INDONESIA INTERNATIONAL INSTITUTE FOR LIFE SCIENCES

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Study report : **PAR-04-220707**
Quotation No : **YAY-2205-00035**

TITLE

In Vitro Cytoprotective Study of X Sunscreen Against Blue Light

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 : December 8th 2021

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1. INTRODUCTION

Nowadays skin damage has been known to not only occur under ultraviolet exposure but also in the high energy visible (HEV) light called blue light that has a wavelength in the range of 400 to 500 nm (Coats et al., 2021). In fact, a paper stated that the effect of visible light exposure that can be emitted from any electronic device may cause hyperpigmentation, DNA damage, and oxidative stress that leads to skin aging (Campiche et al., 2020). Due to its proven risk of damaging the skin, some sunscreen products have claimed photoprotective capability for their product to not only be able to protect against ultraviolet exposure but also to protect the skin from exposure to blue light by reflecting and scattering the light. This increase in claims has been increasing alongside the current rise in online activity which results in increased usage of blue light-emitting electronic devices and exposure to blue light. Due to these increasing claims, there is a need to validate them through a scientifically proven and validated method, such as *in vitro* assay using immortalized keratinocyte cells called HaCat cells.

AIM OF STUDY : to prove the claims of the tested sunscreen product for its protective ability against blue light through in vitro cell viability measurement using HaCat cells.

2. MATERIALS AND METHODS

a. In vitro model/Subject

- Model : HaCat (Immortalized human keratinocyte) cells
- Culture conditions: 37°C, 5% CO_2
- Culture medium : DMEM supplemented with
  L-glutamine 2mM
  Penicillin 1% - Streptomycin 1%
  Fetal Bovine Serum (FBS) 10%

b. Test Compound (Groups of study or experimental design)

Explain the product tested and the comparison group as well as the product storage

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

The *in vitro* assay using HaCat cells for determining the cytoprotective ability of sunscreen against blue light was chosen in this experiment as the previous research study from Sadowska, Narbutt, and Lesiak (2021) found that blue light is toxic for keratinocyte cells as there was a decrease in the cells after irradiation every 24 hours in three consecutive days. The duration and distance tested in this experiment for blue light optimization are according to Togni *et al.* (2019), which then be adjusted according to the available equipment in the lab. Besides that, the utilization of UV lamps for sample sterilization was also used based on the previous experiment that studied the effectiveness of UV lamps in inactivating various bacteria and can become one of the methods for sterilization (Mori *et al*., 2007). Then the cytoprotective method especially the one that does not make direct contact between the cells and the sample was initiated by Tyagi *et al*., 2016.

c.2 Method Principle

The whole experiment was utilizing *in vitro* cell culture method using HaCat cell. Each part of the experiment was conducted by triplication with 3 wells in the same well plate.In the first step of the experiment, blue light optimization was conducted to determine at which distance and duration the blue light exposure to the cells may result in the decrease of cell viability by about 50%. Besides that, the cytotoxicity test with various concentrations of the sunscreen product, base, and API was also done to find the highest concentration of the sample that is not cytotoxic to the cells, which is marked by having cell viability of more than 70%. After all requirement parameters were determined, the cytoprotective assay was done to determine at which concentration the sunscreen product was able to provide the cytoprotective ability to the HaCat cells.

c.3 Description of the method

c.3.1 HaCat Cell Culture

In culturing the HaCat cells, every 2-3 days the cells were subcultured using a T25 flask once the confluency reached 80-90%. The media for washing the cells was DMEM only (Dulbecco's Modified Eagle Medium), while the passaging process was utilizing the complete DMEM that is supplemented with

1% penicillin and streptomycin, and 10% of FBS (Fetal Bovine Serum). The cells were stored at 37°C in the CO2 incubator with a 5% humidified atmosphere.

c.3.2 Miscibility Test

The sunscreen samples tested contain materials that are insoluble in waters. Therefore, a miscibility test was necessary to be conducted. According to Mizuno *et al.*, 2016, the recommended sunscreen used to the face is 2 mg/cm2. When converted into a 96-well plate scale, the requirement for sunscreen to be used is 0.6 mg. Hence the sample concentration that being prepared was 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.3125 mg/ml. To start the miscibility test, the 10 mg/ml of the sunscreen product, 1% w/w base, and API only were made with water in the centrifuge tube separately. Before being mixed with the water, the sample was put in the water bath at 60°C and the water was added gradually to the sample with continuous stirring. If the sample is still immiscible, the sample was sonicated with continuous checking every 1 hour with vortexing the tube until it became homogeneous.

