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Grau de Química

Treball Final de Grau

Establishment of an *in vitro* biological photoassay to identify chemical photosensitizer

Bases per a la posada a punt d'un assaig fotobiològic *in vitro* per a identificar productes químics fotosensibles

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"La ciència es composa d'errors,
que a la vegada son els passos cap a la veritat".

Jules Verne

A la Dra. Montserrat Mitjans Arnal per tot el seu esforç i dedicació, per ajudar-me durant tot el transcurs de l'estudi, per ensenyar-me des de zero totes les bases per posar a punt un assaig *in vitro* i per donar-me l'oportunitat de formar part, durant aquest semestre, del seu grup de projecte.

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REPORT

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1. SUMMARY

Over the last few years, there has been an increase on the incidence of cases of individuals with skin problems, which has led to an accentuation of studies on medicines, cosmetics, or pharmaceutical products, with the aim of preventing or predicting the behaviour of these chemicals when they meet the skin simultaneously with solar radiation. In addition, the limitations that have emerged on the use of animal models and the tightening of the ethical aspects involved in working with them have caused an increase in the development of alternative methods including the *in vitro* techniques, which ensure the integrity and welfare of the animals. For this reason, the aim of this project is to carry out an *in vitro* photobiological test in order to be able to identify chemical products with photosensitising properties.

In this study, we focus on the role of keratinocytes, as these cells can help to activate photoallergic and phototoxic reactions. Specifically, we will work with the commercial cell line of human keratinocytes called HaCaT and we will evaluate the phototoxic behaviour of compounds of different characteristics such as Chlorpromazine (CPZ), Sodium Dodecyl Sulphate (SDS).

We perform a cytotoxic study as a previous step of the phototoxic assessment. The establishment of UVA irradiation dose conditions (J/cm^2) that should be applied to the cells has been set up and, thus, we have been able to evaluate the phototoxic potential of the chemicals studied. This potential is determined by calculating a photoirradiation factor (PIF) with the concentration that reduced cell viability 50% (IC_{50}) in irradiated and non-irradiated conditions and applying a prediction model. From our results, CPZ shows a very high PIF, indicating a phototoxic behaviour, whereas SDS shows similar cytotoxic activity in both situations.

In conclusion, our assay shows that can identify properly CPZ as a phototoxic chemical and SDS as a non-phototoxic one. Further chemicals with well-known phototoxic behaviour must be tested to validate the present assay.

Keywords: skin, solar radiation, *in vitro,* cytotoxicity, phototoxicity, keratinocytes, HaCaT, CPZ, SDS, Ibuprofen, Ketoprofen, IC_{50}.

2. RESUM

En aquests darrers anys s'ha produït un augment en la incidència de casos d'individus amb problemes cutanis, aquesta qüestió ha provocat que s'accentuïn els estudis sobre medicaments, cosmètics o productes farmacèutics, amb l'objectiu de prevenir o predir el comportament d'aquests químics a l'entrar en contacte amb la pell, alhora que s'exposen a la radiació solar. A més, les limitacions que han anat sorgint sobre l'ús de models animals i l'enduriment dels aspectes ètics que comporta treballar amb ells, han provocat un augment en el desenvolupament de mètodes alternatius incloent les tècniques *in vitro*, que vetllen per la integritat i el benestar dels animals. Per aquesta raó, aquest treball té el propòsit de fer un assaig fotobiològic *in vitro* amb la finalitat de poder identificar productes químics que presentin un caràcter fotosensibilitzant.

En aquest projecte volem centrar-nos en el paper que presenten els queratinòcits, ja que aquestes cèl·lules poden ajudar a activar reaccions fotoal·lèrgiques i fototòxiques. Treballarem amb la línia cel·lular comercial de queratinòcits humans HaCaT i avaluarem el comportament fototòxic que presenten diferents compostos com la Clorpromazina (CPZ) i el Dodecil Sulfat de Sodi (SDS). Per fer-ho, prèviament es realitza l'estudi citotòxic que posteriorment permet dur a terme l'estudi fototòxic. S'han establert les condicions de dosi d'irradiació UVA (J/cm^2) que cal aplicar a les cèl·lules que han permès avaluar el potencial fototòxic dels productes en estudi. Aquest potencial es determina amb el càlcul del factor de fotoirritació (PIF) mitjançant la concentració que redueix al 50% la viabilitat cel·lular (IC_{50}) en condicions d'irradiació i no irradiació i aplicant un model de predicció. Els resultats mostren que CPZ presenta un valor elevat de PIF, i per tant capacitat fototòxica, mentre que la citotoxicitat de l'SDS és similar en totes dues condicions.

Per concloure, el nostre assaig identifica de manera adequada CPZ com a fototòxic i SDS com a no fototòxic. Cal continuar assajant productes químics amb capacitat fototòxica coneguda per poder validar l'assaig descrit al present treball.

Paraules clau: pell, radiació solar, *in vitro,* citotoxicitat, fototoxicitat, queratinòcits, HaCaT, CPZ, SDS, Ibuprofè, Ketoprofè, IC_{50}.

3. INTRODUCTION

Development of medicines, cosmetics, and pharmaceutical products, implies not only efficacy but also safety evaluation of their components.

Among the different endpoints to be evaluated, photosafety testing has become a mandatory regulatory requirement for all medical and consumer products. Photosafety aims to prevent adverse effects of products and minimise the risks associated with their use [1].

Factors such as phototoxicity or photoallergy are adverse reactions that can come out when our skin is exposed simultaneously to sun light and photosensitive compounds (European Medicines Agency, 2015, EMA/CHMP/ICH/752211/2012). This ICH guideline provides information regarding the time at which photosafety testing should be carried out before the product can be exposed to a larger number of individuals and applies mainly to photodynamic therapy products, for new excipients of dermally applied on clinical formulations and active pharmaceutical ingredients [2].

To manifest phototoxic and/or photoallergic behaviour, a substance must absorb radiation, belong to the natural sunlight range (290-700 nm) and must be able to generate some reactive species when it is exposed to UV-visible light. If a chemical does not show any of these characteristics, it is most probably that the compound does not present a direct phototoxicological problem [2].

Over the years, many patients with skin pathologies have appeared due to the use of products such as halogenated salicylanilide. The number and characteristics of photosensitizsers is large and heterogeneous including sulfonamides, tetracyclines, thiazides, quinolones, sulfonylureas, phenothiazines, furocoumarins and non-steroidal anti-inflammatory drugs (NSAIDs). Moore have described the chemical characteristics that share those chemicals as low molecular weight, but the most important property is that they can absorb ultraviolet and/or visible light [3]. Moreover, topical reactions are attributed to sunscreens or cosmetic components as well as NSAIDs, being one example benzophenone-3 [4].

Traditionally, animal tests on rats, guinea pigs and mice have made possible to study the phototoxicological properties of the ingredients of products. For this reason, toxicological evaluation was carried out using laboratory animals, but in 2010, the 7th Amendment to the Cosmetics Directive (Directive 76/768/EEC) was introduced with the aim of replacing the use of animals by other techniques with the same validity [5].

Because of that the use of *in vitro* techniques using cell cultures has become more important during the last few years, due to the limitations on the use of animal models and the hardening of the ethical aspects of working with laboratory animals [6].

The *in vitro* term comes from Latin, it means inside the glass and is based on performing a specific experiment under certain controlled conditions which are carried out outside the living organism [7].

3.1. The skin

The skin is considered the most extensive organ of the human body, covering an area of approximately 1.6 square metres. We should also mention that it is the only organ that under physiological conditions and from UVB radiation can produce 7-dehydrocholesterol into calcitriol. Calcitriol is the active form of vitamin D and it is also responsible for the growth and differentiation of keratinocytes. If we order the two layers of the skin from the outermost to the innermost, we can talk about:

- **Epidermis**

Is the outer part of the skin, and it consists of squamous epithelium. It is made up of four different types of cells: keratinocytes, melanocytes, Merkel cells and Langerhans cells. Keratinocytes are the main cell of the epidermis forming four layers which are the basal layer, the stratum spinosum, the stratum granulosum and the stratum corneum. The outermost layer of the epidermis is the stratum corneum and it is formed by dead keratinocytes replenished by mature keratin [8].

- **Dermis**

It consists of fibro-elastic tissue, which is composed of a network of collagen and elastic proteic fibres synthetised by fibroblasts. In this layer we found the dermal vasculature, lymphatics, nervous cells and fibres, sweat glands, hair roots and small quantities of striated muscle [8].

Thanks to the fact that the skin is constituted by a high diversity of cells and structures, this organ has a great number of functions such as: immune, barrier, reparative, vascular and communication and attention.

Among all the functions that the skin can perform, we can emphasise the immunological function, which is responsible of preventing infections and diseases and the barrier function, which prevents the entry of harmful substances or organisms from the outside and acts as a filter for ultraviolet radiation [9].

3.2 Cytotoxicity and Phototoxicity

Cytotoxicity evaluates the ability of a molecule or compound to cause damage to cells. The adverse effects that can occur when cells come in contact with cytotoxic compounds could be alteration in the integrity of the cell membrane, synthesis and degradation of cellular components, cell division and modification of their metabolism [10]. In this work, the level of cytotoxicity of each compound is studied by exposing different concentration applied to the cells and calculating the concentration that reduces cell viability by half, named IC_{50}.

Phototoxicity evaluates the toxic response of different chemical compounds when skin cells come in contact with sunlight or when cells that have been previously treated with a chemical are irradiated [10]. In the case of phototoxic compounds, cells are damaged because the chemical is activated after exposure to radiation. This assay will also evaluate the concentration and radiation that reduces the viability of the cells by half, IC_{50} and, we will work with Ultraviolet light (UV) to evaluate the phototoxic response of our chemicals.

