INDONESIA INTERNATIONAL INSTITUTE FOR LIFE SCIENCES

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In Vitro Cytoprotective Study On HaCaT Cells Against Pollution Models

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FOREWORD

This protocol book was written to serve as a working guideline for determining the cytoprotective activity of a certain compound or product against pollution exposure. This protocol was written following the recent trend of the increased pollution from particulate matter exposure from different types of sources. and how it will affect human health. As technology has been advancing rapidly over the past decades, the use of motorized vehicles and industrial machines has increased sharply. As a result, the exhaust from these machines is also immense, causing pollution to the air that will directly affect the skin, which is the first line of defense against pollution.

The information and guidelines presented in the protocol focus on the study and assessment of compounds and products for their pollution-cytoprotective activity. In addition, several basics related to cell culture, cell seeding, optimization, and cytotoxicity assay are also provided to further aid the experiment.

It's necessary to note that although the basics of cell culture experiments have some similarities, the conditions and cell lines can vary according to the culture condition, as well as the product type. Therefore, it is recommended to familiarize yourself with cell culture and skincare products of interest, to achieve the best results of the objectives.

Date : 15 May 2022

Sincerely,

Authors,

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1. INTRODUCTION

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 the 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).

In this research project, a cosmetic from a well-known cosmetic company 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 covers 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.

AIM OF STUDY: to measure the cytoprotective capability of the cosmetic against pollution models such as cigarette smoke extract and H_2O_2 using HaCaT cells

2. MATERIALS AND METHODS

a. 3. 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)

Test compound	Storage
Product tested	4-8°C
Base/excipient	4-8°C
Active Pharmaceutical ingredients (API)	4-8°C
API without ZnO + TiO_2	4-8°C
Product without ZnO + TiO_2	4-8°C
Internal control	4-8°C
Positive control (Ascorbic acid)	4-8°C

NOTE: these test groups can be adjusted as needed.

c. Methods

c.1 Method origin

The skin is the first line of defense against external pollutants. For this reason, the experiments are done on a human immortalized skin cell line, HaCaT cells, as it represents up to 95% of the outermost skin barrier, which is the epidermis (Colombo et al., 2017). In other words, the experiment was done on the outermost barrier of the front line defense barrier of the body. The utilization of UV lamps for sample sterilization was performed according to a previous experiment that investigated the effectiveness of UV lamps in inactivating various bacteria which may be considered one of the methods for sterilization (Mori et al., 2007).

Determination of the pollution protection performance employing 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 cosmetic 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.

In this experiment, the samples tested might contain materials that are insoluble in water. Therefore, a miscibility test was necessary to be conducted. According to Mizuno *et al*., (2016), the recommended products used on the face are usually in the amount of 2 mg/cm_2. When converted into a 96-well plate scale, the requirement for the product to be used is 0.6 mg. Hence the sample concentration that was prepared in this experiment was 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.3125 mg/ml. Some adjustments according to Liu *et al.* (2012), Warinhomhoun *et al.* (2021), and Zhang *et al.* (2020) were done for the concentration of H_2O_2 and CSE, thus concentration range of 200-1.5625 mM and 16-0.125% respectively, were tested.

c.2 Method Principle

The present method consists in evaluating the protection brought by a cosmetic product against the pollution models such as H_2O_2 and CSE, using MTS assay and spectrophotometric method, using the 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 can 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). A 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

c.3.1. HaCaT Cell Passaging/Subculture (Figure 1)

1. The confluence of the cells was checked under the inverted microscope
2. Remove the media from the T-25 flask
3. Wash the cell monolayer using 2 mL DMEM only
4. Add 1 mL Trypsin-EDTA solution into the flask
5. Incubate for 6 minutes at 37℃
6. Check the cell under an inverted microscope
7. Add 1 mL of cDMEM to deactivate the trypsin
8. Transfer the cell media suspension to 1.5 mL microcentrifuge tubes
9. Centrifuge at 2000 ppm for 4 minutes
10. Discard the supernatant without disturbing the cell pellet
11. Resuspend with 1 mL cDMEM
12. Add 4 mL cDMEM to the flask and incubate at 37°C, 5% CO2