c.3.3 Blue light Optimization

For the optimization of blue light, the HaCat cells were seeded into a 96-well plate with a density of 1 x 10^4 cells per well. After being seeded for 24h, the cells were put inside the blue light box and exposed to blue light. The optimization was conducted by comparing the distance between well-plate to a blue light source (10 cm and 15 cm) and comparing exposure time (6 h and 24h). The control of the experiment is the cells that were not exposed to blue light. The observation of the cells was recorded before and after the irradiation by taking a photo using an inverted microscope, and the cell viability was measured with the MTS assay. The optimum time point and distances that were chosen for future treatment in cytotoxicity and cytoprotective study were based on conditions in which the confluency of the cells was reduced by at least 50%, and or it had lower cell viability.

c.3.4 Samples sterilization with UV Light

For the cytotoxicity test, a 1% w/v for sunscreen product and 1% w/w for base and API sample were prepared with DMEM as the solvent. All samples were serial diluted up to 6 concentration in total including 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, and 0.3125 mg/ml. This experiment was utilizing UV light sterilization since heat and filtration sterilization was not feasible. After the sample solution was prepared, 150 uL of it was transferred to 96-well plates and sterilized for 15 minutes under dark conditions with a distance of 9 cm.

c.3.5 Cytotoxicity test of samples

Before the test was conducted, all sterile sample solutions including product, API, and base (vehicle) were prepared into a total of 6 concentrations (10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, and 0.3125 mg/ml). Subsequently, 100uL of each sample was transferred into each well, with the well-plate arrangement as attached in Figure 1. The negative control was cells without treatment and the blank was DMEM solution without cells. All samples were done in triplicate. This cytotoxicity test was conducted at a duration of either 6 or 24 hours depending on the time point chosen after Bluelight optimization.

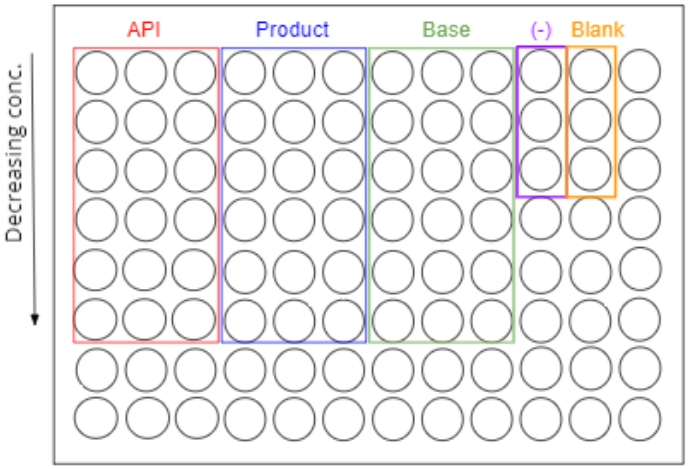

Figure 1. 96 well-plate arrangement for sample cytotoxicity test

c.3.6 Cytoprotective ability measurement against blue light exposure

After the optimization of suitable distance, time, and sample concentration for blue light irradiation study on the HaCat cells, the blue light cytoprotection ability of the product was conducted. The first method was which the sample get in contact directly with the cells. The HaCat cells were first seeded into a 96-well plate with a density of 1 x 10^4 cells per well and incubated for 24h at 37°C in a 5% CO2 humidified atmosphere. On the next day, the sterilized sunscreen product, base, and API samples with optimum concentration will be transferred 100uL into each well. Besides the samples, a negative control was cells without treatment and blank was DMEM solution without cells. All samples were measured in triplicate. Following the treatment, the MTS assay was conducted to determine the blue light protective ability of the product.

For the second method, after the HaCat cells were seeded into a well plate and incubated for 24 hours, the sunscreen product and base were applied with a dosage of 2 mg/cm^2 on the back lid of the well plate. Then the plates were exposed to the blue light and followed by the MTS assay.

c.3.7 MTS Assay

In the MTS assay, first the media in each well was discarded and the cells were washed with DMEM prior to the measurement. A 15ul of MTS reagent in cDMEM was added afterward under dark conditions. Cells were then incubated at 37oC for 3 hours in a 5% CO2 humidified atmosphere. After incubation, the result was measured with a plate reader to record the absorbance at 490 nm. The cell viability was calculated using the formula below (Sjafaraenan, Johannes, and Wulandari, 2019).

$$\%\ Cell\ Viability\ = \frac{a-b}{b-c}\ x\ 100\%$$

A = Absorbance of treatment cell
B = Absorbance of control media (DMEM only)
C = Absorbance of control cell (cell + DMEM)

c.3.8 Cell Viability Test with Trypan Blue

After the irradiation of the cells, the trypan blue will be added to the cell suspension and left for approximately 3 minutes. The stained wells were then observed under the inverted microscope and in each well, there will be 4 photos collected from different areas in the wells. The cell viability percentage was determined by using an ImageJ application by calculating the % area average of the live cells from each well.

c.4 Materials and Equipment used

The HaCat cell used in this experiment was obtained as a gift from Prof Ng Kee Woei from the School of Materials Science and Engineering, Nanyang Technological University. In culturing the cells,

the media was DMEM, a high glucose powder that contains L-glutamine and pyridoxine hydrochloride inside. There are total of three samples were used including the sunscreen product, base, and API only. The API samples were containing Bifilda Ferment Lysate, Bisabolol, Pongamia Glabra, Propanediol, Water, Artemisia Capillaris Flower extract, Saccharide Isomerate, Aqua, Citric Acid, Sodium Citrate, Titanium Dioxide, Dimethicone, Silica, Zinc Oxide, and Triethoxycaprylylsilane. For determining the cell viability, the MTS reagent was using the CellTiter 96® AQueous One Solution Cell Proliferation Assay while the cell counting was using a trypan blue.

