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## **Treball Final de Grau**

Comparing the cytotoxicity and genotoxicity induced by compounds with different phototoxic properties. Comparativa de la Citotoxicitat i Genotoxicitat Induïda per Compostos amb Propietats Fototòxiques Diverses.

Eloi Reig Plazas (June 2024)





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## IDENTIFICATION AND REFLECTION ON THE SUSTAINABLE DEVELOPMENT GOALS (SDG)

In September 2015, the General Assembly of the United Nations adopted the 2030 Agenda for Sustainable Development and its 17 Sustainable Development Goals (SDGs), creating a universal call to action to end poverty, protect the planet, and ensure peace and prosperity for all by 2030. This framework is being adopted by industry, governments, and many organizations worldwide.



Figure 1: List of the 17 Sustainable Development Goals (https://sdgs.un.org/goals)

The chemistry enterprise extends widely into technology, the economy, and human health. Because of it, chemistry is key to achieving a wide variety of these goals.

Due to our dedication to both science and society, and to take part and contribute to this community welfare movement, this study can engage in on the SDG #3, Good Health and Well-Being, aligning also with the P for People.

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## **1. SUMMARY**

The increased use and development of new drugs and cosmetics each year has led to an interest in the evaluation of phototoxicity. Regulatory authorities require an assessment of their phototoxic potential. A need to develop *in vitro* assays has arisen due new regulations emphasizing ethical considerations towards animal testing.

The aim of this study was to identify a reliable *in vitro* assay for photocytotoxicity testing, as well as another for assessing photogenotoxicity, using a commercial human keratinocyte cell (HaCaT) line. This study has focused mainly on chlorhexidine (CHX), though four more chemicals with known phototoxic properties were also tested: chlorpromazine (CPZ), sodium dodecyl sulfate (SDS), benzophenone (BZ) and 8-methoxypsoralen (8-MOP). Cells were incubated with the test chemicals for 1 hour, following an irradiation of 4 J/cm<sup>2</sup> UVA. After irradiation, the solution with the chemicals was removed and fresh medium was added. Cell viability was measured by MTT and LDH assays, and a comet assay was performed 24 hours after irradiation at non-cytotoxic concentrations of the compounds. Methyl methanesulfonate (MMS), a known alkylating agent, was used as a positive control for the comet assay.

CHX was classified as non-phototoxic and non-genotoxic, it was also the second agent with the lowest IC<sub>50</sub>. Other chemicals tested, aside from SDS, showed Photo-Irritation-Factor (PIF) values greater than 5. No increase in DNA damage was observed 24 hours after irradiation, likely due to DNA repair during this period. LDH assay showed inconsistent results due to the short exposure time of cells to the assay reagent, and further studies are needed to determine its reliability in photocytotoxicity testing. MTT and comet assay promise to be simple method to identify both photocytotoxic and photogenotoxic substances, which could improve the safety assessment of new pharmaceutical and cosmetic products.

Keywords: Phototoxicity, cytotoxicity, genotoxicity, in vitro, skin, HaCaT.

## 2. RESUM

L'augment de l'ús i el desenvolupament de nous fàrmacs i cosmètics cada any ha generat un interès creixent per l'avaluació de la fototoxicitat. Les autoritats reguladores exigeixen una avaluació del seu potencial fototòxic. La necessitat de desenvolupar assajos *in vitro* a causa de noves regulacions que emfatitzen les consideracions ètiques respecte a les proves animals.

L'objectiu d'aquest estudi era identificar un assaig *in vitro* fiable per avaluar la fotocitotoxicitat, així com la fotogenotoxicitat, mitjançant una línia comercial de cèl·lules de queratinòcits humans (HaCaT). Aquest estudi s'ha centrat principalment en la clorhexidina (CHX), tot i que també es van provar quatre productes químics més amb propietats fototòxiques conegudes: clorpromazina (CPZ), dodecil sulfat de sodi (SDS), benzofenona (BZ) i 8-metoxipsoralen (8-MOP). Les cèl·lules es van incubar amb els productes químics de prova durant 1 hora i després d'una irradiació de 4 J/cm<sup>2</sup> UVA. La solució amb els productes es va substituir per medi fresc. La viabilitat cel·lular es va mesurar mitjançant els assajos de MTT i LDH i es va realitzar l'assaig cometa 24 hores després de la irradiació a concentracions no citotòxiques dels compostos. El metanosulfonat de metil (MMS), un agent alquilant conegut, es va utilitzar com a control positiu per a l'assaig cometa.

La CHX es va classificar com a no fototòxic i no genotòxic, també va ser el segon agent amb la IC<sub>50</sub> més baixa. Els altres productes químics provats, excepte el SDS, van mostrar un Factor de Foto-Irritació (PIF) superiors a 5. No es va observar cap augment del dany a l'ADN 24 hores després de la irradiació, probablement a causa de la reparació de l'ADN durant aquest període. L'assaig de LDH va mostrar resultats inconsistents probablement a causa del curt temps d'exposició de les cèl·lules als compostos. Es necessiten estudis addicionals per determinar la seva fiabilitat en proves de fotocitotoxicitat. Els assaigs de MTT i cometa prometen ser mètodes senzills per identificar tant les substàncies fotocitotòxiques com fotogenotòxiques, cosa que podria millorar l'avaluació de la seguretat de nous productes farmacèutics i cosmètics.

Paraules clau: Fototoxicitat, citotoxicitat, genotoxicicitat, in vitro, pell, HaCaT.

### **3. INTRODUCTION**

The use of photosensitizing drugs and the emergence of photosensitive skin reactions have been recognized and utilized in medicine for thousands of years, dating since to the first civilization in Mesopotamia and Egypt.

The increase of photosensitization can be attributed to various factors such as, exponential increase of new drugs, chemicals, and cosmetics each year, damage to the ozone layer allowing greater UV radiation to reach the earth, and changes of social and cultural habits which led to an unusual exposure to natural sunlight and artificial sun lamps. These developments have led to an increased concern and interest in photosensitivity by dermatologists, patients, and the scientific community [1].

