 12.30
12.5	117.41% ± 4.53
6.25	129.45% ± 9.09

Table 3. MTS protective assay viability of HaCaT cells after treatment with sunscreen samples and 0.5% CSE. Internal control represents cells treated with 0.5% CSE only

Groups	% Viability
Internal control	62.50% ± 10.65
Untreated	100.00 ± 8.59
0.125% Product	50.89% ± 2.7
0.125% Base	103.17% ± 17.81
0.125% API	24.10% ± 4.48
0.125% API without $ZnO+TiO_2$	96.95% ± 10.38
0.125% product without $ZnO+TiO_2$	140.75% ± 13.64

Table 4. MTS protective assay viability of HaCaT cells after treatment with ascorbic acid and 0.5% CSE. Internal control represents cells treated with 0.5% CSE only

Concentration (ppm)	% Viability
Internal control	51.35% ± 14.07
Untreated	100.00 ± 8.59
50	102.83% ± 9.35
25	111.95% ± 12.30
12.5	117.41% ± 4.53
6.25	129.45% ± 9.09

	Untreated cells	Cells treated pretreated with sunscreen samples and co-treatment with 6.25 µM H_2O_2	Internal control (cells treated with 6.25 µM H_2O_2)
0h			

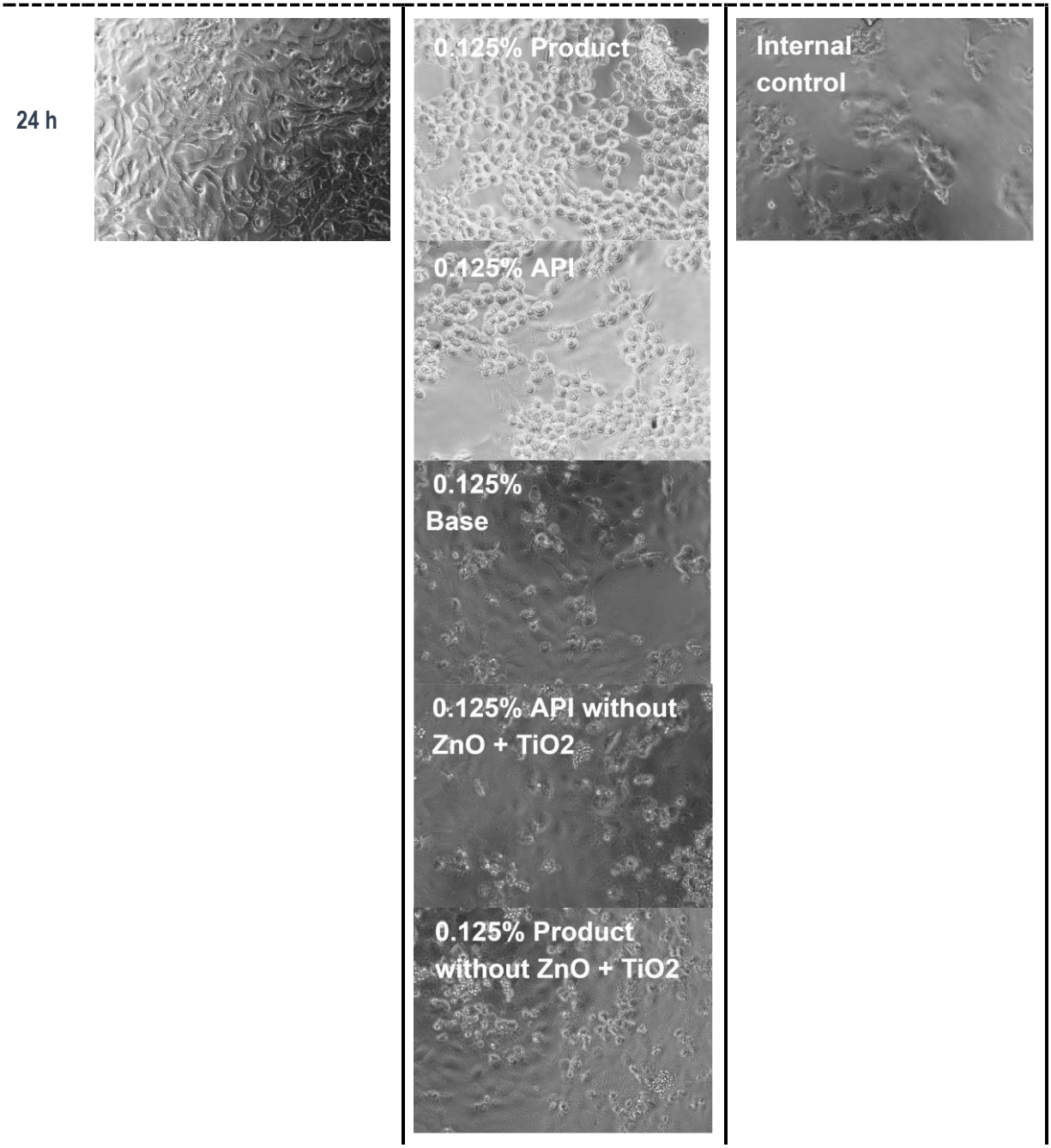
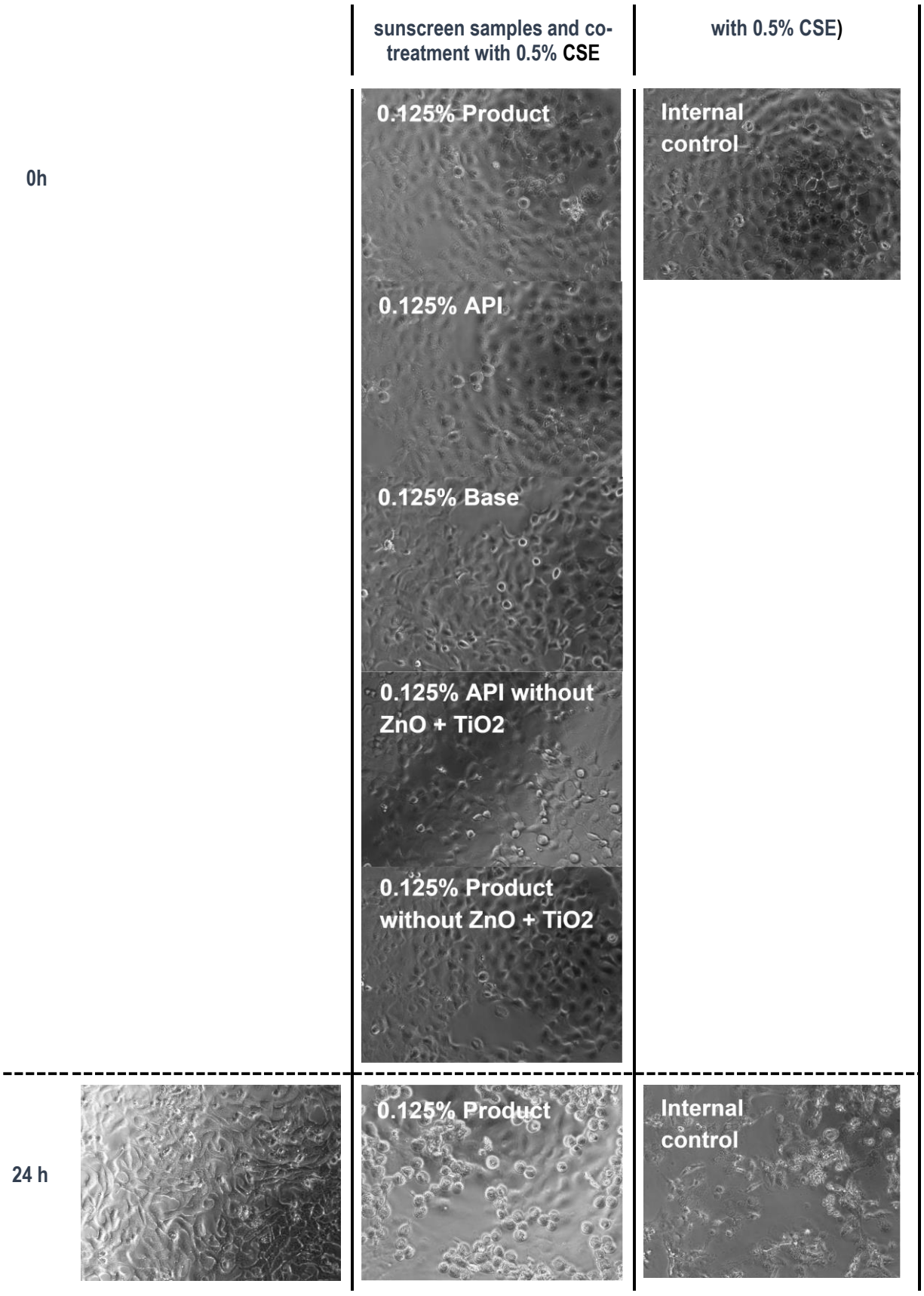