In the incubation of the HaCat cells, the incubator that was used is a CO_2 incubator with a 5% humidified atmosphere, at 37°C. The sonicator that was used for making the sample solution miscible was the ultrasonic bath in the i3L's lab. during the UV sterilization, the light utilized was a 10-watt UVA light source with a wavelength of 352 nm. After the treatment, all cells were observed under the inverted microscope and for the MTS assay, the absorbance was measured using a microplate reader.

d. Data Management, Calculation and Statistical Analysis

All data obtained from the experiment was recorded and calculated using the formula in the method section. After that, the statistical analysis was performed in GraphPad Prism 8.0.1 by using the one-way ANOVA in cytotoxicity results data, and the two-way ANOVA method in cytoprotective results.

3. RESULT

After all tests were performed and the data has been calculated, the summary of the results data was collected and presented below using a graph. In the blue light optimization test, the cells that were exposed to the blue light for 24 hours show a very low % cell viability (<5%). Therefore, the duration parameter chosen for further experiments was 6 hours. On the other hand, for the second parameter in blue light optimization, the 15cm distance from cells to the blue light source was chosen because it results in lower cell viability with a smaller percent error. The lower cell viability was preferred for a better comparison with the treatment groups in the further cytoprotective test.

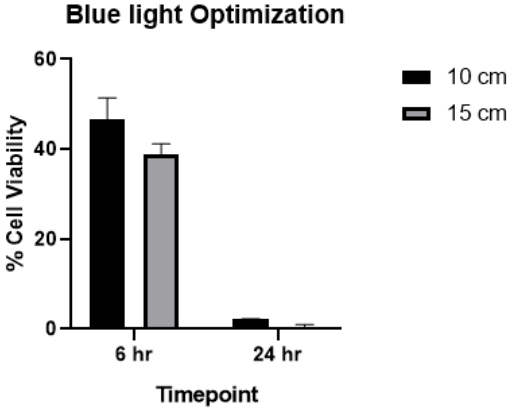

Figure 3.1 Blue light Optimization Graphic Results

In the cytotoxicity test graphic results (**Figure 3.2**), it can be seen that the base sample was already safe in a concentration below 0.5% as their cell viability percentage was already reached 100% above. Meanwhile, in the product sample, the concentration was safe starting from 0.125% below with the cell viability of 87.75% ± 10.26 above. As for the API sample, the cell viability of all six concentrations used in this experiment was showing low cell viability below 50%.

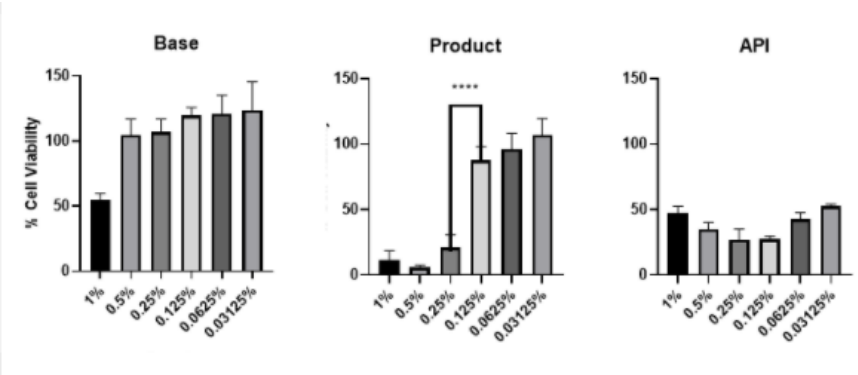

Figure 3.2 Cytotoxicity Test Graphic Results of Sunscreen product, base, and API

With the results from the cytotoxicity test, the sample percentages used for the cytoprotective test were 0.125%, 0.25%, and 0.5%. As it can be seen in **Figure 3.3**, the base sample in the plate treatment has cell viability for about 30%, which is similar to the cell viability of the internal control. Although the base concentration of 0.5% and 0.125% shows a little bit higher percentage than the control, their result is not significant so with those three concentrations, they are showing that the base did not have any cytoprotective ability towards the cell.