Photosensitivity can be caused by the absorption of solar light energy by these compounds in the skin, which can lead to the production of reactive intermediates and reactive oxygen species, causing a wide range of photoallergic and phototoxic reactions.

Consequently, regulatory authorities have requested an assessment of the phototoxic capacity of developed drugs and cosmetic products. This evaluation pertains to compounds that absorb light energy within the sunlight spectrum and reach cell in the skin or the retina either through systematic distribution or by topical application [2-4].

Traditionally, these assessments to the phototoxic capacity of drugs relied on animal test (or *in vivo* tests) like guinea pigs, rats, and mice. However, in 2010, the 7th Amendment to the Cosmetics Directive (Directive 76/768/EEC) was introduced to advocate for the replacement of animal testing with alternative techniques of equal validity. As a result, the use of *in vitro* techniques using cell cultures has got a lot more attention in recent years [5, 6].

This study focuses on Chlorhexidine as the chemical agent under investigation. Phototoxic potential will be determined using the HaCat cell line comparing the cytotoxic and genotoxic effect of the chemical with and without exposure to UV light. Moreover, various known phototoxic agents will be tested as a reference. These being chlorpromazine (CZ), sodium dodecyl sulfate (SDS) 8-methoxypsoralen (8-MOP) and benzophenone (BZ).

#### 3.1. THE SKIN

The skin is the largest organ of the human body, covering its entirety and comprising a total of 16% of its body weight. [7]

Human skin is a remarkably specialized organ with notable properties and diverse functions essential for the body. These include external protection, regulation of body temperature, sensation, absorption, and secretion of substances. [8]

The skin consists of three layers, ordered from its outer layer to the innermost, we can name.

#### • Epidermis

Is the outermost layer of the skin consisting of squamous epithelium, a high concentration of epidermal cells that are actively dividing and self-renewing the dying cells of the skin.

The epidermis is the major source of vitamin D for the body. Thanks to the ultraviolet radiation (UV) of the sun, the 7-dehydrocholesterol located in this layer is changed to vitamin D.

Vitamin D, more specifically its active form (calcitriol), is the responsible for the growth and differentiation of the major cell type of the layer, the keratinocytes. [7-9]

#### Dermis

Being the middle layer, the dermis supports the epidermis. It is composed mainly of collagen fibers and elastic fibers intertwined in a gelatinous matrix.

This matrix gives this layer hardness and resistance properties that allow it to protect the body against mechanical injuries, while providing a certain degree of elasticity to the skin. [8]

#### Hypodermis

Is the innermost layer of the skin and is characterized by tightly packed cells containing a considerable amount of fat. The subcutaneous fat layer provides thermal insulation for the conservation of body heat when blood flow to the skin is restricted. [8]

#### 3.1.1. Cell line: HaCaT

Human Adult Low Calcium High Temperature (HaCaT) is a spontaneously immortalized aneuploid human keratinocyte line with the same characteristics of basal epidermal keratinocytes.



Figure 2. Photograph of HaCaT cell morphology in culture

Due to its resemblance of keratinocytes found in the epidermis, exhibiting normal differentiation, and having the capacity of being used as an *in vitro* model for its higher proliferation capacity, HaCaT is extensively employed in assays and scientific investigations of skin biology [10, 11].

HaCaT will be the focus of this study, subjecting the cells to various pharmaceutical compounds. Subsequently, assays will be conducted to evaluate the cytotoxicity and genotoxicity of these compounds.

#### **3.2. CYTOTOXICITY, GENOTOXICITY AND PHOTOTOXICITY**

Cytotoxicity refers to how a substance can be toxic to cells. Adverse effects produced by the exposition of a cytotoxic agent to a cell can lead to necrosis or apoptosis, also known as programmed cell death, by the disruption of the cell membrane, reducing the cell viability [12]. There are several methods used for quantifying the cytotoxicity on cells, such as MTT, LDH or Neutral red uptake (NRU) assays. In this study, the decrease of cell viability will be quantified by exposing different concentrations of multiple cytotoxic compounds to cells and calculating the half-maximal inhibitory concentration (IC<sub>50</sub>). This IC<sub>50</sub> indicates the drug concentration required to reduce cell viability by 50%.

Genotoxicity can be defined as how a substance can induce damage to the DNA of the cell. This damage can lead to the development of carcinogenic malignancies if the cell fails to undergo apoptosis to prevent the expression of those mutations [13]. Damage of the genetic material can be assessed with the comet assay, as well as other assays [14]. Phototoxicity is the acute toxic response that occurs after the initial exposure of certain substances and a subsequent exposure to sunlight [15]. For phototoxic agents, cell damage is observed because the compound is activated after exposure to radiation. In this study, to decide whether the chemical is phototoxic or not, a Photo-Irritation Factor (PIF) could be calculated, being the ratio of IC<sub>50</sub> of non-irradiated over irradiated samples (Equation 1) [16].

Table 1: Prediction model of phototoxicity by PIF

IC <sub>50</sub> (UV-)	
IC <sub>50</sub> (UV+)	

Equation 1: Photo-irritation Factor (PIF) formula

PIF < 2	NO
	Phototoxic
2 < PIF < 5	PROBABLE
	Phototoxic
5 < PIF	Phototoxic

Although sunlight is the cause for phototoxicity, all sun radiation is not equally reactive for skin and phototoxic agents. Sunlight is composed by ultraviolet rays (200-400 nm), visible light (400-700 nm), and infrared rays (700 nm to 1000  $\mu$ m). The former is known for its capacity to induce phototoxic reactions and is the general focus of studies, although recently visible light is also getting recognition as having some of the same photoreactive properties [17].