3.3 Light sources

Sun radiation provides us with different energies such as light and heat. Solar radiation is composed by visible light, infrared rays, and ultraviolet rays. Generally, studies have focused on the action of ultraviolet light although recently visible light (400-700 nm) is also considered an inducer of phototoxic reactions [11].

UV rays play a fundamental role on the skin and can cause significant damage depending on the type of radiation and the time of exposure. Contact with ultraviolet light can produce adverse health effects like lead to pigmentation, the formation of burns, skin ageing or even alterations and damage to deoxyribonucleic acid (DNA).

Ultraviolet light (200-400 nm) can be classified into three different types of radiation:

- **UVA (315-400 nm):** It constitutes the 95% of solar radiation that arrives at the equator and can pass through the dermis of the skin [12].
- **UVB (290-315 nm)**: This type of radiation constitutes only the 5% of solar radiation that reaches the equator. It only could pass through the outermost layer of the skin, known as the epidermis. Major part of UVB is absorbed by the atmosphere and only some radiation reaches earth [12].
- **UVC (200-290 nm):** This type of radiation is the most dangerous and toxic for the organism, however, most of this radiation is blocked by the absorption of atmospheric oxygen [12].

3.4 Current phototoxic assays

Currently, the main validated tests for assessing the photoreactivity of chemicals are the following:

- **3T3 Neutral Red Uptake (NRU):** This test is used to identify the phototoxic nature of a chemical after being exposed to UVA light. The assay is based on measuring the relative reduction in the viability of cells that have been exposed to a chemical in the absence and presence of light and it is the most frequently employed in vitro phototoxicity test [10].
- **Reactive Oxygen Species (ROS):** is an assay that helps to predict the photoreactive behaviour of a chemical by measuring the production of reactive species when photoreactive chemicals absorbs UV or visible light. It is described that generation of ROS is determinant to cause direct phototoxicity. Specifically, the method determines the production of superoxide anion (SA) and singlet oxygen (SO) by colorimetric assays [13].
- **Reconstructed Human Epidermis (RhE):** is a recently validated *in vitro* assay based on the use of reconstructed human epidermis which mimics the physiological and biochemical properties of the epidermis. This assay uses untransformed human keratinocytes as a source of cells to reconstruct it. The test is based on the quantification of cell viability by the MTT assay and their relative reduction when exposed to light compared to the non-irradiated samples [14].

4. OBJECTIVES

There has been an increase on the development of adverse reactions in the body due to substances that were contained in some household products, body care products and in products such as medicines.

This factor has caused an increase of skin and allergic diseases, which has ended up with a major health problem. Several studies revealed by the patch test that almost 30% of the European population develops allergic reactions and for that reason society must pay attention to the increase on exogenous photodermatosis and allergic dermatitis [15].

The pollution generated, which includes many chemicals that could be photosensitizers, and electromagnetic radiation can also produce adverse effects on our body, such as phototoxicity or photoallergy.

Due to the importance of these harmful effects on our organism it is important to perform phototoxicity and photoallergy tests before bringing these products to the market, to assure the safety when using them [16]. For this reason, there is a need to develop novel *in vitro* assays as a tool to both minimize the use of laboratory animals but also to improve the safety evaluation.

The main goal of the research group project (PID2020-113186RB-I00) is to develop novel alternative *in vitro* methods to identify and discriminate between phototoxic and photoallergenic compounds. **The specific objective of the present study** is to establish the conditions of an *in vitro* phototoxicity assay using a keratinocyte cell line being the subobjectives:

- To stablish the cytotoxic concentration range of different chemicals with phototoxic and no phototoxic properties in the keratinocyte cell line HaCaT.
- To determine the optimal UVA light dose to discern the phototoxic potential of different chemicals by exploring the sensitivity of cells.
- To identify phototoxic chemicals by comparing the concentration of test chemical that reduces viability to 50% in UVA light and dark conditions using a photo irritation factor.

Proyecto PID2020-113186RB-I00 financiado por MCIN/AEI/10.13039/501100011033

5. MATERIAL AND METHOD

5.1 Reagents

5.1.1 Chemicals studied

In Table 1 we can see the different chemicals studied in this work, all of them purchased at Sigma-Aldrich (Madrid, Spain).

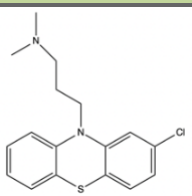
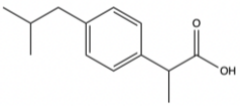
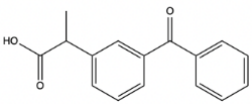
Chemical	Structure	Compound characteristics
Chlorpromazine **(CPZ)**	 **$C_{17}H_{19}ClN_2S$** 318,86 g/mol	Phenothiazine used to treat psychotic disorders. Is a first-generation antipsychotic drug that stimulates dopaminergic receptors [17].
Sodium Dodecylsulfate **(SDS)**	 **$NaC_{12}H_{25}SO_4$** 288,38 g/mol	Surfactant found in a big variety of personal care products [18].
Ibuprofen	 **$C_{13}H_{18}O_2$** 206,29 g/mol	Non-steroidal anti-inflammatory drug that blocks prostaglandin synthesis by inhibiting COX-1 and COX-2 cyclooxygenases [19].
Ketoprofen	 **$C_{16}H_{14}O_3$** 254,281 g/mol	Non-steroidal anti-inflammatory drug that blocks prostaglandin synthesis by inhibiting COX-1 and COX-2 cyclooxygenases [20].

Table 1 Chemicals studied. Structure and characteristics. All the structures have been carried out by the Chemdraw software.

5.1.2 Cell line: HaCaT

Keratinocytes are the most predominant cells in the epidermis and make up approximately 80% of the total layer, in addition they have the capacity to produce keratin and cytokines.

HaCaT (Human Adult Low-Calcium-High-Temperature Keratinocytes) cells are an aneuploid keratinocyte cell line derived from adult human skin that has been spontaneously transformed and immortalised [21].

HaCaT cells are extensively used in research because, in contrast to epidermal cells from mouse skin, human skin keratinocytes are more resistant to transformation via *in vitro* techniques and have a higher capacity for proliferation and differentiation. HaCaT cells are defined as immortalised cells and during the first studies immortalisation was achieved using the SV40 virus, but this method generated cell lines with altered differentiation [22]. This problem was solved when Petra Boukamp's research group established an epithelial cell line of human origin, which could be spontaneously transformed from adult skin, maintaining the capacity for normal epidermal differentiation. And they are called immortal because they can endure more than 40 passes (APPENDIX 1) unchanged their properties, and although they present a phenotype that is modified with the use of the *in vitro* technique, it is non-tumorigenic and therefore not harmful.

This cell line grows as a monolayer because they are adherent cells that need the presence of a surface to develop, and the reason for this is that they come from a solid tissue.

To prevent alterations on the viability of these cells, specific temperature and humidity conditions are required, as well as a specific culture medium that provides them with the nutrients that they need for their correct growth [23]. The main characteristics of the HaCaT cells are described in Table 2.

ORIGIN	They come from human skin cells, forming part of keratinocytes.
GROWTH	They grow as a monolayer; therefore, they are adherent cells that require a surface to be able to develop.
CULTURE MEDIUM	The cells are maintained in Dulbecco's Modified Eagle Medium (DMEM), which also contains 10% of fetal bovine serum (FBS), 1% L-glutamine and 1% Antibiotic.
MAINTENANCE	They are kept in an incubator at 37ºC and 5% CO_2 and humidity.

Table 2 Conditions and main characteristics of HaCaT cells [24].

5.1.3 Other reagents

Sterile phosphate buffer saline solution (PBS), which does not contain Calcium or Magnesium, supplied by Lonza (Verviers, Belgium).

Thiazolyl Blue Tetrazolium Bromide (MTT), Neutral Red Solution (NRU), Trypan Blue Solution and Ethanol and Acetic Acid, used to prepare the extinction solution, all of them were acquired from Sigma-Aldrich (Madrid, Spain).

Dimethyl sulfoxide (DMSO), which was supplied by Sigma-Aldrich (Madrid, Spain), was used to dissolve all the hydrophobic compounds.

5.1.4 Culture medium

Cells were grown and maintained in DMEM (as indicated in Table 2) 4.5 g/L glucose, supplemented with L-glutamine 2 mM, and penicillin-streptomycin solution (10,000 U/mL penicillin and 10 mg/mL streptomycin), all purchased at Lonza (Verviers, Belgium) and enriched with 10% Hyclone FBS (BioLab, Barcelona, Spain). For cytotoxicity assays, DMEM 5% FBS was used and in colorimetric assays DMEM 0% FBS without phenol red. A summary is presented in Table 3.

Medium	Composition	Application
DMEM 0%	1% L-glutamine, Antibiotic	To prepare 5% and 10% medium.
DMEM 5%	5% FBS, 1% L-glutamine and 1% Antibiotic	Cytotoxicity studies and prevents potential interferences with the products to be studied.
DMEM 10%	10% FBS, 1% L-Glutamine and 1% Antibiotic	Growing and maintaining cell culture.
DMEM without dye	1% L-Glutamine and 1% Antibiotic	To perform colorimetric assays.

Table 3 The different DMEM medium used, their composition and application

FBS, provides growth factors and hormones to cells and L-glutamine (L-Glu) is an essential amino acid that facilitates cell growth. On the other hand, antibiotics are not an essential component of the media but prevents from potential contamination especially when many users work in the same place. Finally, all media include phenol red, an organic compound that is used

as a pH indicator, to easily monitor the metabolic status of cell culture and thus cell growth. In good conditions, cell medium must be pink or lightly orangish while a yellow medium indicates an acidic ambient and a purple a basic one.