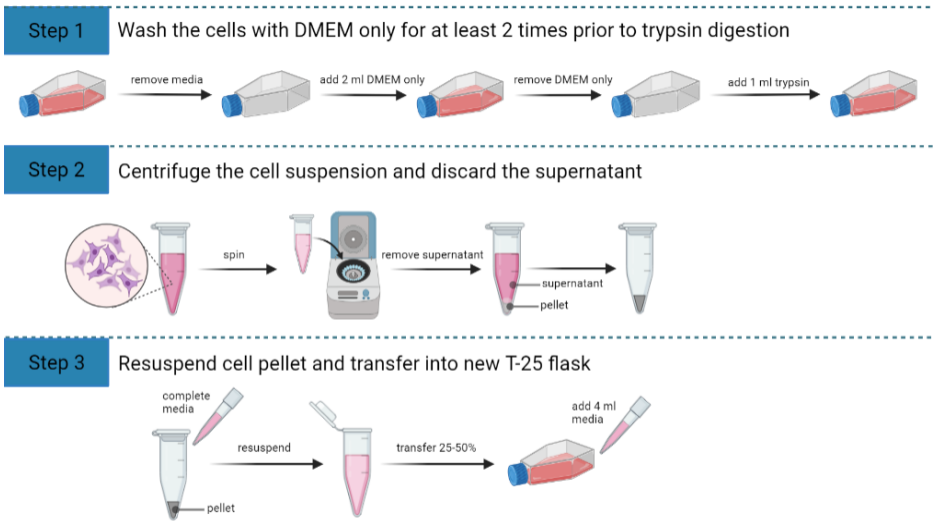

Figure 1. Overview of cell passaging/ subculture

c.3.2. HaCaT Cell Counting (**Figure 2**)

1. If cell counting is desired, aliquot 10 µl of the cell suspension to a parafilm (refer to step 10 of c.3.2.1. Primary HaCaT Cell Passaging/Subculture)

2. Dilute with 10 µl of the Trypan Blue, mix well
3. Insert 10 µl of the mixture into a hemocytometer
4. Locate the hemocytometer grid in 4x magnification
5. Locate the quadrants using 10x magnification
6. Count the number of viable cells for each quadrant, shown by transparent color
7. Determine the number of cells by using the following formula

N (cells/mL) = ½ x total cell count (4 quadrants) x 10^4

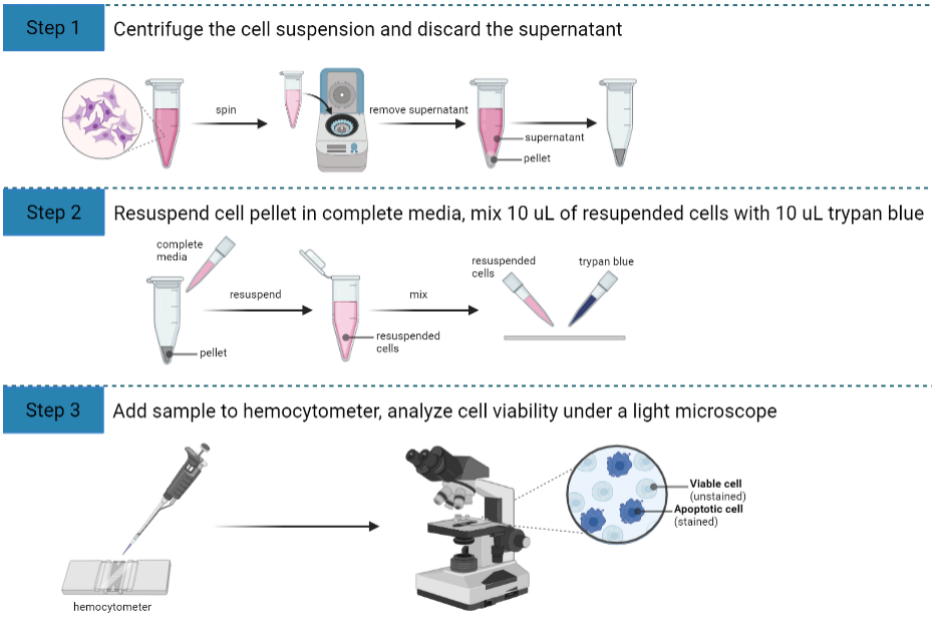

Figure 2. Overview of cell counting

c.3.3. Miscibility Test

NOTE: The miscibility test is conducted as needed, in which the sample or product should be soluble in the cell media. Do not use DMEM directly, use water instead (DMEM is water-based)