Ultraviolet light can be classified in three different groups of radiation:

- UVA (320-400 nm): Constituting of 95% of solar radiation that arrives the earth, UVA radiation can pass through the epidermis and dermis of the skin. Is also the source of radiation used in this study [18].
- UVB (280-320 nm): Being the last 5% of solar radiation that reaches earth, mostly absorbed by the atmosphere, UVB is only capable of pass through the epidermis, and because it has higher energy levels than UVA, UVB is the main source of sunburns [18].
- UVC (200-280 nm): This radiation does not reach earth as it is blocked by the absorption of atmospheric oxygen, however, UVC is the most toxic and dangerous radiation for the human organism, for its energy levels are the highest in this group [18].

#### **3.3. PHOTOTOXIC ASSAYS**

At present, the main validated *in vitro* assays for quantifying the phototoxicity of chemicals are the following:

- The 3T3 Neutral Red Uptake (NRU) assay is used to determine the phototoxic nature of a chemical succeeding an exposure to UVA light. The assay involves the measuring of the cell viability after being exposed to a chemical both with and without light presence. Cytotoxicity being proportional to the reduction of the absorption of the Neutral Red dye [19].
- The Reactive Oxygen Species (ROS) assay predicts the photoreactive behavior of a chemical by quantifying the production of reactive species after the irradiation of visible light or UV, more specifically, generation the of superoxide anion and singlet oxygen, using colorimetric assays. The generation of these ROS being indicative of phototoxic potential [20].
- The Reconstructed Human Epidermis (RhE) assay mimics the biochemical and physiological properties of the epidermal human skin, using reconstructed human epidermis tissues, such as HaCaT. To quantify the photoreactivity of chemicals an MTT assay is performed. It quantifies the cell viability determining the relative reduction of the MTT when exposed to light opposed to those not irradiated [21].
- The Comet assay uses an electrophoresis instrument, enabling the measurement of DNA damage of eukaryotic human cells subjected to phototrauma. After a lysis step for the cells, damaged DNA fragments migrate further and faster than those undamaged. Thus, creating the comet-like structures that can be observed through a fluorescence microscopic, using fluorescent dye to stain the DNA [15].

There are also other not validated, but equally effective and popular assays, like the Lactate dehydrogenase (LDH) assay, that are also commonly used to perform *in vitro* assays for quantifying the phototoxicity of chemicals.

In this study we perform the MTT, LDH and comet assay to assess the cytotoxicity and genotoxicity of the chemicals used.

#### 3.3.1. MTT assay

The MMT assay is a colorimetric assay based on the reduction of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, turning to the yellow tetrazole salt to the purple hydro-insoluble formazan crystals, seen in figure 3. This chemical reaction takes place inside the mitochondria of metabolically active cells [22]. Being the reaction:





Therefore, the quantification of cell viability is carried out spectrophotometrically, where the absorbance is directly proportional to the number of live cells.

#### 3.3.2. Lactate dehydrogenase assay

L-Lactate dehydrogenase (LDH) is an enzyme present in the cytoplasm. During the glycolysis, catalyzes the conversions of pyruvate to L-lactate and NADH to NAD+, and the reverse reactions during the Cori cycle. In response to cellular damage, either through endogenous mechanisms or external trauma, LDH is released from the cytoplasm into the extracellular matrix [25].

Experimentally, LDH activity is typically determined by utilizing a coupled enzymatic reaction, where LDH oxidizes lactate to pyruvate, which subsequently reacts with iodonitrotetrazolium chloride (INT) to form formazan. Formazan, same as the MTT assay, is water soluble and can be easily detected using colorimetry, measuring the absorbance at 490 nm. For this reason, any increase in the production of formazan in the culture supernatant is directly correlated with cell death [25].

#### 3.3.3. Comet assay

The *in vivo* alkaline single cell gel electrophoresis (comet) assay is a common technique to measure genotoxicity. In this assay, cells exposed under low concentrations of phototoxic agents are used, this is because the trauma to the cell should be moderate in order not to kill many cells and to be able to observe the damage in the genetic material. Suitable chemical concentrations are those ranging from 10% to 30% induced mortality.

Damaged cells are collected and immobilized in slides using the gel properties of agarose. Cells are treated with a lysis buffer to remove cellular and nuclear membrane, following an exposure to a strong alkali solution to allow DNA unwinding and release of relaxed DNA loops and fragments. An electrophoresis step takes place to migrate the negatively charged DNA fragments, because small (damaged) fragments migrate faster and further than those big (undamaged), a comet-like structure with the genetic material is formed. Being the "head" of the comet the undamaged genetic material, and the "tail" the damaged ones. These comets, after staining the DNA with a suitable fluorescent dye, can be observed under a fluorescence microscopy.

Genotoxicity can be quantified by to parameters of the comet structure: the tail intensity (%) and the tail moment. The latter parameter is the product of tail percentage and tail length [15].

#### **3.4 CHLORHEXIDINE**

Chlorhexidine (CHX) was discovered by a group of scientists in the late 1940's seeking to create antimalarial agents. Although never being employed in the treatment of malaria, CHX has been used since the early 1950's for disinfection of the skin and for treating burns. And from 1959 to the present day, it has been used orally, mainly for the control of dental plaque [26].

CHX is a cationic biguanide antiseptic active against gram-positive and gram-negative bacteria, bacterial spores, facultative anaerobes and aerobes, molds, viruses, and yeast. CHX presents effectiveness against a broad range of microorganisms present in caries lesions, it also has been widely employed as an antimicrobial agent for topical preoperative disinfection, skin wounds (including burns), general skin cleansing, and as soap for surgical hand scrub [27, 28].

From these different studies, CHX appears to have a potential cavity cleanser with its promising properties. Nevertheless, high concentration of CHX can lead to toxic effects. With the increase of CHX concentration, cytoplasmic contents precipitate, leading to cell death. A range of studies has shown that CHX has toxic effects on eukaryotic cells, Hidalgo and Dominguez (2001) [27] showed, through the measurement of intracellular ATP levels and succinate dehydrogenase activity, that the presumed cytotoxicity mechanism is by mitochondrial injury.