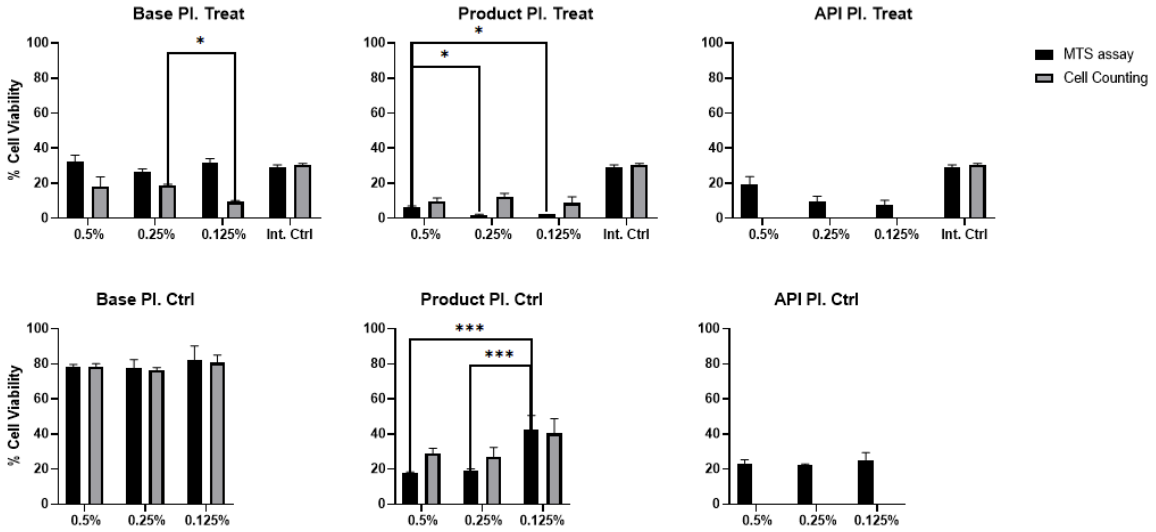

Figure 3.3 First Cytoprotective Assay Graphic Result

For the product sample, those three concentrations result in very low cell viability, indicating that the sunscreen product doesn't have a cytoprotective ability towards the cells. This low value was also affected by the cytotoxicity of the samples. Meanwhile in the API graphic results, it shows that in the concentration of 0.5% it may provide higher cell viability than the other concentration, but it still doesn't provide the cytoprotective ability because its value is still lower than the internal control. However, as the three concentrations of API sample in the plate control have similar viability and after blue light exposure only 0.5% API that can maintain the viability in approximately 20%, therefore it can be suggested that in the higher concentration, the API containing Titanium dioxide and Zinc oxide may provide a cytoprotective ability, although on the other hand it also increases the cytotoxicity effect towards the cells.

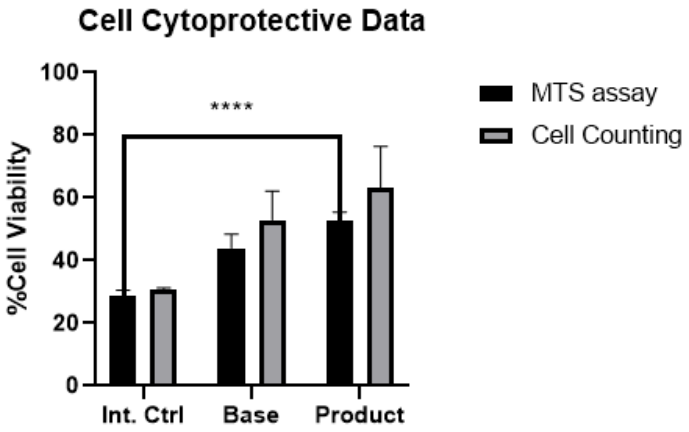

Figure 3.4 Second Cytoprotective Assay Graphic Result

When utilizing another method of a cytoprotective assay where the cells were not in direct contact with the samples, the product shows a significant increase compared to the internal control, indicating that the sunscreen product may provide a cytoprotective ability against the blue light. From the MTS assay, the product can increase the cell viability by 85% or 1,9x compared to non-treated groups, while the cell counting result shows that the product is able to increase the cell viability by about 106% (2.1x).

4. CONCLUSION

From the cytotoxicity test, the sunscreen product was safe at a concentration of 0.125%. However, the product can't provide a cytoprotective ability towards the cell in that concentration. Meanwhile, when the sunscreen product was not in contact with the cells, **the product was able to provide cytoprotection against blue light exposure by increasing the cell viability by almost 1.9x compared to non-treated sample groups, using the recommended amount of usage for sunscreen (2 mg/cm²)**.