Figure 1. Representative images of *In vitro* cytoprotective evaluation of 0.125% sunscreens samples against H2O2 at 0 h and 24 h.
(Objective lens 20x)

Untreated clls	Cells treated pretreated with	Internal control (cells treated


sunscreen samples and co-treatment with 0.5% CSE
with 0.5% CSE)
0h
0.125% Product
Internal control
0.125% API
0.125% Base
0.125% API without ZnO + TiO2
0.125% Product without ZnO + TiO2
24 h
0.125% Product
Internal control

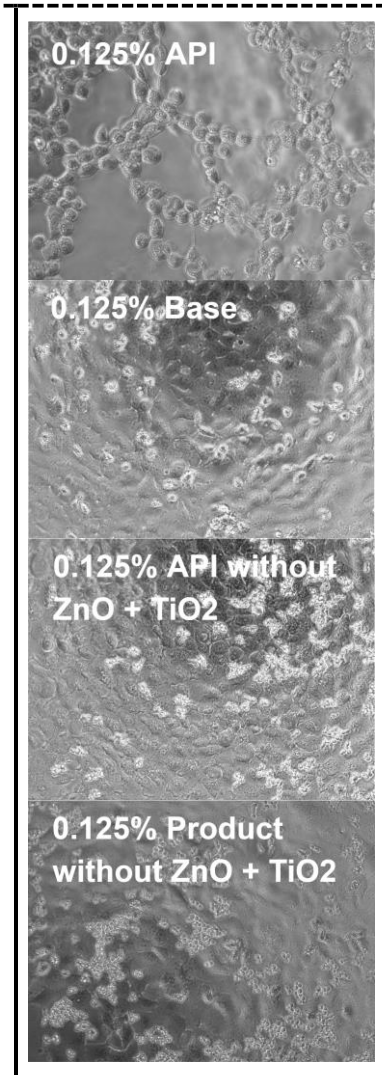


Figure 2. Representative images of *In vitro* cytoprotective evaluation of 0.125% sunscreens samples against CSE at 0 h and 24 h.
(Objective lens 20x)