Lucarotti et al. (1990) [29] found 100% cytotoxicity in human fibroblast when exposed for 24h with 0.0005% (0.005 mg/mL) CHX determined by methylene blue exclusion dye; Babich et al. (1995) [30] reported cytotoxicity after 1, 24 and 72 h in human gingival fibroblasts using the supravital dye neutral red.

Although the cytotoxicity of CHX is proven by the researches above, this study will be assessing its phototoxicity and genotoxicity. Struwe et al. (2007) [2] showed that CHX in a range of concentrations of 0.025-0.0016 mg/mL using mouse lymphoma cells, no photocytotoxicity and no photogenotoxicity were observed using the Alamar Blue assay and comet assay, respectively.

The range of concentration for CHX used was based on other studies [28-32], as well with the approximate solubility of CHX in DMSO (25 mg/mL) and the lowest concentration of DMSO that is not cytotoxic (approximately 0.5% DMSO). The use of DMSO as the solvent for the stock solution of CHX is due to the low solubility of CHX in water (1 g/L) [33].

## 4. OBJECTIVES

This study is part of a research project (PID2020-113186RB-I00). Its main goal is to develop novel *in vitro* assays to assess both new and common chemical compounds with phototoxic capacity.

The objectives of this specific study are the following:

- Evaluate the cytotoxicity of chlorohexidine using the MMT and LDH assays and evaluate its genotoxicity with the comet assay. The measured values will be compared with different chemicals with known phototoxic properties.
- Determine the concentration of chlorhexidine that reduces cell viability by 50% (IC<sub>50</sub>).
- Compare the IC<sub>50</sub> values of assays under UVA light irradiation and in dark conditions, using the Photo-Irritation Factor to estimate the phototoxic potential of chlorhexidine.
- Identify a reliable method for evaluating photocytotoxicity, as well as another for assessing photogenotoxicity.

## **5. EXPERIMENTAL SECTION**

All procedures were performed under aseptic conditions to avoid contamination of the cell culture. This was achieved by working under class II vertical laminar flow cabinet, using sterile plastic and glass material, and using 70° ethanol to sanitize and disinfect both the cabinet surfaces and materials before and after utilization. [34]

#### **5.1 MEDIUM AND REAGENTS**

Unless otherwise stated, all materials have been acquired from Sigma-Aldrich (Barcelona, Spain).

Compound	Composition	Description/Uses
FBS <sup>(a)</sup> *	Complex mixture of biomolecules that includes growth factors, proteins, trace elements, vitamins, and hormones.	Provides the necessary elements for cell proliferation and culture growth.
DMEM <sup>(b)</sup> 10%	10% FBS, 1% L-Glutamine*, 1% Antibiotic <sup>(c)*</sup> in DMEM** solution	Medium for growing and maintaining a cell culture.
DMEM without dye**	DMEM medium without phenol red	For colorimetric assays (MTT assay).
PBS <sup>(d)</sup> *	137 mM NaCl, 2.7 mM KCl, 10 mM Na <sub>2</sub> HPO, 1.8 mM KH <sub>2</sub> PO4 in H <sub>2</sub> O	Non-toxic solution used in cell mediums. Prevents cells rupture or dehydration due to osmosis.
Tris 1M	48.45 g Tris <sup>(e)</sup> in 1 L H <sub>2</sub> O, adjusted to pH 7.5 with HCl	To wash and adjust the pH in the comet assay
Tris-HCI	$0.2\ M$ Tris in $H_2O,$ adjusted to pH 8.2 with HCl	To prepare Buffer A, Buffer B and MPMS supplement.

Table 2. List of reagents used in the study

Buffer A	4 mM INT <sup>(f)</sup> in Tris-HCl	To prepare the assay reagent of LDH assay.
Buffer B	6.4 mM NAD <sup>(g)</sup> , 320 mM sodium lactate in Tris- HCl	To prepare the assay reagent of LDH assay.
MPMS <sup>(h)</sup> supplement	75 mM MPMS in Tris-HCI	To prepare the assay reagent of LDH assay.
Acetic acid 1M	1M Acetic acid in H <sub>2</sub> O	To stop the assay reagent in the LDH assay
МТТ	(3-(4,5-Dimethylthiazol-2-yl)-2,5- Diphenyltetrazolium Bromide)	Compound used in colorimetric assays due to its reduction to Purple Formazan.
MMS	Methyl methanesulfonate	Positive control in the comet assay.
Triton 100-X	2-[4-(2,4,4-trimethylpentan-2- yl)phenoxy]ethanol	Cell-lysis compound. To prepare lysis solution. Used also as positive control in LDH assay.
Lauryl Sarcosine	Sodium (N-methyldodecanamido)acetate	To prepare lysis solution.
Lysis solution	Stock: NaCl 2.5 M, Na <sub>2</sub> EDTA 100 mM, Tris 10 mM in H <sub>2</sub> O, adjusted to pH 10 with NaOH Before using: Triton 100-X up to 1%, Lauryl Sarcosine up to 1%	To perform cell lysis in the comet assay.
Electrophoresis buffer	Na2EDTA 1 mM, NaOH 300 mM in H2O	To unwind DNA in the comet assay.
DAPI	4,6-diamino-2-phenylindole dihydrochloride	Fluorochrome compound that attaches to DNA.

- (a) Fetal Bovine Serum
- (b) Dulbecco's Modified Eagle Medium
- (c) 10,000 U/mL penicillin, 10 mg/mL streptomycin
- (d) Phosphate buffered saline
- (e) 2-Amino-2-(hydroxyethyl)-1,3-propanediol)
- (f) lodonitrotetrazolium Chloride
- (g) B-Nicotinotinamide Adenine Dinucleotide Sodium Salt
- (h) 1-Methoxyphenamine methosulfate

\* Acquired from BioLab (Barcelona, Spain)

\*\* Acquired from Lonza (Verviers, Belgium)

#### 5.2 CELL CULTURE

#### 5.2.1 Growth and maintenance

As mentioned before, we used the human keratinocyte cell line HaCaT as subject of our studies. HaCaT cells grow in a 75 cm<sup>2</sup> culture flask (T75). The cell culture flask is stored in a humidified incubator at 37°C with 5% CO<sub>2</sub> and 95% air to keep it within the physiological range. Cells feed on the DMEM 10%, multiplying, and spreading throughout the entire surface of the flask. Once they reach approximately 80% confluence, meaning cells cover 80% of the surface, we must perform a procedure known as cell passing to mitigate excessive differentiation and safeguard against nutrient depletion and cellular damage. This occurs usually twice every week.