5.2 Cell culture

5.2.1 Growth and maintenance

We used the human keratinocyte cell line HaCaT (Eucellbank, Celltec UB, Universitat de Barcelona, Barcelona, Spain).

Eucellbank preserves the cells in nitrogen tanks at temperatures of approximately -196ºC. This entity supplied us the cells in a vial, and they were kept in a DMEM medium that also have FBS, antibiotic and DMSO.

Cells were acquired in a frozen vial containing DMSO as a cryopreservative, but when this substance is at temperatures above 4ºC it is toxic to the cells. For this reason, the defreezing process should be carried out as quickly as possible, to prevent that DMSO can cause the death of the cells [25].

The unfreezing process starts with a bath at 37 ºC, and once the cells are defrosted, they are transferred to a falcon tube containing 10% FBS DMEM to dilute the DMSO. Then cells are centrifuged (1200 rpm, 5 minutes) and finally, supernatant is discarded, and cells resuspended in the adequate volume of complete DMEM and transferred to 75 cm^2 flask.

After defrosting, cells were grown and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/mL:100 U/mL streptomycin-penicillin mixture (10% FBS-DMEM) at 37°C in a 5% carbon dioxide humidified incubator. Cells were regularly checked and, when they reach 80-90% confluence (cells touch to each other), subcultured into another 75 cm^2 flasks after detaching cells with Trypsin-EDTA, that was supplied by Lonza (This process is called trypsinisation and is explained in more detail in APPENDIX 2).

Aseptic techniques were followed in all the processes to prevent contaminations such as working in class II vertical laminar flow cabinet, using sterile plastic material, and using 70° ethanol to clean and disinfect cabinet surfaces and materials before and after their use [26].

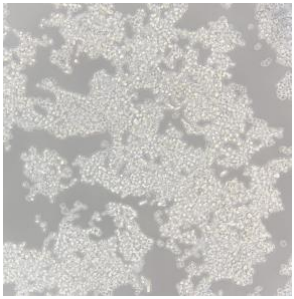


Figure 1 Microphotography of HaCaT monolayer (x100 magnification). Image made by me during the study.

5.2.2 Cytotoxicity study: treatment of the cells

For this project, we have followed the 3T3 Neutral Red Uptake guideline [10] with different adaptations after checking protocols carried out by other authors [5, 6].

Cytotoxicity is carried out over three days, during the first day the cells are seeded on the plates, during the second day cells are treated with the compound on study, and on the last day the live cells are quantified (APPENDIX 3).

Cytotoxic behaviour of SDS, CPZ, Ibuprofen and Ketoprofen was studied at a cell density of $1x10^5$ cells/mL. Previously, a cell suspension was obtained by detaching cells, when they are approximately 80% confluent, by applying Trypsin-EDTA for 6-8 minutes and inactivating it by adding fresh media to prevent cell digestion.

Then, an aliquot of the cell suspension was stained with the vital dye Trypan Blue and, thanks to a Neubauer chamber (Figure 2), living cells were counted and cell suspension was adjusted to a cell density of $1x10^5$ cells/mL.

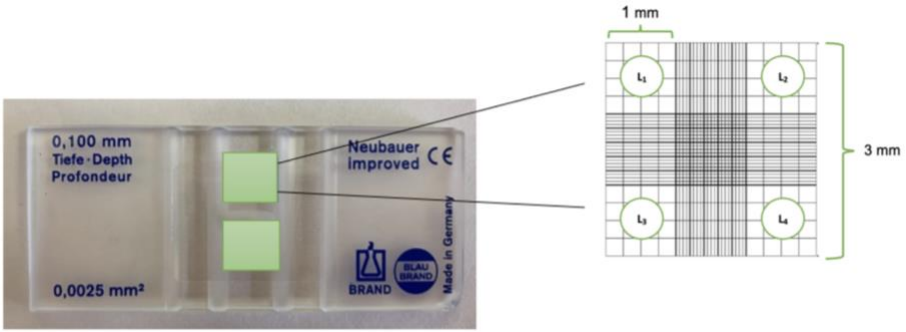


Figure 2 Graphical representation of the Neubauer chamber. Green areas indicate the squares used to count cells amplified on the right, where we can see te quadrants L1, L2, L3 and L4.

To determine cell density of our flask, we use the following equation:

$$\boldsymbol{Concentration\ of\ suspension\ (Cells/mL)} = \frac{L1 + L2 + L3 + L4}{4} \times 10^4$$

To know more about the cell counting process and the interpretation of last equation, see APPENDIX 4.

Finally, 100 µL of cells were seeded (APPENDIX 5) in 96 well plates and incubated at 37ºC and 5% CO_2. After 24 hours of incubation, cell medium was discarded, and cells were exposed to different concentrations of chemicals studied (Table 4). In each plate, untreated cells were included as controls of cell viability. Concentration of chemicals were obtained from serial dilutions in DMEM 5% FBS of fresh concentrated stock solution in DMEM 5% FBS in the case of SDS and CPZ or DMSO for Ibuprofen and Ketoprofen.

Range Concentration (µg/mL)				
CHEMICAL	**FISRT ASSAY**	**SECOND ASSAY**	**THIRD ASSAY**	**FOURTH ASSAY**
SDS	**500 - 7.81**	**150 - 12.5**	**150 - 12.5**	**150 - 12.5**
CPZ	**150 - 12.5**	**30 - 0.23**	**30 - 0.23**	**30 - 0.23**
Ibuprofen	**100 - 0.781**	**500 - 3.9**	**--------**	**--------**
Ketoprofen	**500 - 3.9**	**--------**	**--------**	**--------**

Table 4 Concentration ranges used to study the cytotoxicity of each chemical and number of assays carried out.

5.2.3 Cytotoxicity tests: determination of cell viability

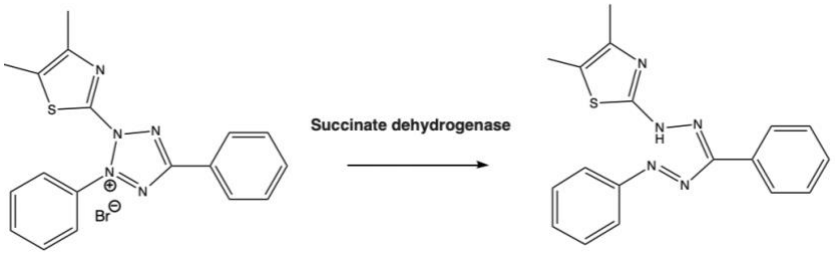
We have used two types of assays to count and evaluate the cytotoxicity of the different products.

MTT ASSAY

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay is used to determine cell survival and growth. The chemical reaction that takes place is the enzymatic reduction of the yellow tetrazolium salt and the formation of formazan, which has a blue-purple colour. The reduction is carried out by the mitochondrial enzyme succinate dehydrogenase.

The quantification of this method is carried out spectrophotometrically, where the absorbance values directly correspond to the number of live cells [27].

The reaction that takes place is:



We have followed the experimental protocol of Mosmann [28] with the modifications reported by Zanette et al. [29]. Briefly, 100 µL of an MTT solution (0.5 mg/mL in 0% FBS-DMEM without phenol red) was added in each well following incubation of the plates for at least 3 hours in cell culture incubation conditions (37°C and 5% CO_2). At the end of incubation, supernatant was replaced for 100 µL of the organic dissolvent dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. The amount of soluble formazan is proportional to the number of cells with optimal mitochondrial activity. Absorbance was measured at 550 nm using a Tecan Sunrise® microplate reader (Männedorf, Switzerland), previous homogenization of the well content by gently shaking each microplate during 10 minutes at 300-400 rpm/min.

NRU ASSAY

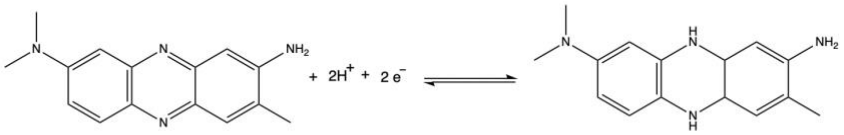
The neutral red uptake assay evaluates the number of live cells in a culture. This technique is based on the ability of living cells to introduce and fix the dye inside the lysosomes.

Neutral red uptake depends on the cell's ability to maintain the pH gradient through ATP production. The reason is that the neutral red uptake has a net charge close to zero when it is at physiological pH, and this fact allows the dye to pass through the cell membrane.

Once the dye is inside the lysosomes, there is a proton gradient that keeps the inside of the cell at a lower pH than the cytoplasm, because of that the dye increases in charge and therefore it is retained inside the lysosomes [30].

The neutral red uptake assay is evaluated spectrophotometrically, where the absorbance values correspond directly to the number of living cells, therefore it is a colorimetric assay.

The reaction that takes place is:



In this case, we followed the Borenfreund and Puerner [31] with some adaptations. Once the incubation time of the cells with the treatments had transcended, the supernatant was extracted from each well and 100 µL of NR solution was applied (0.05 mg/mL in DMEM 0% FBS without phenol red). After three hours, the supernatant was removed by inversion from the plate and 100 µL of the developer NR solution consisting in an acidic ethanol solution [32] was added. In a similar way than in the case of MTT assay, plates were stirred 10 minutes and absorbance was obtained at 550 nm (Tecan Sunrise® microplate reader, Männedorf, Switzerland).

5.2.4 Phototoxicity study

Phototoxicity is defined as a toxic reaction that occur when our body is exposed topically or systemically to specific chemicals and then exposed to light.

Here, the phototoxicity tests are based on our previous results on cytotoxicity studies, being the experimental process very similar. In this case, in the laboratory we have followed the guideline number 432 published by Organization for Economic Co-operation and Development (OECD), making some modifications [10].