1. Calculate the concentration and the amount of the experimental design according to recommended usage.
2. Weigh the ingredients for each sample according to the recommended range of usage in different falcon tubes.
3. Add 5 mL of DW Type 3 to all the cosmetic samples in each falcon tube
4. Vortex and sonicate the samples continuously to disperse the samples homogenously

c.3.4. Cytotoxicity test of samples (MTS Assay)(**Figure 3**)

1. Seed the HaCaT cells inside 96 well plates. Each well contains 1 x 10^4 cells in 100 µl of DMEM medium. Close the lid and incubate the plate for 24h at 37°C in a 5% CO_2 humidified atmosphere.
1. When it reaches 60-80% of confluency, wash the cells, 100uL of the experimental design were added to each well in triplicate. Several concentrations of these groups are preferable, as a comparison to each other.
2. The 96-well plate is then incubated at 37°C with 5% CO_2 for 24 hours,
3. After the incubation, the cell viability is assessed with an MTS assay.
    a. Each well was washed and 100 µL cDMEM with 20 µL MTS reagent, CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, USA), were added.
    b. The plate was covered with aluminum foil and further incubation for 4 hours at 37°C, 5% CO_2.

c. The cell viability was determined by absorbance (abs) measurement using TECAN NanoQuant Infinite M200 Microplate Reader System at 490 nm
d. Determine the cell viability percentage