Cell passing consists in aspirating the DMEM culture medium and making two washes with 8 mL of PBS solution, adding 2.5 mL of trypsin 0.05%, and incubating (at 37°C and 5% CO<sub>2</sub>) for 6 minutes so the trypsinization process takes place. Trypsin detaches the cell from the surface of the flask.

After the 6 minutes, and after few small hits on the side of the flask, cells in suspension are seen. 7.5 mL of DMEM 10% FBS is added to deactivate the trypsin and the solution inside the flask is transferred to a sterile 14 mL tube.

A volume between 0.5-1.5 mL is added to a new T75 flask and make level up to a volume of 18 mL with DMEM 10% FBS. The new flask must be identified by its user, including the cell line, date, and pass number.

It is worth noting is that an excess of the exposure of the cell line to the trypsin solution (about 10 min) can be dangerous to the cell DNA, altering its structure and decreasing their viability. Also, the serum and calcium found in the medium inhibit the trypsin, therefore the importance in washing thoroughly with PBS before adding the trypsin. [35, 36]

Besides cell passing, every 48 hours the medium is changed so that cells always have nutrients, and the pH is stable in the medium. Using a pipette, about 14 mL of the old medium is removed (leaving approximately 3 mL remaining) and 15 mL of new medium previously poached for 30 minutes at 37°C is added.

#### 5.2.2. Cell counting

To achieve a known concentration of cells into a 96-well plate, a previous cell counting is required.

The cell counting methodology consists in diluting the cellular suspension 1:10. In our case, 10  $\mu$ L of cell suspension is added to 80  $\mu$ L of PBS with 10  $\mu$ L of 0.4% Trypan Blue solution in a 0.5 mL microtube. Trypan Blue solution will stain only dead cells blue [37].

A 10  $\mu$ L aliquot of the solution is micro-pipetted into a Neubauer chamber. It is applied between the chamber and the edge of a cover slip, and the solution enters by capillary action, filling one of the chambers.



Figure 4: Neubauer chamber and a representation of the microscopic quadrants

Live cells in spaces L1 to L4, see figure 4, are counted under an optical microscope. And the following equation is used to calculate the cell concentration.

Cell concentration 
$$\left(\frac{\text{cells}}{\text{mL}}\right) = \frac{\text{L1+L2+L3+L4}}{4} \text{x10}^4 \text{x10}$$

Equation 2: Cell concentration formula for counting the cells in a mL

The factor of 10<sup>4</sup> considers the volume of the Neubauer chamber, and the 10 factor is to reverse the initial dilution.

For our phototoxicity assays, 100  $\mu$ L of cells, at a density of 10<sup>5</sup> cells/mL, is seeded into the 60 inner wells in two 96-well plate (plate 1 and 2). The external 36 other wells are filled with 100  $\mu$ L of PBS to ensure humid conditions. These plates are incubated overnight at 37°C and 5% CO<sub>2</sub>. During this time, cells will attach to the surface of the wells (see Appendix 1).

#### 5.3 EXPOSURE OF THE COMPOUNDS OF INTEREST

The exposure of the phototoxic agents into our cells is performed at the following range concentrations:

Chemicals	Concentration range
СНХ	1.953 – 125 µg/mL
CPZ	0.234 – 15 μg/mL
SDS	0.585 – 37.5 µg/mL
8-MOP	39 – 2500 µg/mL
BZ	1.172 – 150 µg/mL

Table 3. Range of concentrations of the chemicals studied

These concentrations were obtained using PBS solutions and, due to the low solubility in water, dimethyl sulfoxide (DMSO) was used for making stock solutions of CHX, 8-MOP and BZ.

Non-treated cells were used as using PBS. For the chemicals where DMSO is used for the first or stock solution, the control solution will consist of PBS and the same concentration of DMSO as the solution with highest concentration of chemical analyzed (C  $\leq$  0.05 % DMSO). A positive substance for the comet assay will be methyl methanesulfonate (MMS) 1000  $\mu$ M solution in PBS.

The medium of the 96-well plate is aspirated and 100  $\mu$ L of the range of chemical concentrations, alongside with the control and positive solutions, is added to our cells and left incubated for 1h (37°C and 5% CO<sub>2</sub>).

Afterwards, only one of the two plates (plate 2) is irradiated using a fluorescent UVA lamp. Reaching a radiation dose of 4 J/cm<sup>2</sup>. Thus, we will evaluate the effect of sunlight on the toxicity of the compound.

Before we change the medium that have our compound of interest, 50  $\mu$ L of the supernatant medium of the first four rows of both plates are transferred to another 96-well plate to perform the LDH assay (plate 3), additionally in 3 wells of control solution, 10  $\mu$ L of 9% of Triton 100-X is added and mixed with the medium, transferring after 50  $\mu$ L of the supernatant of these 3 wells on the plate 3. Triton 100-X will act as a control in the LDH assay since it makes the cells lysed.

Lastly, the medium with the different ranges of concentrations of the phototoxic agent on the two original plates is aspirated and replaced with 100  $\mu$ L of DMEM 10% FBS medium, incubating the 1 and 2 plates overnight. The whole procedure is shown in Appendix 1.

#### **5.4 CYTOTOXICITY DETERMINATION**

#### 5.4.1 LDH assay

The procedure followed in this study is the one described by Kaja et al. (2017) [25].

The LDH assay is made the same day we expose the cells to the phototoxic agent, rather than the following day like the MTT and comet assay.