This test is based on comparing the cytotoxicity induced by a chemical in conditions of light irradiation and non-irradiation of non-cytotoxic UV light [10]. For this reason, a fundamental step before study the phototoxicity caused for a chemical is to establish the adequate dose of light that must be applied to the cells. According to OECD TG 432, the quality criteria that have been established up to now, is to obtain a viability of the untreated cells exposed to light higher than 80% in relation to the untreated ones not irradiated [10].

The scheme of our phototoxic study is summarized in Figure 3 and more explained in *APPENDIX 6.*

DAY 1	DAY 2	DAY 3
1. ***Trypsinisation*** 2. ***Cell counting*** 3. ***Cell seeding***	*Treatment with the product to be studied and exposition to UVA light* ***(CPZ and SDS)***	*Evaluation of cell viability:* • *MTT* • *NRU*

Figure 3 Schematic representation of phototoxic study.

The first day, we seed the cells in 96 well plates in a similar way as explained in sections 5.2.1 and 5.2.2. In this case we prepare two plates in parallel for each chemical in study, one will be kept in dark (-Irr) and the other exposed to light (+Irr). Afterwards, cells are incubated at 37ºC and 5% CO_2 for 18-24 hours.

On the second day, cells were exposed to chemicals for one-hour at 37ºC and 5% CO_2, and then one plate is exposed to light and the other maintained in dark by covering it with aluminium foil. Concentrations assessed were based on the previous cytotoxicity studies.

Solutions of chemicals were freshly prepared in PBS to avoid the potential reactions to UVA light of DMEM components.

We performed the irradiation of the cells in a home-made chamber equipped with three fluorescent UVA lamps (Actinic BL TL/TL-D/T5; Philips, 43 V, 352 nm, 15 W), as described in Martinez et al. 2013 [1]. We have regularly been determined the lamp irradiance before samples exposure with the help of a photoradiometer Delta OHM supplied with a UVA probe (HD2302 – Italy) to stablish UV dose, using the following equation:

$$E\ (J/cm^2) = t\ (s) \times P\ (W/cm^2)$$

where E represents the UV dose, P stands for the lamp potency and t means the time expressed in seconds. Cells were irradiated with a dose of 3.3-2.8 mW/cm^2 to obtain a final exposure of 4 or 5 J/cm^2 [1].

After irradiation of plates, media was discarded and replace by fresh DMEM 10% FBS and subsequently incubated at 37ºC and 5% CO_2 overnight. Finally, next day cell viability was determined by the MTT and NRU assays as explained in previous sections.

6. RESULTS AND DISCUSSION

6.1 Cytotoxicity of chemicals

Cytotoxicity studies are designed to predict the toxicity of a substance when is exposed to cells, in this case HaCaT cells.

To determine the cytotoxic behaviour of each substance, we have used the MTT assay, which evaluates the metabolic activity of the mitochondria, and the NRU assay, which evaluates the integrity of the lysosomal membrane. Cytotoxicity studies show different results depending on the product and the concentrations used.

MTT is considered the gold standard method in many cytotoxic studies [33]. However, there exists many other assays that provide similar information such as the NRU assay or the lactate hydrogenase release assay (LDH). Moreover, the existence of potential interferences, linearity, sensitivity, and reproducibility of the assay can underestimate or overestimate cell viability. For this reason, using more than one endpoint is recommended elsewhere [34].

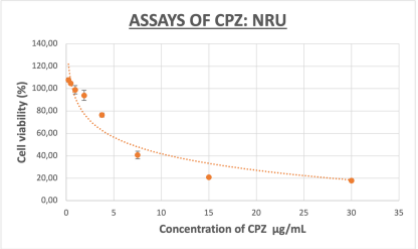
Graphically, the effect of cytotoxicity is shown as a function of the decrease on cell viability that can be adjusted to a logarithmic or linear function depending upon cell behaviour and range of the concentrations assessed. In any case, for each assay the best adjustment was chosen to calculate the concentration that induces the 50% of mortality or IC_{50}.

6.1.1 CPZ

To study the cytotoxicity of CPZ, a total of four independent experiments were performed, where in each test there were almost three for each condition assessed.

OECG TG 432 [10], describes an IC_{50} of CPZ in dark or non-irradiated conditions between 7.0 to 90.0 µg/mL in the case of 3T3 cell line (murine fibroblasts). According to this, we studied the cytotoxicity induced by this chemical at concentrations in the range of 300 to 12.5 µg/mL in the first assay. However, results obtained indicate to much cytotoxicity to calculate IC_{50}, thus cell viability recorded at the lowest concentration was almost 25% in the case of NRU and 17% in the case of MTT.

For this reason, we readjust the range of concentrations to study that was set at 30 - 0.23 µg/mL, as we can see in Figure 4.



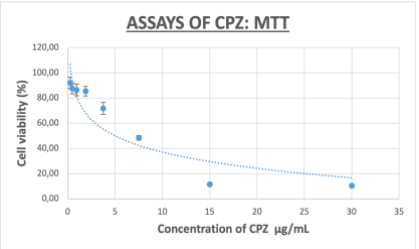


Figure 4 Cytotoxic behaviour of CPZ on HaCaT cells obtained by NRU (left) and MTT (right) assays. Viability expressed as percentage of viable cells related to untreated (control) cells. Results are expressed as mean ± ES of three independent experiments.

For each test, IC_{50} was calculated by the equation obtained after plotting the set of points by the best fitting, with the exception of the first assay. In Table 5, we can see that the values obtained for each assay and method (MTT or NRU) are very similar.

IC_{50} CPZ [µg/mL]						
ASSAY	FISRT	SECOND	THIRD	FOURTH	AVERAGE	STANDARD ERROR
NRU	------	6,8	6,2	7,2	6,7	0,3
MTT	------	4,3	4,9	6,1	5,1	0,5

Table 5 CPZ IC_{50} values obtained for each experiment and method.

In short, if we express the IC_{50} value of the CPZ compound calculating the average and the standard error of the results, we obtained:

$$\mathbf{IC_{50\ (NRU)} = 6,7 \pm 0,3\ \mu g/mL}$$

$$\mathbf{IC_{50\ (MTT)} = 5,1 \pm 0,5\ \mu g/mL}$$

6.1.2 SDS

For the cytotoxicity study of the SDS compound, a total of four experiments were performed, where in each test there were three replicates for the MTT assay and three replicates for the NRU assay.

During the first test, we worked at a concentration range of 500 - 7.81 µg/mL. In this assay, the graph showed that as the concentration of the compound increased the cell viability decreases logarithmically, as we can see in Figure 5.

However, we wanted to reduce the range of concentrations, with the aim of working in the area that presented the steepest slope in the previous graph. The purpose because we reduce the concentration range is that we want to see a change in the viability of the cells in a more progressive way and not in a suddenly way. For this reason, we worked in the range of 150 - 12.5 µg/mL. A total of three different experiments were carried out for this last concentration range, which are grouped together on the Figure 6. In both of them, we can see that above a concentration of 70 µg/mL the graph is completely flat and during the interval with the steepest slope is in the concentration range of 20-70 µg/mL. As we can see in our results, we obtain a value quite simitars between the results of the two tests (MTT and NRU), which adds more validity to our results.

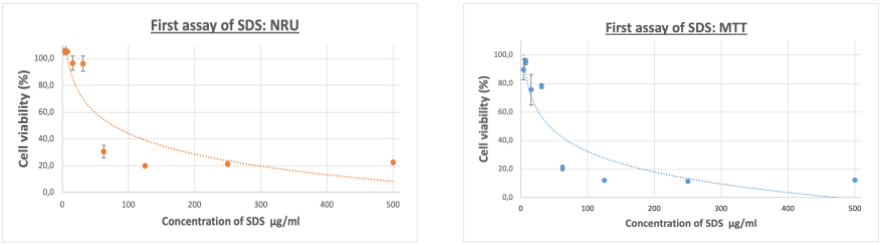


Figure 5 Cytotoxic behaviour of SDS on HaCaT cells obtained by NRU (left) and MTT (right) assays. Viability expressed as percentage of viable cells related to untreated (control) cells. Results are expressed as mean ± ES of three replicates.

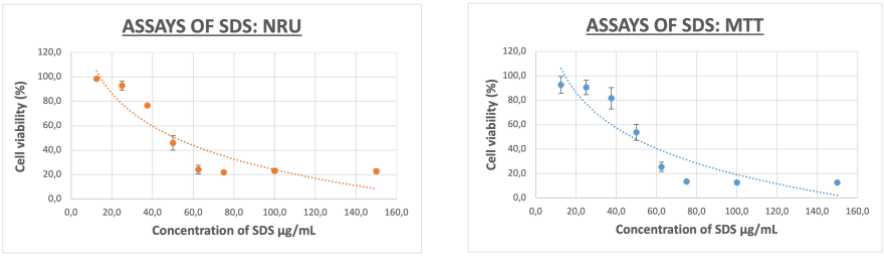


Figure 6 Cytotoxic behaviour of SDS on HaCaT cells obtained by NRU (left) and MTT (right) assays. Viability expressed as percentage of viable cells related to untreated (control) cells. Results are expressed as mean ± ES of three independent experiments.

For each of the four tests, we calculated the concentration of the compound by which the cell viability is decreased by a 50% (IC_{50}). We obtained this value from the logarithmic equation of the regression line of each graph.

IC_{50} SDS [µg/mL]						
ASSAY	FISRT	SECOND	THIRD	FOURTH	AVERAGE	STANDARD ERROR
NRU	76,5	48,4	48,8	57,2	57,7	6,5
MTT	42,9	53,3	28,0	44,8	33,8	5,3

Table 6 SDS IC_{50} values for each experiment and method.