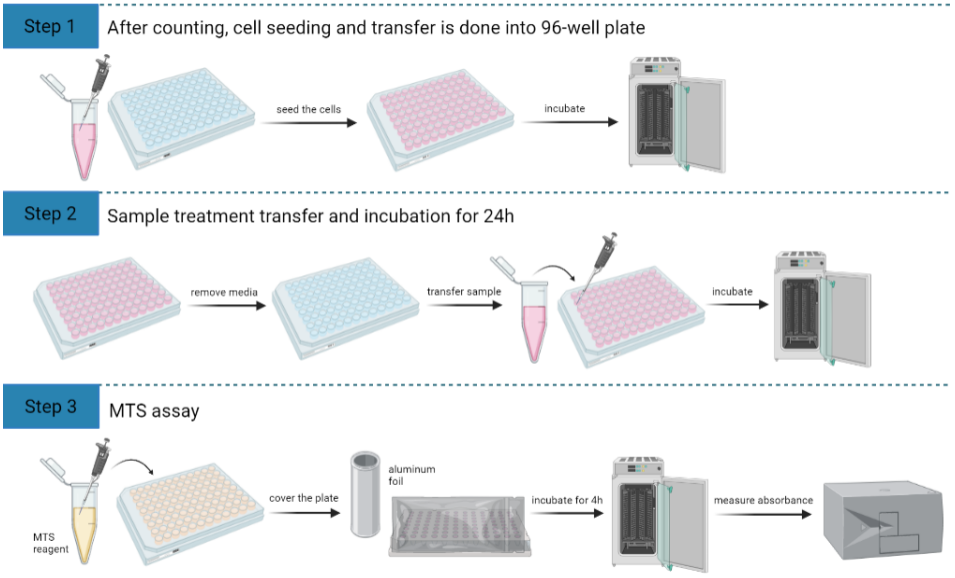

Figure 3. Overview of cytotoxicity study

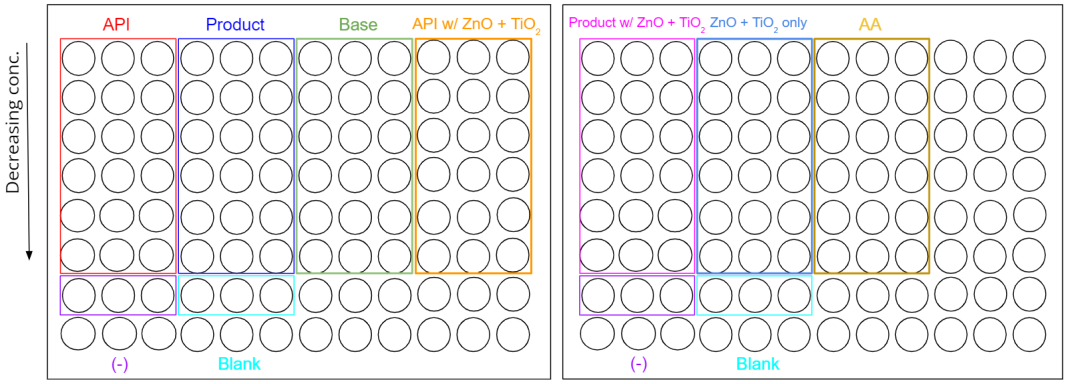

Figure 4. Well-plate layout for the experimental design

c.3.5. Cigarette smoke extraction and CSE solution preparation

1. The apparatus was made with a falcon tube, Pasteur pipette, and nylon tubing constructed together with epoxy glue as illustrated in Figure 5.
2. 10 mL of 1x phosphate-buffered saline (PBS) solution was utilized for every cigarette (Dji Sam Soe, Indonesia) to make 100% CSE solution.
   NOTE: PBS was made by dissolving 1 PBS tablet in 100 mL DW Type 3 and filtered with a 0.22 µm PES filter
3. 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. A duration of 5-10 minutes was required for the complete extraction of the cigarette.
4. Sterilization was carried out by subjecting the CSE solution to successive filtration processes by vacuum membrane filtration using Whatman filter paper no. 1 and sterile syringe filtration through a 0.22 µm-pore filter.

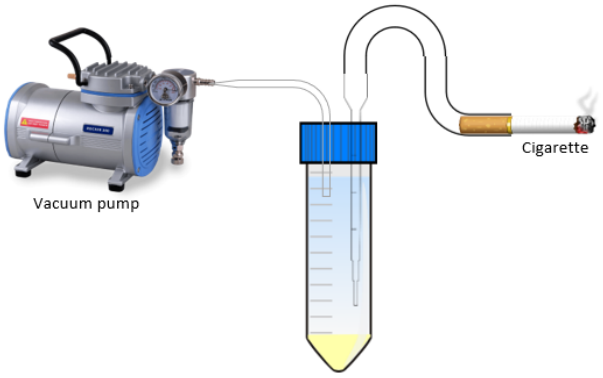

Figure 5. Cigarette smoke extraction apparatus

c.3.6. Determination of H_2O_2 and CSE concentration as pollution models (MTS Assay)

1. Seed the HaCaT cells inside 96 well plates. Each well contains 1 x 104 cells in 100 µl of DMEM medium. Close the lid and incubate the plate for 24h at 37oC in a 5% CO2 humidified atmosphere.
2. When it reaches 60-80% of confluency, wash the cells, 100uL of the pollution model solutions were added to each well in triplicate. Several concentrations of these groups are preferable, as a comparison to each other.
3. The 96-well plate is then incubated at 37°C with 5% CO_2 for 24 hours,
4. Following the incubation period, an MTS assay is performed (refer to step 3 of c.3.4. Cytotoxicity test of samples (MTS Assay))

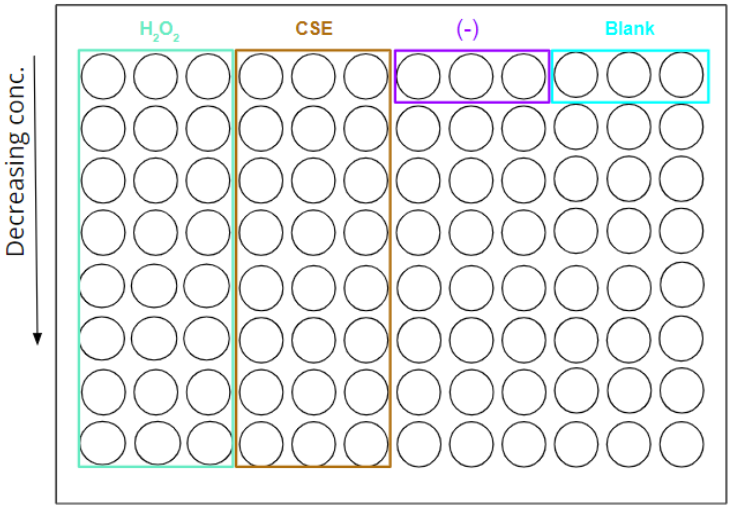

Figure 6. Well-plate layout for the Pollution models (H_2O_2 and CSE)

c.3.7 *In vitro* Cytoprotective Evaluation against Pollution Models (MTS Assay)