A solution made of 2.5 mL of Buffer A, 2.5 mL of Buffer B and 1  $\mu$ L of MPMS supplement is prepared. 50  $\mu$ L of this assay reagent is added to each well of plate 3 and left in the dark and at 37°C for 30 min. 50  $\mu$ L of a solution of acetic acid 1M is then added to the wells to deactivate the assay reagent. And the plate, previously shaken at 300-400 rpm/min for 15 seg, is taken for absorbance reading at 492 nm with a 690 nm background using a Tecan Sunrise<sup>®</sup> microplate reader (Männedorf, Switzerland).

#### 5.4.2 MTT assay

The procedure followed in this study is the one originally described by Mosmann (1983) [23], with the modifications in the procedure reported by Zanette et al. (2011) [24].

The supernatant of the 4 first rows of cells in plates 1 and 2 is aspirated. 100  $\mu$ L of an MTT solution (0.5 mg/ml, in DMEM without dye) is placed in each well, following an incubation of 1h. At the end of incubation, the MTT solution of each well is discarded, and replaced with 100  $\mu$ L of DMSO to dissolve the water-insoluble formazan crystals. The plates are gently shaken for 10 minutes at 300-400 rpm/min. The absorbance of the wells of both plates is then measured at 550 nm using a Tecan Sunrise<sup>®</sup> microplate reader.

#### 5.5 GENOTOXICITY DETERMINATION: COMET ASSAY

The procedure followed in this study is the one described by the Organization for Economic Co-operation and Development (OECD), test No. 489: In Vivo Mammalian Alkaline Comet Assay (2016) [15].

#### 5.5.1 Slides preparation and cell adhesion

To see the DNA damage in the cells a Nikon TS100 epifluorescent microscopy is used. Thus, a solid invisible support for the cells is needed.

A solution of 1% of normal melting point agarose is prepared and heated to 50 °C in a thermostatic bath. Slides are submerged into the agarose solution, and one side of the slide is whipped so only one of the faces of the slide has agarose and left to dry for at least 24 h. Agarose is a linear polymer that dissolves in boiling water conditions (90-95 °C), but it has the capacity to gel upon cooling. [38]

The cells collected were those present in the two end rows of plates 1 and 2, and the cell collection procedure is similar to that of cell passaging. The supernatant of these rows is aspirated. Two washes of 50  $\mu$ L of PBS are made, following a volume of 30  $\mu$ L of trypsin 0.05%. After leaving the plates for 6 minutes in incubation conditions (37 °C and 5% CO<sub>2</sub>) 60  $\mu$ L of DMEM 10% FBS is added, and the solution inside the wells of the same columns is transferred in microtubes of 0.5 mL.

Two individual samples are gathered per concentration of chemical exposed, and each sample is distributed into two slides. Hence, four slides are prepared for each concentration to guarantee result replicability.

A solution of 80  $\mu$ L of cellular suspension and 160  $\mu$ L of a solution of 0.9% aqueous low melting point agarose solution at 40 °C of each microtube is prepared, and drops of 5  $\mu$ L of each solution are placed into the pre-made slides with the agarose layer. The slides are left to rest 10 min at room temperature, followed by 6 min in the freezer. After removing the slides form the freezer, cells will be attached to the agarose layer of the slides.

#### 5.5.2 Cell lysis, DNA unwinding and electrophoresis

The slides are submerged in the lysis solution overnight at 2-10 °C. This solution will disrupt the cell membrane and expose the cell DNA. After the lysis step, slides are immersed into the electrophoresis buffer inside the electrophoresis cuvette for 40 min at 2-10 °C to unwind and denature the DNA chains. [39]

A constant current intensity of a 300 mA is set to achieve an electric field of 0.7 V/cm throughout all 30 min of the electrophoresis step. This will make smaller DNA fragments to

migrate further than bigger DNA fragments, making the comet figure that will be seen under the microscope.

Finally, 3 washes of 5 min each of Tris 1M solution are made to neutralize the slides. Once this process is completed, the slides can be dried and stored indefinitely since the DNA loops and fragments composing the comet remain stable after neutralization.

#### 5.5.3 Comet observation

Before the observation of the comets, slides must be hydrated by submerging them 10 min in Milli-Q water. 20  $\mu$ L of 5  $\mu$ g/mL DAPI solution is added to dye DNA, as this compound binds to the adenine-thymine regions of the DNA, acting as a fluorochrome. This compound has a maximum absorption wavelength around 340 nm (UV) and a maximum emitting wavelength at 448 nm (blue). [40]

Under a Nikon TS100 epifluorescent microscope with a 100 W mercury lamp, alongside the Comet Assay IV (Instem, UK) software installed in a computer connected to the microscope, enables an efficient observation and scoring of the comet irradiating the slides with UV light.

#### 5.5.4 Statistical analysis methodology

60 cells are scored per analyzed chemical concentration, and both in light and dark conditions. The five extreme values for tail intensity and tail moment are discarded to ensure a valid representation of the DNA damage, in doing so reducing the high cell-to-cell variability in each slide.

A two-tailed Student's t-test, at a 95% confidence level, is performed between control solvent and MMS positive control to ascertain if the test has been done successfully. An analysis of variance is also performed at a confidence level of 95% to compare the different analyzed concentrations and the different conditions to which the cells have been exposed to (UV or no UV irradiation) to determine the statistically significance between them.

## 6. RESULTS AND DISCUSSION

#### 6.1. Рнотосутотохісіту

To avoid false-positive results in the assays, relative cell viability/cytotoxicity has been determined. Control solvent is set as 100% viability in the MTT assay, and positive control in the LDH assay is set at 100% cytotoxicity (or 0% cell viability).

#### 6.1.1 MTT assay results





Figure 5 shows a decreasing trend of viability with increasing CHX concentration in both the irradiated and non-irradiated cell.

When the equation of the tendency line is obtained, the  $IC_{50}$  and the PIF can be calculated for each replica. Table 4 summarizes these values on the different replicas on both in light and dark conditions, as well as the average values and the standard deviation of each one.