In short, if we express the IC_{50} value of the SDS compound calculating the average and the standard error of the results, we obtained:

$$\mathbf{IC_{50\ (NRU)} = 57,7 \pm 6,5\ \mu g/mL}$$

$$\mathbf{IC_{50\ (MTT)} = 33,8 \pm 5,3\ \mu g/mL}$$

As we can see in Table 6, in the first assay, evaluated by the NRU test, we have obtained a value higher than the rest, but if we see their MTT test, the result is between the values of the others assays and the same happens with the third assay, where the test evaluated with MTT is different from the others, but their NRU follows the trend of the other assays.

Because of that, all the values must be considered to calculate the average, as a value cannot be discarded if there is not a statistical basis. Cytotoxic behaviour of SDS in the keratinocyte cell line NCTC 2544 was described by Sanchez et al 2004 [35] and Martínez et al 2006 [36] using MTT. These authors find IC_{50} values of 30.2 ± 8.5 and 43.6 ± 1.5 which are very similar to those find in this work.

6.1.3 Ibuprofen

To study the cytotoxicity of ibuprofen, we performed a total of two experiments, each one had three replicates for the MTT assay and three replicates for the NRU assay.

The problem is that this compound is insoluble in water, therefore it was not possible to perform the dilutions in PBS. Considering the solubility of ibuprofen, we could only dissolve it in organic solvents, such as DMSO. The problem arises because this organic solvent is highly toxic to cells, so we had to dissolve ibuprofen considering that cells can tolerate a maximum of 0.2% DMSO.

During the first trial we worked in a range of 100 - 0.781 µg/mL, where the first dilution presented 0.2% DMSO, but as can be seen in the APPENDIX 7, the graphs that we obtained

showed intermittent increases and decreases in cell viability. Because of that it was not possible to obtain sufficient data to calculate IC_{50} as viability does not depend on ibuprofen concentration.

For this reason, we decided to carry out a second test with a wider range of concentrations, applying a percentage of DMSO greater than 0.1% in the first three dilutions, with the aim of dissolving a higher amount of ibuprofen. In this case, a concentration range of 500 - 3.9 µg/mL was used. However, as it is shown in the graphics enclosed in APPENDIX 7 it was not possible to obtain homogenous data to calculate IC_{50}. We think that maybe we were using a very low quantity of DMSO to avoid killing the cells, and because of that the ibuprofen was not being dissolved properly.

In any case, we included a DMSO control in each plate, where cells were treated with DMEM containing the maximal amount of DMSO (0.2%) to discard the possible cellular toxic effects of this organic solvent. Other solvents need to be explored for a better evaluation of this chemical.

6.1.4 Ketoprofen

To study the cytotoxicity of ketoprofen, we performed only one experiment with three replicates for the MTT assay and three replicates for the NRU assay.

We only performed one test because we had also solubility problems and that was because Ketoprofen comes from the Ibuprofen family. Therefore, it could not react with the cells and for that reason the viability of the cells did not decrease drastically, as we can see in the APPENDIX 7. Because of that it was not possible to obtain sufficient data to calculate IC_{50} as viability does not depend on ketoprofen concentration.

6.2 Phototoxicity of substances

Phototoxicity studies assess the acute toxic response that occurs when the skin contacts with certain chemicals and then is exposed to light. When we study phototoxicity, the product can be transformed by the action of light, causing an increase in their cytotoxicity.

We only carried out the phototoxic study of CPZ and SDS, because the phototoxicity study required a previous cytotoxicity study, and these two compounds were the only ones whose concentration range was delimited. Moreover, as CPZ is a well-known phototoxic compound and SDS is a non-phototoxic one, those chemicals are important standards to set up the conditions of the assay.

By the other side, the results that we had expected to obtain for Ketoprofen and Ibuprofen were that they were photosensitising compounds, because both of them belong to the group of AINES drugs, in other words, non-steroidal anti-inflammatory drugs, but unfortunately this information has not been corroborated.

The dose of light to be applied for each experiment must be calculated at the time that the treatment is going to start, because the dose of UV light that must be administered to the plates depends on the intensity of the lamp and the time that it is exposed to it. It must be considered that with the course of the time, the UV light fluorescents lose intensity, because of that is necessary to measure it just before the exposition. The instrument used to measure the light intensity was the Delta OHM fluxmeter (HD 2302.0 LigthMeter).

Base on the OECD guideline it can be accepted that the test fulfils the quality criteria if irradiated control compounds present a viability higher than 80% compared to non-irradiated control compounds [10].

6.2.1 CPZ

For all CPZ phototoxicity tests we worked with the concentration range of 30 - 0.23 µg/mL, established previously with the cytotoxicity studies. The only factor that was modified between the different assays, was the irradiation dose, expressed in J/cm^2 to set the best dose for our studies.

In a first experiment (Figure 7) we worked with an irradiation dose of 5 J/cm^2 as it was the established in the OECD TG. Viability of untreated cells and exposed to light was compared with the untreated ones that remain in dark, to evaluate their sensitivity to UVA light. When viability was determined by the NRU test, untreated irradiated cells show a cell viability of 85.7% which is an acceptable value (recommended > 80%). However, in the case of MTT this value diminishes to 67.0% and this interferes in the assay because we can classify erroneously a chemical due to light cytotoxicity. For this reason, a second trial was performed decreasing UV light dose to 4 J/cm^2.

In the second experiment (Figure 8) cells were exposed to 4 J/cm^2 as explained before and cell viability of untreated exposed cells improve. In this case we obtain a cell viability value of 86,1% for the NRU test and 84,7% for the MTT assay. The rest of experiments were performed at this irradiation UVA dose.

Results of the third test are presented in Figure 9. Viability of the light controls with respect to the dark controls gave values of 85.9% for the NRU test and 88.3% for the MTT test.

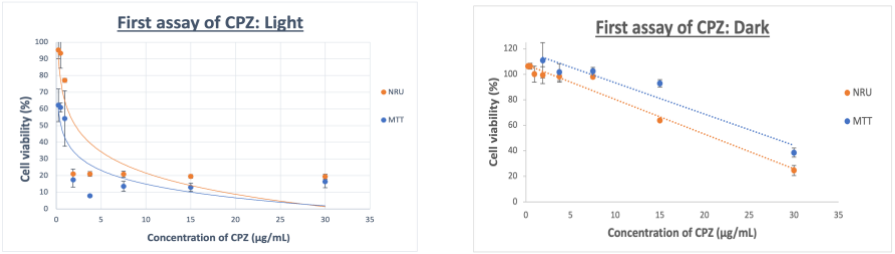


Figure 7 Phototoxic (left) and cytotoxic (right) activity of SDS in HaCaT obtained by NRU (orange) and MTT (blue) assays. Viability expressed as percentage of viable cells related to untreated (control) cells. Results are expressed as mean ± ES of three replicate. Cells were irradiated at a dose of 5 J/cm^2 after 1 hour incubation with the chemical.

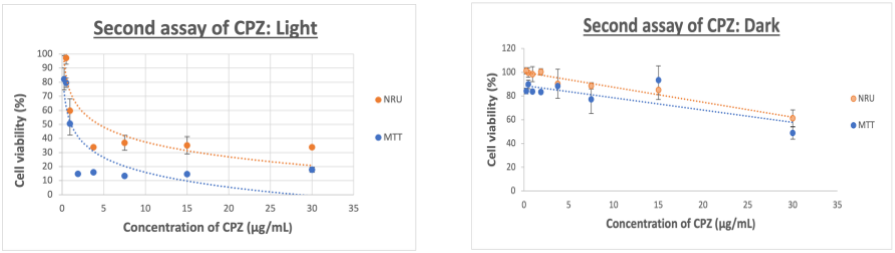


Figure 8 Phototoxic (left) and cytotoxic (right) activity of CPZ in HaCaT obtained by NRU (orange) and MTT (blue) assays. Viability expressed as percentage of viable cells related to untreated (control) cells. Results are expressed as mean ± ES of three replicate. Cells were irradiated at a dose of 4 J/cm^2 after 1 hour incubation with the chemical.

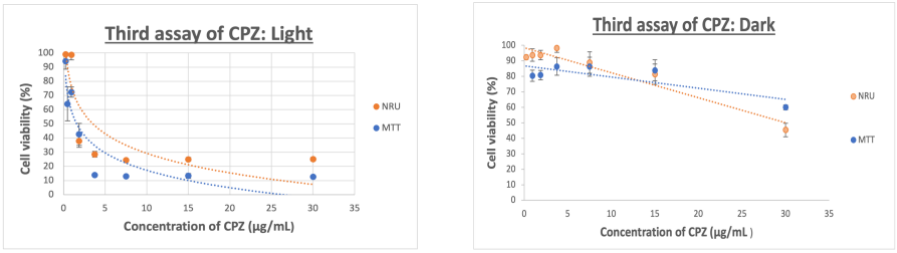


Figure 9 Phototoxic (left) and cytotoxic (right) activity of CPZ in HaCaT obtained by NRU (orange) and MTT (blue) assays. Viability expressed as percentage of viable cells related to untreated (control) cells. Results are expressed as mean ± ES of three replicate. Cells were irradiated at a dose of 4 J/cm^2 after 1 hour incubation with the chemical.

For the three phototoxicity assays performed we can finally calculate the IC_{50} in both conditions (irradiated and non-irradiated) after fitting graphically the set of plots.

IC_{50} CPZ [µg/mL]						
ASSAY	FISRT		SECOND		THIRD	
CONDITIONS	Light	Dark	Light	Dark	Light	Dark
NRU	2,1	21,1	3,4	39,8	0,7	30,0
MTT	0,5	27,7	1,1	37,6	0,6	50,8

Table 7 CPZ IC_{50} values obtained by NRU and MTT assays in light and dark conditions.