1. Seed the HaCaT cells inside 96 well plates. Each well contains 1 x 104 cells in 100 µl of DMEM medium. Close the lid and incubate the plate for 24h at 37°C in a 5% CO_2 humidified atmosphere.
2. When it reaches 60-80% of confluency, HaCaT cells are pretreated with the chosen concentration of cosmetic samples and ascorbic acid as a positive control
3. An incubation time of 1 hour at 37 °C with 5% CO_2 was done
4. Subsequent co-treatment with the chosen concentration of pollution models (H_2O_2 or 0.5% CSE) for 24 hours was given
5. MTS assay is performed (refer to step 3 of c.3.4. Cytotoxicity test of samples (MTS Assay))
6. The protection percentage is calculated

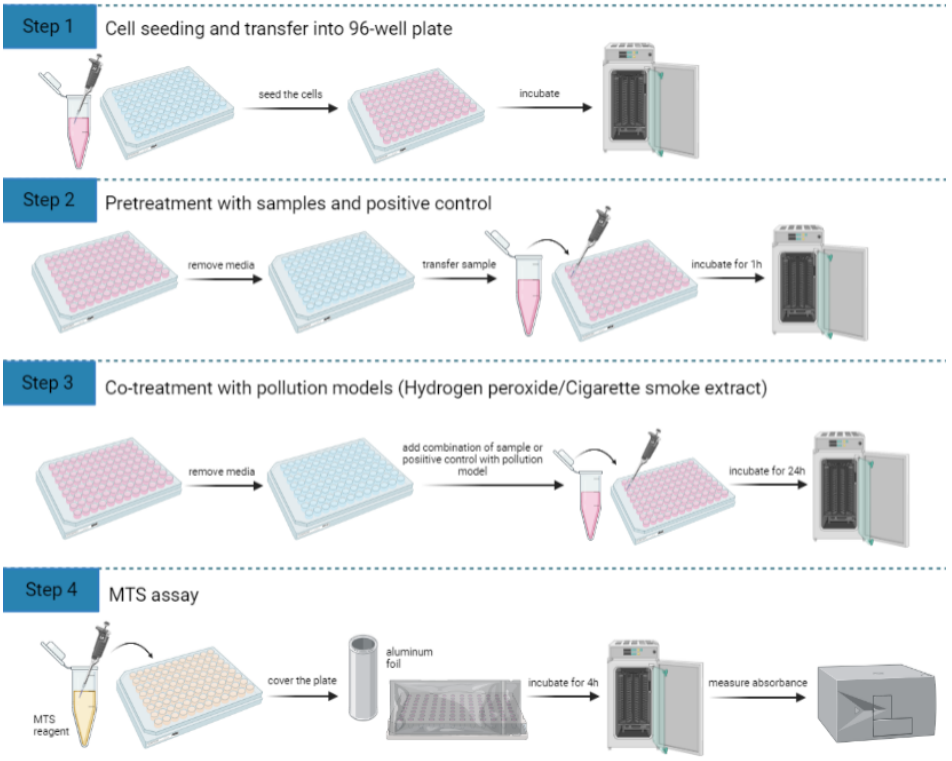

Figure 7. Overview of *in vitro* cytorprotective evaluation against pollution models

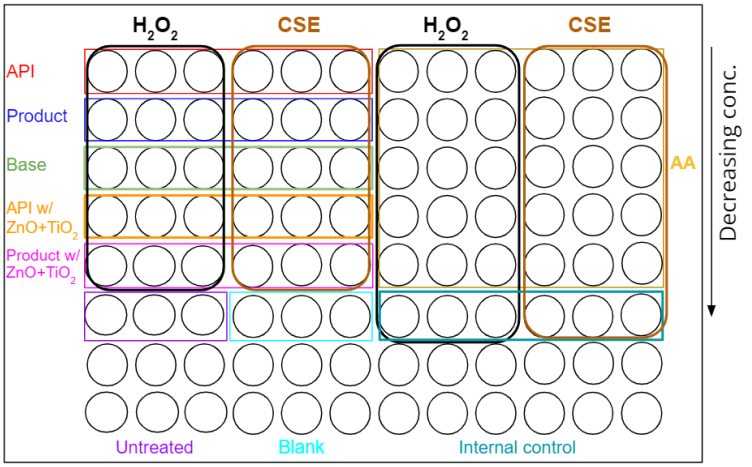

Figure 8. Well-plate layout for the *In vitro* Cytoprotective Evaluation against Pollution Models