MTT assay results	First replica	Second replica	Third replica	Average values	Standard deviation
IC₅₀ UV (µg/mL)	18.6	13.2	16.9	16.2	2.3
IC₅₀ NO UV (μg/mL)	19.2	13.8	53.3	28.8	17.5
PIF	1.0313	1.0491	3.1533	1.7	0.99

Table 4. Results obtained in the MTT assay of CHX

Table 5. Comparison of MTT results of all phototoxic agents. Cells with a dash (-) mean not calculable. N/A stands for not applicable, as the value cannot be calculated.

MTT assay results	CPZ	SDS	8-MOP	BZ	СНХ
IC₅₀ UV (μg/mL)	1.801	-	168.6	30.33	16.2
IC₅₀ NO UV (μg/mL)	-	-	-	-	28.8
PIF	8.333	N/A	14.82	4.945	1.7

The representation of cell viability over CHX concentration of light and dark conditions are quite similar in table 4, same can be said with the  $IC_{50}$  values. With the representation and the  $IC_{50}$ , a non-phototoxic effect on CHX can be predicted. This is further corroborated with the value of PIF < 2 obtained, which confirms that CHX is not phototoxic.

Table 5 compares the IC<sub>50</sub> and PIF values of the reference agents with CHX. IC<sub>50</sub> values with a dash (-), are because it has been not possible to calculate. As seen in Appendix 2, tendency lines do not reach 50 % viability.

In all cases, PIF was obtained by dividing the largest concentration used in the assay by the  $IC_{50}$  UV. When the PIF is calculated in this way, if PIF > 1, the chemical is considered phototoxic. With SDS, as it has no calculable  $IC_{50}$  value, PIF cannot be obtained either.

Based on the experimental conditions, this study coincides with the assessment of Campos et al. (2007), Lessa et al. (2010), López-García et al. (2014), and other studies, that the CHX solutions administered to cultured HaCaT cells demonstrated a dose-dependent cytotoxicity. And coincides with Struwe et al. (2007) by classifying CHX as non-phototoxic.

#### 6.1.2 LDH assay results

LDH assay has presented some problems throughout the study. For the four reference agents that were analyzed at the beginning of the study, the 50  $\mu$ L of the supernatant medium of the first four rows of both plates that are transferred to plate 3, later to perform the LDH assay, was collected after the medium containing the chemical agent was replaced by DMEM medium and left to the incubator overnight, that is, on day 3 (see Appendix 1).

After evaluating the results of the first four assays, it was determined that the LDH released due to the cell membrane damage was aspirated during the medium replacement process, leading to misleading values for the assessment of cytotoxicity. Consequently, the LDH assay results for the four reference agents are not included in this study.

Hereafter, for CHX LDH assays, the 50  $\mu$ L of the supernatant medium was only collected on day 2, before medium replacement.

Moreover, the experimental procedure for the LDH assay in the first replicate was incorrect regarding of the incubation time with the assay reagent solution, being 60 min instead of the 30 min stated in the actual experimental procedure and followed for the next two replicates.



Figure 6. Effects of CHX on LDH release in light and dark conditions for all replicas. First replica was left with assay reagent for 60 min as opposed to the 30 min of second and third replicas. Response values in the graphs are mean values  $\pm$  SD (n=3).

Statistical analysis of replicas cannot be done as the experimental procedure in which both assays were implemented slightly differ from one another, but a trend of LDH release with increasing CHX concentration is apparent in figure 6.

	Table 6. Comparison of IC <sub>50</sub> and PIF	values of the first replica obtained b	y MTT and LDH assays
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Assay utilized	MTT assay	LDH assay
IC₅₀ UV (µg/mL)	18.6	19.1
IC₅₀ NO UV (µg/mL)	19.2	18
PIF	1.0313	0.9435

Albeit by mistake, and due to the longer exposure time of the assay reagent, only the first replica allows to calculate the  $IC_{50}$  from its tendency line, as well of the PIF value. These values can be compared to the MTT assay of the first replica, and the similarities can be appreciated in table 6.

LDH assay reiterates the non-phototoxic behavior of CHX with the value of PIF < 2, and also gives almost identical IC<sub>50</sub> values to those obtained by the MTT assay.

#### 6.2. PHOTOGENOTOXICITY

In all concentration groups, both in light and dark conditions, a total of 60 cells were scored to obtain a valid representation of the DNA damage, since this assay presents a distinctly high cell-to-cell variability observed in each slide. The five maximum and minimum values are discarded to eliminate further variability in the results, giving a total of 100 cells scored per concentration, 50 for each light or dark condition.



Figure 7. Tail intensity and moment for both in light and dark conditions for CHX. Response values in the graphs are mean values of the averages ± SD (n=3).

To verify if the comet assay has been performed correctly, a Student's t-test of two tails was performed using Microsoft Excel between the control solvent and the MMS positive control. This t-test confirms, with 95% confidence, that the comet assay has been carried out correctly, as the control solvent and MMS treatment have a significant difference in the results. This difference can be observed in figure 7 and 8, as MMS intensity and moment values of the analyzed comets are considerably higher than those of the control.

Figure 7 shows that no correlation between tail intensity/moment and CHX concentration. Though an increase in tail intensity and moment in the UV conditions, seen in figure 7 and 8, may suggest a phototoxic effect. A two-factor analysis of variance (ANOVA) with several samples per group, using Microsoft Excel, has been carried out for the different concentrations of CHX (control to 0.0078  $\mu$ g/mL) and the exposure, or absence, of UV. In all cases, with 95% confidence, no significant difference of the values is observed. This is interpreted as CHX being neither genotoxic nor photogenotoxic, refuting the previous statement.



Figure 8. Images of comets, stained with 5  $\mu$ g/mL DAPI solution, exposed to control solvent (A), 19.53  $\mu$ g/mL CHX (B), 19.53  $\mu$ g/mL CHX (C) or MMS 1000  $\mu$ M (D) without (top) or with UV (bottom).