As a summary of the different assays, the average IC_{50} values for CPZ and the standard error are presented below:

- Dark plate
  - **$IC_{50\ (NRU)}$= 30,3 ± 5,4 µg/mL**
  - **$IC_{50\ (MTT)}$= 38,7 ± 6,7 µg/mL**
- Light plate
  - **$IC_{50\ (NRU)}$= 2,1 ± 0,8 µg/mL**
  - **$IC_{50\ (MTT)}$= 0,7 ± 0,2 µg/mL**

As can be seen in the phototoxicity studies the NRU and MTT values are quite different from each other. In chemistry, in most cases, when a standard error is greater than 5%, the experiment is considered invalid. Because of that, we cannot apply chemistry standards in biology because we must consider that in this case we are working with cells and there are a lot of factors that influence their viability. It should be noted that errors in cell culture are usually lower than when we work with animals, but even though the dispersion is still high.

For this reason, when we work with cell cultures, errors lower than 10% are considered acceptable. Moreover, each assay evaluates a different cellular function that can account to different sensitivity.

With cell cultures, we must take into account the following factors: the conditions of the cells, the changes that the cells may suffer during passages, the technical errors (counting, seeding, dilutions...), and other variables that cannot be controlled. In the case of phototoxicity, when the

cells are irradiated with UV light, the temperature of the environment increases significantly and depending on how the plates are positioned, they will present a determinate irradiation in function of the fluorescent's orientation. For this reason and many other causes, it is to be expected that the results will vary relatively between them.

One of the objectives of this project was to determine if the substances studied were phototoxic when they were exposed to UV light. In order to evaluate their phototoxicity, we have calculated the PIF (Photo-Irritation-Factor) by the following equation:

$$PIF = \frac{IC50\ (-Irr)}{\mathbf{IC50(+Irr)}}$$

As we can see in the last formula, we need to know the value of IC_{50} to determine it. PIF is a factor produced by comparing two cytotoxic concentrations, IC_{50}, of the test substance acquired in the presence and in the absence of non-cytotoxic irradiation with UV-A light.

Thanks to the result of the validation study [37], we can make a prediction of the behaviour of the compounds based on their PIF, as we can see in Table 8, to determine the phototoxicity of our compounds, we must see in which range the PIF obtained belong.

PREDICTION	*PIF*
No Phototoxicity	*PIF < 2*
Equivocal Phototoxicity	*PIF ≥ 2 and > 5*
Phototoxicity	*PIF ≥ 5*

Table 8 Phototoxic prediction model according to the PIF value [37].

As we can see in Table 9 all the PIF values for the different assays, independently of the test used (MTT or NRU) give a PIF value greater than five. For this reason, we can conclude that CPZ presents a phototoxic behaviour when it is irradiated with UVA light.

PIF			
ASSAY	**FISRT ASSAY**	**SECOND ASSAY**	**THIRD ASSAY**
NRU	9,92	11,15	44,70
MTT	52,96	32,23	88,59

Table 9 PIF values obtained for CPZ for NRU and MTT assays.

Due to the tests that we have carried out, we have been able to demonstrate that CPZ has phototoxicity, which can be justified through his mechanism. This drug has photochemical activity because it contains a chlorine substituent in his structure, therefore when the chemical comes into contact with UV radiation there is a dissociation of this substituent, leading to free radical reactions with lipids, DNA and proteins [38].

Studies by Grant and Green indicated that when CPZ is in solution it becomes as promazine and when it is exposed to sunlight it becomes 2-hydroxypromazine [39].

6.2.2 SDS

To carry out the SDS phototoxicity study, we performed a single test, due to the time that we had available. However, thanks to the cytotoxicity studies that we completed by triplicate and the studies described in Martinez et al. 2013 [1], we knew that this chemical must have a non-phototoxic behaviour. For this reason, although we did not present replicates, we could consider our results valid if they agree with previous studies.

We worked with a dose of 4 J/cm^2 and we obtain a value of 99,0% for the NRU test and 81,2% for the MTT assay (Figure 10). Due to these results, we were able to validate the test because it met the criteria that the viability of the controls untreated on the plate irradiated compared to the ones of the non-irradiated plate was higher than 80%. Therefore, we could accept that we were working with the correct irradiation dose.

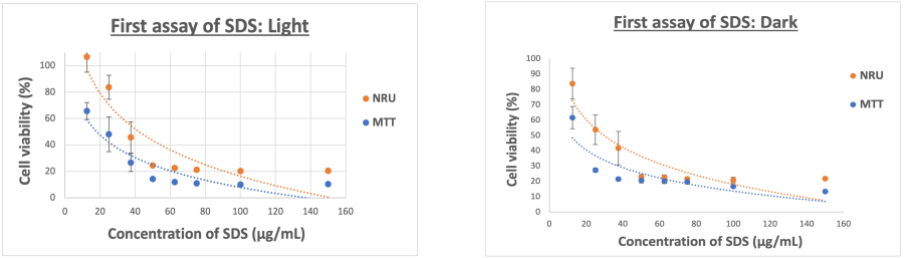


Figure 10 Phototoxic (left) and cytotoxic (right) activity of SDS in HaCaT obtained by NRU (orange) and MTT (blue) assays. Viability expressed as percentage of viable cells related to untreated (control) cells. Results are expressed as mean ± ES of three replicates. Cells were irradiated at a dose of 4 J/cm^2 after 1 hour incubation with the chemical.

Thanks to the equations of the logarithmic regression lines that we obtained for each graph, we could calculate the value of IC_{50}, which are in Table 10.

IC_{50} SDS [µg/mL]		
ASSAY	**FISRT ASSAY**	
CONDITIONS	LIGHT	DARK
NRU	42,2	29,8
MTT	18,0	11,4

Table 10 SDS IC_{50} values obtained by NRU and MTT assays in light and dark conditions.

If we compare the PIF values obtained for SDS (Table 11) with the prediction model (Table 8), we can classify SDS as a no phototoxic chemical because the PIF is lower than 2 in both tests.

PIF	
ASSAY	**FISRT ASSAY**
NRU	1,4
MTT	1,6

Table 11 PIF values obtained for SDS for NRU and MTT assays.

7. CONCLUSIONS

- Related to the cytotoxicity study, it was possible to establish the concentration ranges for SDS (150-12.5 µg/mL) and for CPZ (30 - 0.23 µg/mL), while for Ibuprofen and Ketoprofen, the concentration range could not be delimited due to solubility problems.

- With regard to the phototoxicity studies, it was possible to establish the optimum dose of UVA light that should be applied to the HaCaT cells, in order to distinguish the phototoxic potential of each compound. For both CPZ and SDS, we determined a light dose of 4 J/cm^2.

- With the help of the photoirritation factor (PIF), it has been possible to determine that the SDS compound is a non-phototoxic substance, while the CPZ chemical has been shown to be phototoxic to cells. On the other hand, the phototoxic behaviour of Ketoprofen and Ibuprofen has not been corroborated.

- This *in vitro* technique based on cell culture is a promising tool to predict the phototoxic behaviour. More chemicals with well-known phototoxic behaviour should be assayed to validate the method and their accuracy.

8. REFERENCES AND NOTES

1. Martínez, V.; Galbiati, V.; Corsini, E.; Martín-Venegas, R.; Vinardell, M.P.; Mitjans, M. (2013). Establishment of an in vitro photoassay using THP-1 cells and IL-8 to discriminate photoirritants from photoallergens. Toxicology in Vitro, 27(6), 1920- 1927.
2. European Medicines Agency, 2012. ICH guideline S10, Guidance on photosafety evaluation of pharmaceuticals, Step 5. https://www.ema.europa.eu/en/documents/regulatory-procedural-guideline/ich-guideline-s10-photosafety-evaluation-pharmaceuticals-step-5_en.pdf
3. Moore, D.E. (2002). Drug-Induced Cutaneous Photosensitivity: Incidence, Mechanism, Prevention and Management. Drug Safety, 25(5), 345–372.
4. Hyoung-June, K.; Eunyoung, L.; Moonyoung, L.; Sungjin, A.; Jungmin, K.; Jingjing, L.; Sun Hee, J.; Jaehyoun, H.; Hong, B.;Tae Ryong, L.; Minsoo, N. (2018). Phosphodiesterase 4B plays a role in benzophenone-3-induced phototoxicity in normal human keratinocytes. Toxicology and Applied Pharmacology, 338, 174–181.
5. Galbiati, V.; Martínez, V.; Bianchi, S.; Mitjans, M.; Corsini, E. (2013). Establishment of an in vitro photoallergy test using NCTC2544 cells and IL-18 production. 27(1), 103-110.
6. Galbiati, V.; Bianchi, S.; Martínez, V.; Mitjans, M.; Corsini, E. (2014). NCTC 2544 and IL-18 production: A tool for the in vitro identification of photoallergens. Toxicology in Vitro, 28(1), 13–17.
7. Real Academia Española y Asociación de Academias de la Lengua Española (2005). «in vitro». Diccionario panhispánico de dudas.
8. Martínez, V. (2007). Marcadores de irritación en modelos celulares y organotípicos como alternativa a los ensayos in vivo, aplicado al estudio de tensioactivos de tipo lipoaminoácido. Tesis Doctoral. https://www.tdx.cat/bitstream/handle/10803/1833/VMO_TESIS.pdf
9. Tortora, G. J. (2018). Principios de anatomía y fisiología (B. Derrickson, Ed.; 15a edición). Editorial Médica Panamericana; Capitulo 5; 144-170.
10. OECD, 2004. OECD Guideline for Testing of Chemicals n° 432. In Vitro 3T3 NRU Phototoxicity Test. Organisation for Economic Co-operation and Development, Paris.
11. Hinton, Andrea N.; Goldminz, Ari M. (2020). Feeling the Burn. Dermatologic Clinics, 38(1), 165–175.
12. Maverakis, E.; Miyamura, Y.; Bowen, M.P.; Correa, G.,; Ono, Y.; Goodarzi, H. (2010). Light, including ultraviolet. J. Autoimmun. 34, J247–J257.
13. OECD, 2019. OECD Guideline for Testing of Chemicals nº 495. Ros (Reactive Oxygen Species) Assay for Photoreactivity.
14. OECD, 2021. OECD Guideline for Testing of Chemicals nº 498: In vitro Phototoxicity - Reconstructed Human Epidermis Phototoxicity test method
15. Diepgen T.L.; Ofenloch R.F.; Buuze M.; Bertuccio P.; Cazzaniga S.; Coenraads P.-J.; Elsner P.; Goncalo M.; Svensson A.; and Naldi L. (2016). Prevalence of contact allergy in the general population in different European regions. Br J Dermatol. 174: 319-29.
16. Concepción Alfonso, A.R; de la Peña Pino, J.; & García Capote; J. (2007). Approaching to ethical-moral thinking of scientists working with experimental animals, Acta Bioethica, 13(1), 9–15.