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6. SUPPLEMENTARY DATA, TABLES AND FIGURES

Table 6.1 Blue light Optimization Results Data

	Distance	
	10 cm	**15 cm**
6 hrs	46.71% ± 4.73	38.74% ± 4.19
24 hrs	2.10% ± 0.19	0.45% ± 0.57

Table 6.2 HaCat cells under microscope for Blue light optimization test - 10 cm

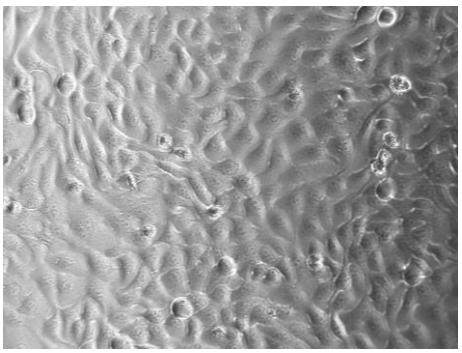
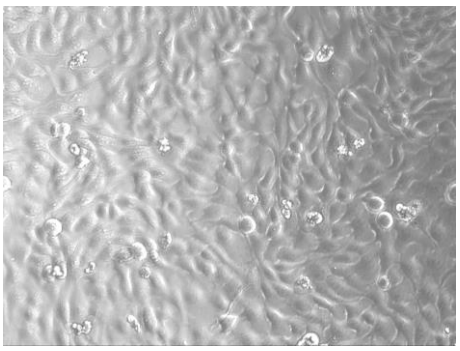
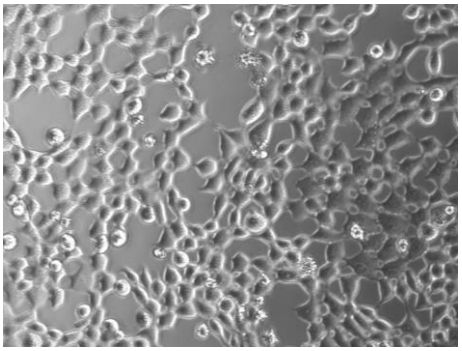
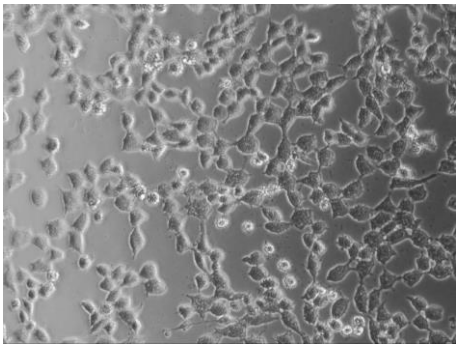
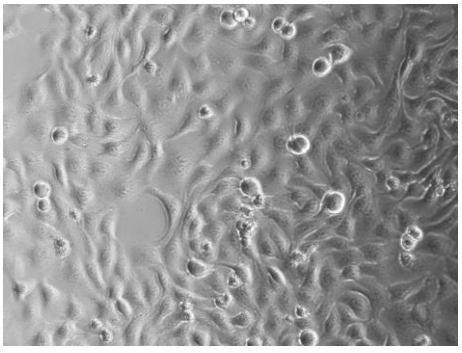
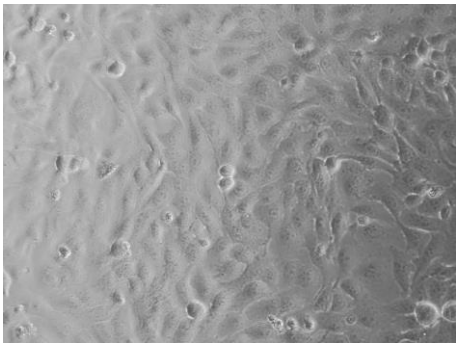
	6 Hours	**24 Hours**
Control		
Treatment		

Table 6.3 HaCat cells under microscope for Blue light optimization test - 15 cm

	6 Hours	**24 Hours**
Control		

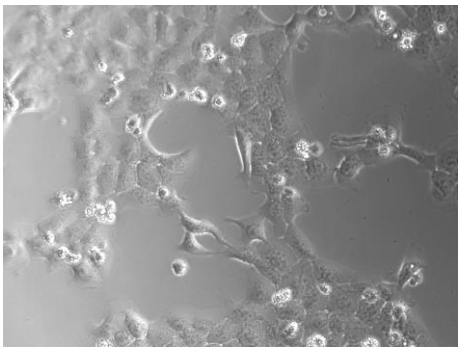
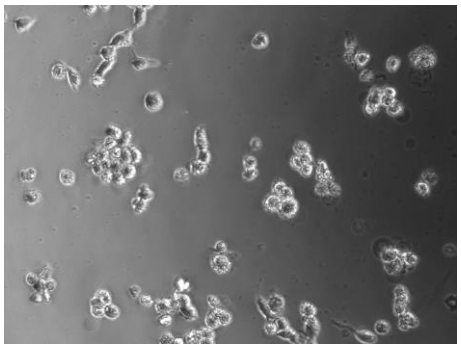
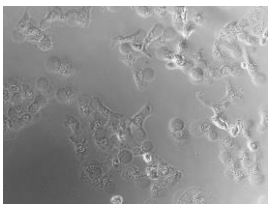
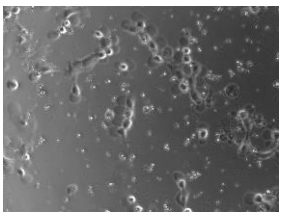
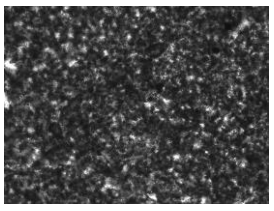
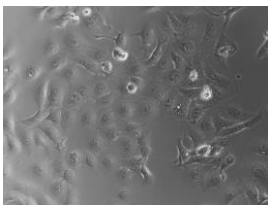
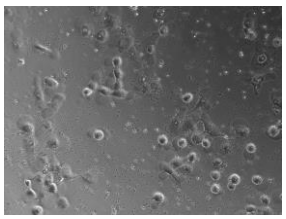
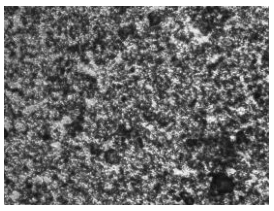
Treatment		
		