c.4 Materials and Equipment used

1. Materials:
    a. HaCat cell from Prof Ng Kee Woei from the School of Materials Science and Engineering, Nanyang Technological University
    b. DMEM only
        i. Sodium bicarbonate
        ii. Penicillin 1% - Streptomycin 1%
    c. Complete DMEM (cDMEM): DMEM only with an addition of Fetal Bovine Serum (FBS) 10%
    d. Sunscreen product
    e. Sunscreen base
    f. Sunscreen API only, containing: *Bifida 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.
    g. MTS reagent: CellTiter 96® AQueous One Solution Cell Proliferation Assa
    h. Trypan blue
    i. 96-well plate
    j. T-25 flask
    k. 20 mL Falcon tubes
    l. Microcentrifuge tubes
    m. Pipette tips 1000, 200, 10 µL
    n. 10 mL syringe
    o. 0.22 µm PES syringe filter
    p. Cigarette smoke extraction apparatus, containing 50 mL falcon tubes, nylon tubing, Pasteur pipette, and epoxy glue.
    q. Vacuum pump
    r. Hydrogen peroxide
    s. Cigarette from Dji Sam Soe, Indonesia
    t. Ascorbic acid
    u.
2. Equipments:
    a. Biosafety cabinet
    b. A 37°C CO_2 incubator with a 5% humidified atmosphere
    c. Analytical balance
    d. Autoclave
    e. Microcentrifuge
    f. Pipette 1000, 200, 10 µL
    g. Ultrasonic bath
    h. 10 watt UVA lamp with a wavelength of 352 nm (Sankyo Denki, Japan)
    i. Inverted microscope
    j. Optical light Microscope
    k. Haemocytometer
    l. UV-Vis Microplate reader

3. DATA ANALYSIS

The absorbance data from the cytotoxicity study and *in vitro* cytoprotective evaluation obtained from MTS assay will be calculated using the formula below, to know the % Cell viability of the HaCaT cells (Mughal *et al.*, 2019).

$$cell\ viability\ (\%) = \frac{At-Ab}{Ac-Ab} \times 100$$

At = Absorbance of treatment cell
Ab = Absorbance of blank (DMEM only)
Ac = Absorbance of the untreated cell (cell + DMEM only)

After that, the statistical analysis should be performed to assess the significance, based on the preferable comparison. It is suggested to assess the data using GraphPad Prism 8.0.1 by using one-way ANOVA in cytotoxicity study and cytoprotective results data.

4. RESULT INTERPRETATION

In the H_2O_2 and CSE concentration determination, the aim is to determine the suitable concentration of each pollution model for the experiment. The main observation of this experiment was to find out at which concentration will result in around 50% of cell viability of the HaCat cell. The resulting cell viability of around 50% is preferable as it may show a more significant difference between the treated and non-treated cells in the cytoprotective assay, taking the standard deviation into consideration. However, the cell viability should not be too close to 0%, which could indicate that the cells are unhealthy, thus not preferable to be used in the following assay.

For the cytotoxicity assay, the aim is to find the highest concentration of the product, base, and API of the sunscreen that is non-cytotoxic (more than 70% cell viability) to the cell. The viability of the cells is directly proportional to the samples' degree of safety. The higher the viability percentage, the higher the number of cells that are metabolically active and considered alive. Statistically, the samples can be said to be safe if there is no significant difference between the viability percentage of the sample groups and the untreated group.

In vitro cytoprotective assay conducted following cytotoxicity study has the aim of determining the cytoprotective ability of the sunscreen product against pollution models to HaCat cells. A significant increase in the cell viability of the cells treated with sunscreen product compared to the internal control (cells exposed to pollution models, but no treatment solution given) may be interpreted as the product having cytoprotective properties against pollution.

In all of the experiments conducted above, all samples were done triplicate to optimize the accuracy of the result obtained in each assay.

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