17. Baumeister, A. (2013). The Chlorpromazine Enigma. Journal of the History of the Neurosciences, 22(1), 14–29.
18. Bhattarai, A.; & Niraula, T.; & Chatterjee, S. (2014). Sodium dodecyl sulphate: A very useful surfactant for Scientific Invetigations. The Journal of Knowledge and Innovation. (2). 111-113.
19. Rainsford, K. D. (2009). Ibuprofen: pharmacology, efficacy and safety. Inflammopharmacology, 17(6), 275–342.
20. Ahmed, M.; Azam, F.; Gbaj, A.; Zetrini, A.E.; Abodlal, A.S.; Rghigh, A.; Elmahdi, E.; Hamza, A.; Salama, M.; Bensaber, S.M. Ester Prodrugs of Ketoprofen: Synthesis, In Vitro Stability, In Vivo Biological Evaluation and In Silico Comparative Docking Studies Against COX-1 and COX-2. Curr Drug Discov Technol. 2016;13(1):41-57
21. Baccarin, T.; Mitjans, M.; Ramos, D.; Lemos-Senna, E.; & Vinardell, M. P. (2015). Photoprotection by Punica granatum seed oil nanoemulsion entrapping polyphenol-rich ethyl acetate fraction against UVB-induced DNA damage in human keratinocyte (HaCaT) cell line. Journal of Photochemistry and Photobiology B: Biology, 153, 127–136.
22. Boukamp, P.; Petrussevska, Rule T.; Breitkreutz, D.; Hornung, J.; Markham, A.; Fusenig, Norbert E. (1 March 1988)."Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line". *The Journal of Cell Biology. 106 (3): 761–77.*
23. Turksen, K. (2014). [Methods in Molecular Biology] Epidermal Cells Volume 1195 || Growth and Differentiation of HaCaT Keratinocytes. (Chapter 42), 33–41.
24. Jaeger, A.; Weiss, Dieter G.; Jonas, L.; Kriehuber, R. (2012), Oxidative stress-induced cytotoxic and genotoxic effects of nano-sized titanium dioxide particles in human HaCaT keratinocytes, Toxicology 296, 27-36.
25. Pegg D.E. (2007). Principles of Cryopreservation. In Cryopreservation and Freeze-Drying Protocols. Ed. Day, John G.; Stacey, Glyn N. ((Chapter 3), 39–57.
26. Rosalie J. Coté. (1998). Aseptic Technique for Cell Culture. In Current Protocols in Cell Biology,. 00 (1): 1.3.1-1.3.10,
27. Grela, E.; Kozłowska, J.; & Grabowiecka, A. (2018). Current methodology of MTT assay in bacteria – A review. Acta Histochemica, 120(4), 303–311.
28. Mosmann, T. Rapid colorimetric assay for cellular growth and survival - application to proliferation and cyto-toxicity assays. Journal of Immunological Methods 1983, 65 (1-2), 55-63.
29. Zanette, C.; Pelin, M.; Crosera, M.; Adami, G.; Bovenzi, M.; Larese, F. F.; Florio, C. Silver. (2011), Nanoparticles exert a long-lasting antiproliferative effect on human keratinocyte HaCaT cell line. Toxicology in Vitro, 25 (5), 1053-1060.
30. Repetto, G.; del Peso, A.; & Zurita, J. L. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nature Protocols, 3(7), 1125–1131.
31. Borenfreund E.; and Puerner J.A. (1985). Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicology Letters 24, 119-124.
32. Riddell, R.J.; Clothier, R.H.; Balls, M. (1986). An evaluation of three In vitro cytotoxicity assays. Food and Chemical Toxicology, 24(6-7), 469–471.
33. Van Tonder, A.; Joubert, A. M.; & Cromarty, A. D. (2015). Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays [Article]. BMC Research Notes, 8(1), 47–47.
34. Nogueira DR.; Mitjans M.; Rolim CMB.; and Vinardell M.P (2014). Mechanisms Underlying Cytotoxicity Induced by Engineered Nanomaterials: A Review of In Vitro Studies. Nanomaterials 2014, 4, 454-484.
35. Sanchez, L.; Mitjans, M.; Infante, M.R.; Vinardell, M.P. (2004). Assessment of the Potential Skin Irritation of Lysine-Derivative Anionic Surfactants Using Mouse Fibroblasts and Human Keratinocytes as An Alternative to Animal Testing. 21(9), 1637–1641.
36. Martinez, V.; Corsini, E.; Mitjans, M.; Pinazo, A.; Vinardell, M.P. (2006). Evaluation of eye and skin irritation of arginine-derivative surfactants using different in vitro endpoints as alternatives to the in vivo assays. 164(3), 259–267.

37. Spielmann, H.; Balls, M.; Dupuis, J.; Pape, W.J.W.; Pechovitch, G.; De Silva, O.; Holzhütter, H.G.; Clothier, R.; Desolle, P., Gerberick, F.; Liebsch, M.; Lovell, W.W., Maurer T.; Pfannenbecker, U.; Potthast, J. M.; Csato, M.; Sladowski, D.; Steiling, W.; and Brantom, P. (1998). The international EU/COLIPA In vitro phototoxicity validation study: results of phase II (blind trial), part 1: the 3T3 NRU phototoxicity test. Toxic. In Vitro 12, 305-327.
38. Moore, D. E. (2002). Drug-Induced Cutaneous Photosensitivity: Incidence, Mechanism, Prevention and Management. Drug Safety, 25(5), 345–372.
39. Chignell, C. F.; Motten, A. G.; & Buettner, G. R. (1985). Photoinduced Free Radicals from Chlorpromazine and Related Phenothiazines: Relationship to Phenothiazine-Induced Photosensitization [Article]. Environmental Health Perspectives, 64, 103–110.

12. ACRONYMS

- **CPZ:** Chlorpromazine
- **DMEM:** Dulbecco's Modified Eagle Medium
- **DMSO:** Dimethylsulfoxide
- **FBS:** Fetal Bovine Serum
- **HaCaT:** Human Adult Low-Calcium-High-Temperature Keratinocytes
- **IC_{50}:** Concentration that reduces cell viability by half
- **MTT:** (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
- **NRU:** Neutral Red Uptake
- **OECD:** Organisation for Economic Co-operation and Development
- **PBS:** Phosphate Buffer Saline Solution
- **PIF:** Photo-Irritation-Factor
- **RhE:** Reconstructed Human Epidermis
- **ROS:** Reactive Oxygen Species
- **SDS:** Sodium dodecyl sulfate
- **UV:** Ultraviolet light

APPENDICES

APPENDIX 1: PASSAGE OF THE CELLS

A passage is defined as the action of transferring a small number of cells to a new flask, where the cells will continuously divide until they occupy the whole available surface area. This process is performed when the cells are at 80% confluence to avoid high differentiation and to prevent the cells becoming depleted of nutrients and damaged. HaCaT cells can withstand more than 40 passages without suffer any alteration in cell characteristics and because of that we use the word immortal to define these cells.

APPENDIX 2: TRYPSINIZATION

Trypsinization is a process that has the aim of unhook the cells from the surface of the flask, and it is used when we want to make a pass or when we want to extract a suspension of cells to perform an assay.

Trypsin is added during the process. It is a protease that acts breaking the peptide bonds by hydrolysis. The reason why the cells remain attached in the flask is because the surface of the recipient contains calcium and magnesium ions, which help the cells to stick together.

The trypsinization process is started by aspirating the DMEM culture medium and it is followed by two washes with PBS. The aim of the PBS is to weaken the adherence of the cells to the surface of the flask and to clean possible traces of serum and calcium, because their presence would inhibit the trypsin that we have to added later.