Table 6.4 Cytotoxicity Test Results Data

	Base	Product	API
1%	54.00% ± 4.77	11.82% ± 6.77	47.46% ± 5.15
0.5%	104.75% ± 11.97	5.69% ± 1.83	34.99% ± 5.11
0.25%	106.99% ± 9.70	20.99% ± 9.75	26.16% ± 8.08
0.125%	119.81% ± 5.85	87.75% ± 10.26	27.16% ± 2.72
0.0625%	121.08% ± 14.13	96.17% ± 12.11	42.84% ± 4.91
0.03125%	123.80% ± 21.79	106.78% ± 12.59	52.78% ± 1.31

Table 6.5 HaCat cells under microscope in Cytotoxicity Test

	Base	Product	API
1%			
0.5%			

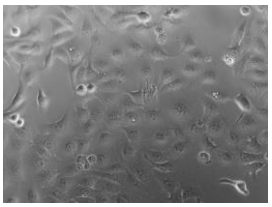
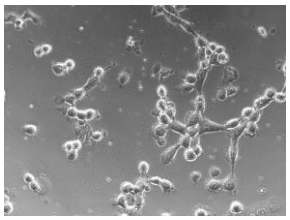
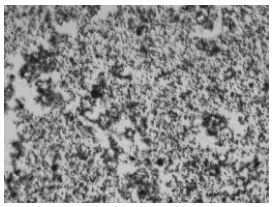
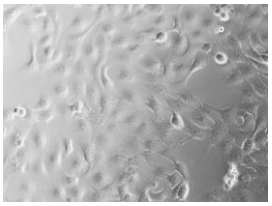
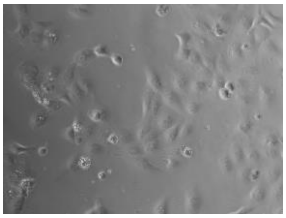
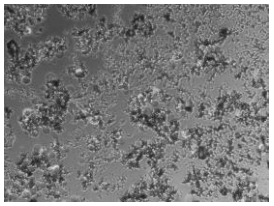
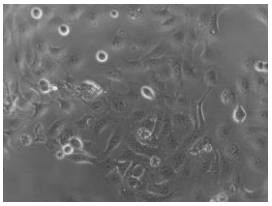
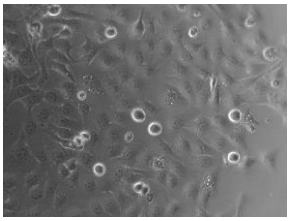
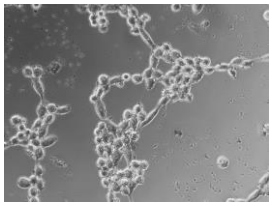
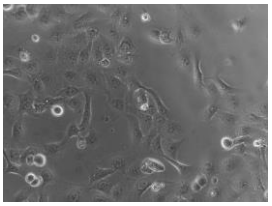
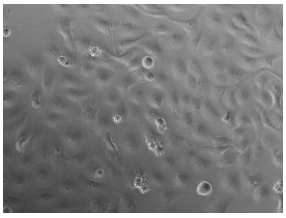
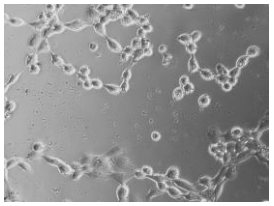
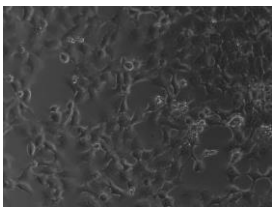
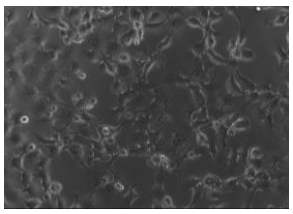
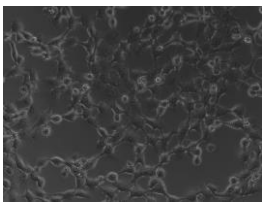
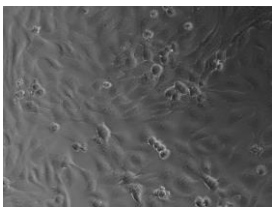
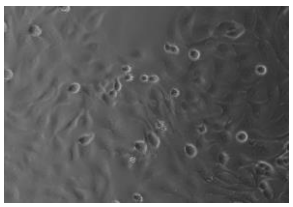
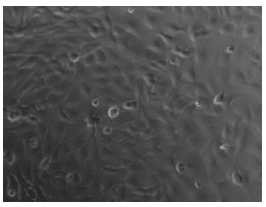
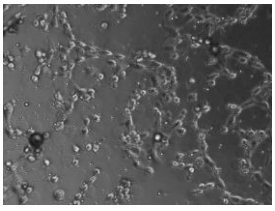
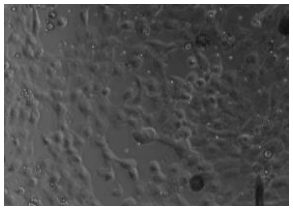
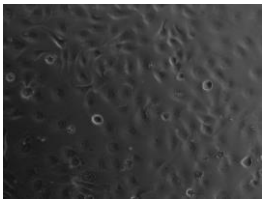
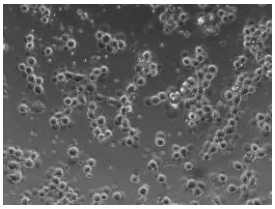
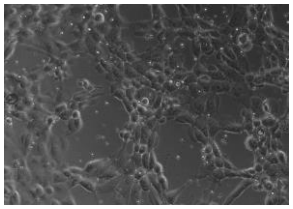
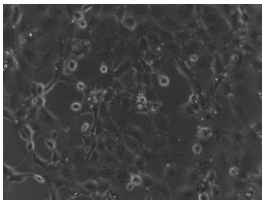
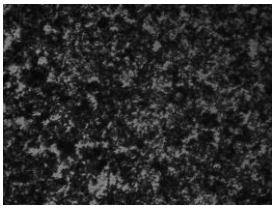
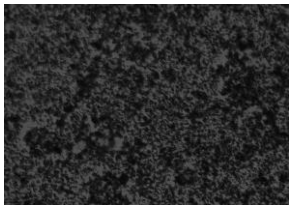
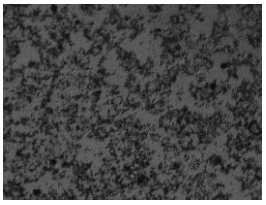
0.25%			
0.125%			
0.0625%			
0.03125%			