For the reference agents, appendix 3 showcases their photogenotoxic behavior. We can see that BZ causes a genetic material damage to the cells as its tail intensity and moment values are relatively high at all concentrations. For the highest concentrations that were irradiated with UVA, the decrease in those values is due to the higher cell mortality that BZ has in light conditions (at 9.38  $\mu$ g/mL and in light conditions, cell viability is 77.5%), and the surviving cells seem to not have been damaged.

SDS is the negative reference agent, and its genetic damage is very low. For 8-MOP, even though very low values of tail intensity and tail momentum are also observed at all concentrations tested, these are probably due to an interstrand cross-linking effect on the DNA,

preventing DNA duplex separation under alkaline or denaturing conditions, behavior also observed by Struwe et al. (2007) [2].

CPZ values appear to be similar to those of BZ, where at higher concentrations or exposure to light, damaged cells die, and the only ones alive and quantifiable are the undamaged ones. But this behavior is not expected for CPZ, as it is known to be a very photogenotoxic agent [2], so it was hypothesized that the cells after irradiation and during the overnight incubation could repair the DNA.

#### 6.2.1. DNA repair evaluation

The assessment was done using CPZ. To determine if DNA is actually repaired, a first comet assay was performed on the same day as the CPZ exposure (Day 1, after irradiation) and a second comet assay was performed the day after the exposure (Day 2), as has been done throughout the study.

A three-factor ANOVA was performed, with a 95% confidence, using GraphPad Prism software, comparing different concentrations, light or dark conditions and test days. Both the ANOVA and the graphs in figure 9 indicate a statistically significant increase in the tail intensity and moment with increased concentrations under light conditions on day 1. However, no statistically significant correlation was observed in tail intensity or moment values and concentration in dark conditions on day 1, as well as in both light and dark conditions on day 2. That suggests that CPZ is a potential photogenotoxic substance, displaying its expected behavior, but only on day 1.

A comparison of the tail values on day 1 and on day 2 shows a statistically difference. In figure 8, a decrease of the values is observed, indicating that the hypothesis was correct and there is indeed an overnight DNA repairing. Even in the control sample, DNA damage occurs due to UV light exposure and the next day the DNA damage is significantly reduced.



Figure 9. Comparison of tail intensity and moment of CPZ in the different concentrations exposed, dark and light conditions, and day 1 and 2. Response values in the graphs are mean values ± SEM (standard error of the mean) (n=50). Where \* indicates differences with the corresponding control, + differences between days and ^ differences between no UV and UV:

\*\*\*\* = Very significant (p < 0.0001) \*\*\* = Significant (0.0001 < p < 0.001)

\*\* = Moderate significance (0.001 < p < 0.01) \* =Marginal significance (0.01 < p < 0.05)

No asterisk = Not significant (p > 0.05)

The only concentration that doesn't follow these patterns is 0.45  $\mu$ g/mL. To determine if this deviation is significant and not merely due to variability, two additional replicates must be performed to ensure accurate analysis, as only one replicate has been completed due to time constrains. Furthermore, DNA repair should also be checked for all other chemicals to ascertain if they exhibit similar behavior

## 7. CONCLUSIONS

 $IC_{50}$  value obtained for CHX using the MTT assay were 16.2 and 28.8 µg/mL for light and dark conditions, respectively. However, this increase is not substantial enough to classify CHX as a potential phototoxic chemical, since the PIF value is < 2. The other chemicals showed PIF values above 5, classifying them as phototoxic, except for SDS as expected.

The LDH assay has shown unsatisfactory results for photocytotoxicity assessment, probably due to a procedural flaw. Further testing should be performed with modifications of the procedure to estimate the value of the assay for determining cell viability.

Regarding the evaluation of photogenotoxicity, CHX does not exhibit photogenotoxic properties. However, neither CHX nor the four reference phototoxic agents have shown increased DNA damage 24 hours after irradiation, possibly indicating DNA repair during this period. Only CPZ has been tested in this context, confirming DNA repair. Complementary tests for the remaining chemicals should be carried out in order to ascertain their DNA repair behavior.

Overall, the data from this study indicate that CHX is cytotoxic to human dermal fibroblasts *in vitro* at concentrations lower than those used in clinical practice (0.05% for burns and wounds; 0.12% as oral rinses [27]), concentrations at which CHX completely eradicates cell viability.

Furthermore, the MTT and comet assays show promise as a straightforward and reliable methods for identifying both photocytotoxic and photogenotoxic chemicals, potentially enhancing the safety assessment of new pharmaceuticals and cosmetic products.

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## 9. ACRONYMS

- 8-MOP: 8-Methoxypsoralen
- ANOVA: Analysis of variance
- BZ: Benzophenone
- CPZ: Chlorpromazine
- DAPI: 4,6-diamino-2-phenylindole dihydrochloride
- DMEM: Dulbecco's Modified Eagle Medium
- DMSO: Dimethyl sulfoxide
- FBS: Fetal Bovine Serum
- HaCaT: Human Adult Low Calcium High Temperature Keratinocytes
- IC<sub>50</sub>: Half-maximal inhibitory concentration
- INT: Iodonitrotetrazolium chloride
- LDH: Lactate dehydrogenase
- MMS: Methyl methanesulfonate
- MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- OECD: Organization for Economic Co-operation and Development
- PBS: Phosphate Buffered Saline Solution
- PIF: Photo-irritation Factor
- **SDG:** Sustainable Development Goals
- SDS: Sodium Dodecyl Sulphate
- T75: 75 cm<sup>2</sup> culture flask
- UV: Ultraviolet

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# **APPENDICES**

## **APPENDIX 1: EXPERIMENT DIAGRAM**



## APPENDIX 2: REFERENCE AGENTS MTT ASSAY CYTOTOXICITY GRAPHS













## APPENDIX 3: REFERENCE AGENTS COMET ASSAY GRAPHS