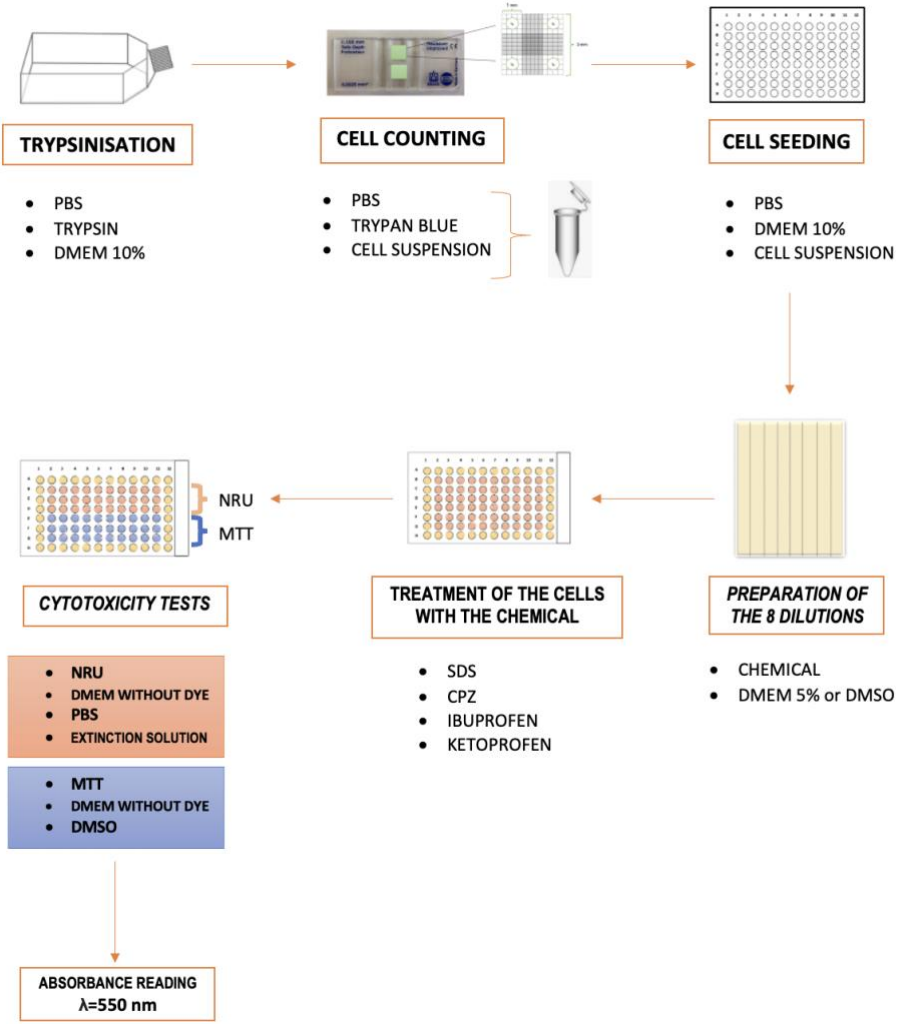
After that, 2.5 mL of trypsin is added and incubated for no more than 10 minutes (each cell line needs different times). It is important not to exceed this interval because it could cause alterations in the DNA structure of the cells, it decreases their viability.

At the end of this time, we can observe that the cells are in suspension, and we need to inactivate Trypsin. This is accomplished by adding a volume of DMEM with 10% FBS three times greater than the volume of Trypsin

Finally, a new 75 cm^2 flask is prepared, which must be identified by the user, the cell line, date, and the pass number. The volume of cell suspension added to the flask depends on the number of cells in the cell suspension and is adjusted to have a 1:5 or 1:10 dilution.

APPENDIX 3: CYTOTOXICITY DIAGRAM

Schematic representation of the different steps t followed to carry out a cytotoxic study. For each step is indicated the reagents that are needed. In total this process has a duration of three days.



APPENDIX 4: CELL COUNTING

The live cell count allows us to estimate the cell density presented in our flask, and we need to know this parameter to carry out a correct seeding.

The quantification method used in the laboratory has been the Neubauer chamber. This method allows us to discriminate between living and dead cells, as we use a dye called Trypan Blue which only stains dead or damaged cells, while the living cells have a perfect membrane and therefore the dye is not able to pass through it.

As we can see in the Figure 2, Neubauer chamber is made up of two identical squares that help to count in duplicate. With the use of the microscope, we can identify that each square is three millimetres by three millimetres long and has a surface area of 9 mm^2. For each of these squares, we can observe four quadrants at the corners (L1, L2, L3 and L4), each of them also divided by a set of 4x4, where each quadrant has a surface area of 1 mm^2.

For cell counting an aliquot of 10 µL of the cell suspension was diluted in PBS and stained with Trypan Blue (1:10) using an Eppendorf tube. Finally, the cells are counted with the help of an optical microscope.

After cell counting, we apply the equation which appears in the section 5.2.2, by obtaining the mean number of cells on the four squares and considering the volume of the sample introduced in the Neubauer chamber (10^4) and their dilution factor (1:10).

APPENDIX 5: CELL SEEDING

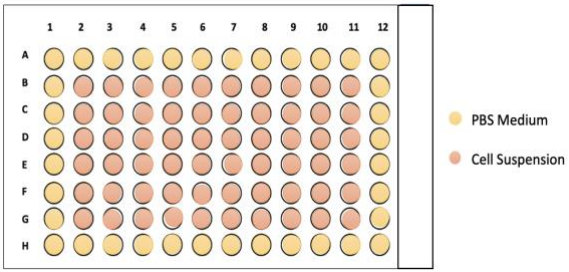
To seed the cells, it is necessary to calculate the final volume (Vf) that we want to obtain, and this value will depend on the number of plates that we have to seed. We worked with a final density (df) of $1 \cdot 10^5$ cells/mL to ensure that after 24 hours of incubation, each well would be 80% confluent.

The initial density (di) is the value obtained by the counting cells with the Neubauer chamber. So once we have the data for di, df and Vf we can calculate the initial volume (Vi) needed, using the following equation:

$$\boldsymbol{di\ x\ Vi = df\ x\ Vf}$$

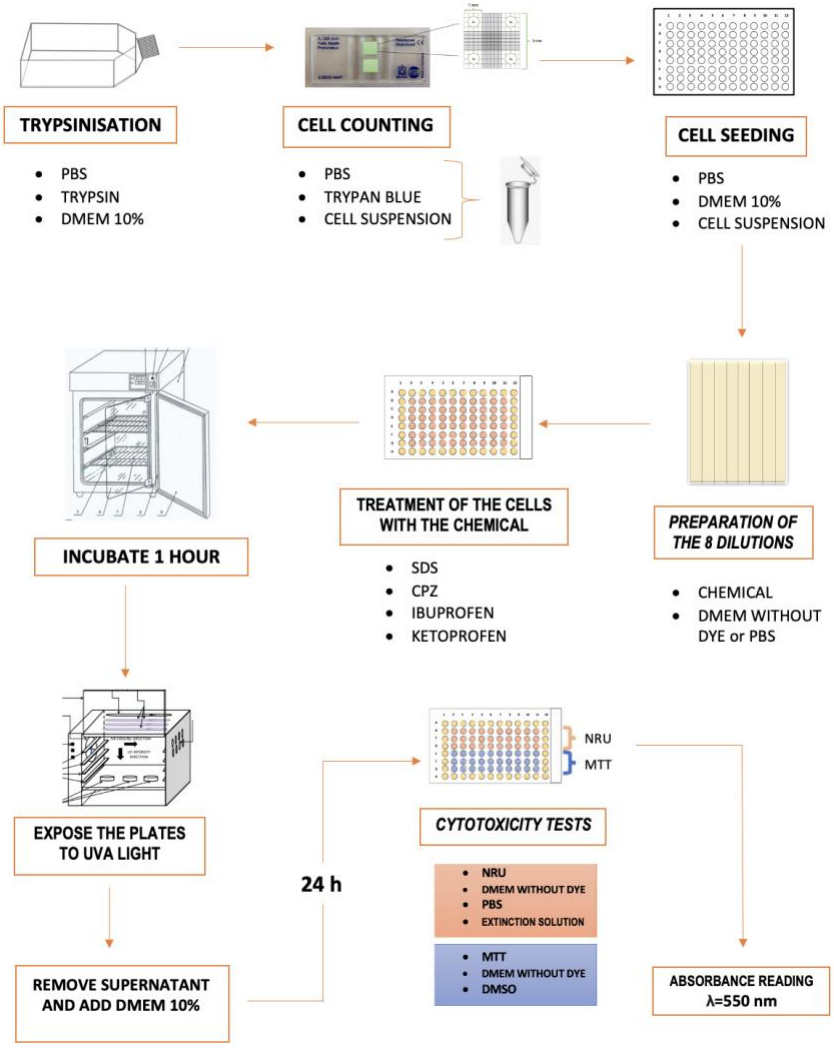
The value of the initial volume will indicate the dilution that we must make to the cell suspension to achieve the final density that we want. Once the dilution is done, cells can be seeded in the plate, but we only seed cells in 60 wells, adding 100 µL of suspension in each well. In the remaining 36 wells, which are those that are around the perimeter of the plate, we add 100 µL of PBS to eliminate the edge effect. The aim is to evaporate the reserve wells and consequently not affect the wells that contain cells, and we use PBS because is cheaper than other mediums. To conclude we want to ensure the uniformity between cells and control humidity. Finally, the plates are incubated at 37°C and 5% CO_2 humidity for a period of 24 hours. The CO_2 is used to maintain the cells in a CO_2 atmosphere.

The 96-well plates, which are made from transparent polystyrene, looks like the following figure:



APPENDIX 6: PHOTOTOXICITY DIAGRAM

Schematic representation of the steps that must be followed to carry out a phototoxic study, where for each step there are indicated the reagents that are needed. In total this process has a duration of three days.



APPENDIX 7: IBUPROFEN AND KETOPROFEN CYTOTOXICITY GRAPHS

Graphical representation of the different assays performed for ibuprofen and ketoprofen. Orange dots correspond to NRU values and blue dots to the MTT ones.

We can see that the behaviour of these two chemicals is different than the observed for CPZ and SDS compounds. The inexistence of relationship of cell viability and chemical dose exposition at the concentrations assayed, makes difficult to calculate a valid IC_{50}.