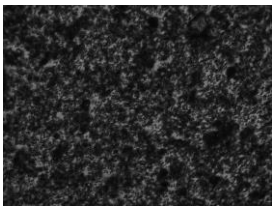
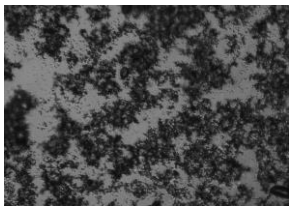
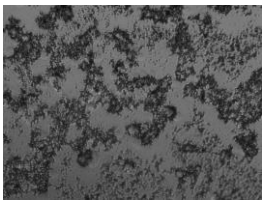
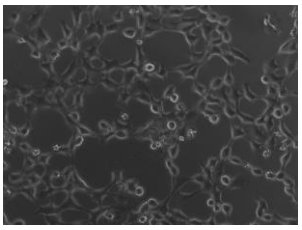
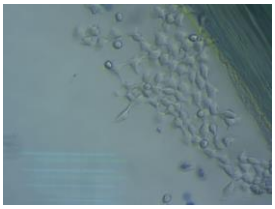
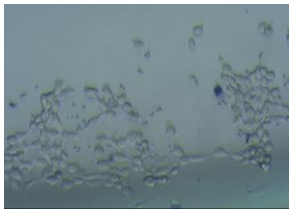
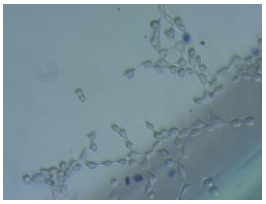
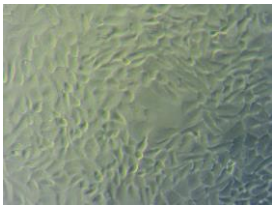
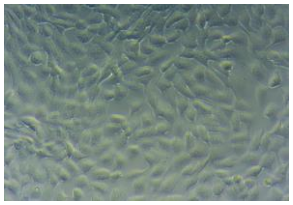
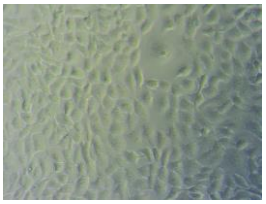
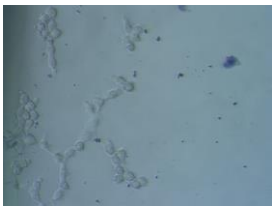
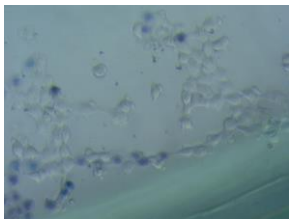
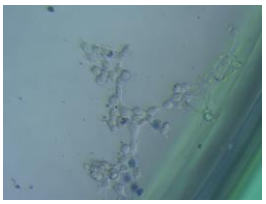
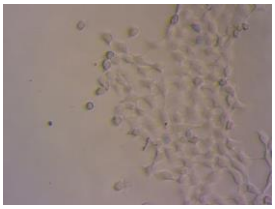
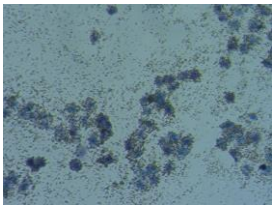
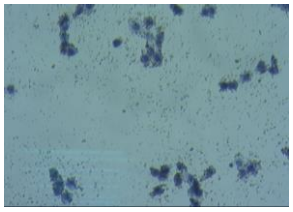
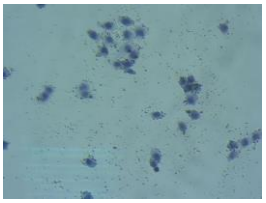
Table 6.6 First Cytoprotective Test Results Data

MTS Assay			
	0.5%	**0.25%**	**0.125%**
Base Treat	32.40 ± 3.55	26.51 ± 1.68	31.48 ± 2.58
Base Control	78.04 ± 1.71	77.37 ± 5.07	82.44 ± 7.82
Product Treat	6.30 ± 0.78	1.87 ± 0.46	2.10 ± 0.08
Product Control	17.53 ± 0.89	19.29 ± 0.86	42.78 ± 7.85
API Treat	19.50 ± 4.29	9.77 ± 2.79	7.63 ± 2.68
API Control	23.27 ± 2.11	22.39 ± 0.66	25.26 ± 4.16
Internal control	33.09 ± 1.02		
Cell Counting			
Base Treat	18.28 ± 5.44	18.62 ± 0.81	9.41 ± 0.74

Base Control	78.53 ± 1.56	76.61 ± 1.32	80.78 ± 5.44
Product Treat	9.75 ± 1.84	12.22 ± 2.06	8.67 ± 3.63
Product Control	27.72 ± 4.41	27.19 ± 5.26	40.50 ± 8.37
API Treat	-	-	-
API Control	-	-	-
Internal Control	30.64 ± 0.66		

Table 6.7 HaCat cells under microscope in First Cytoprotective Test

MTS Assay			
	0.5%	**0.25%**	**0.125%**
Base Treat			
Base Control			
Product Treat			
Product Control			
API Treat			

API Control			
Internal control			
Cell Counting			
Base Treat			
Base Control			
Product Treat			
Product Control			
API Treat			

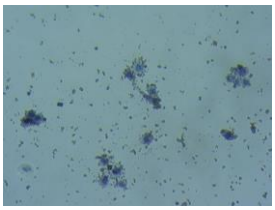
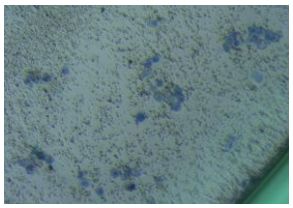
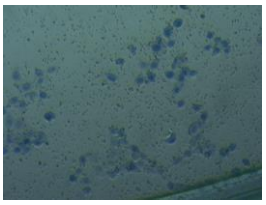
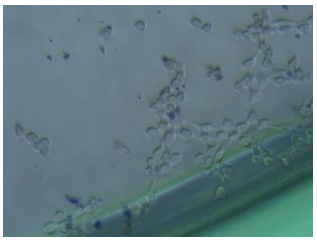
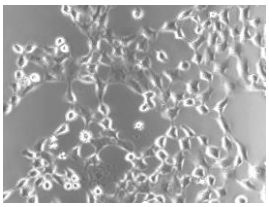
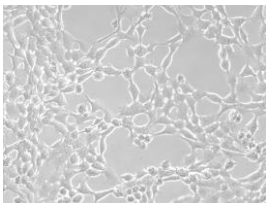
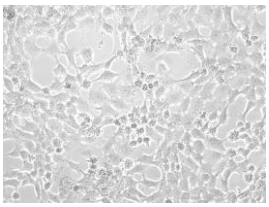
API Control			
Internal Control			

Table 6.8 Second Cytoprotective Test Results Data

	Internal Control	Base	Product
MTS assay	28.61% ± 1.70	43.96% ± 4.40	52.98% ± 2.46
Cell Counting	30.64% ± 0.66	52.99% ± 9.24	63.36% ± 12.99

Table 6.9 HaCat cells under microscope in Second Cytoprotective Test

	Int. Control	Base	Product
MTS assay			
Cell Counting