2013 International Nuclear Atlantic Conference - INAC 2013
Recife, PE, Brazil, November 24-29, 2013
ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR - ABEN
ISBN: 978-85-99141-05-2

Development and construction of a specific chamber for phototoxicity test

Bianca S. Sufi[1], Natália M. Esteves-Pedro[2], Patrícia S. Lopes[3], Telma Mary Kaneko[2] and Monica B. Mathor[1]

[1] Instituto de Pesquisas Energéticas e Nucleares (IPEN / CNEN - SP)
Av. Professor Lineu Prestes 2242
05508-000 São Paulo, SP
biancasufi@usp.br
mathor@ipen.br

[2] Faculdade de Ciências Farmacêuticas da Universidade de São Paulo (FCF-USP)
Av. Professor Lineu Prestes 580
05508-000 São Paulo, SP
nataliamenves@yahoo.com.br
tsakuda@usp.br

[3] Universidade Federal de São Paulo (UNIFESP) – Campus Diadema
Rua Prof. Artur Riedel, 275 – Jd. Eldorado
09972-270 Diadema, SP
patricia.lopes@unifesp.br

ABSTRACT

Phototoxicity corresponds to the acute toxic response induced after skin exposure “in vivo” and “ex vivo” to certain chemicals and subsequent exposure to irradiation. Phototoxicity “in vitro” assay is determined by viability of fibroblasts BALB/c 3T3 exposed to chemicals in the presence and absence of light. Substances identified as phototoxic are susceptible to “in vivo” phototoxicity (OECD 432, 2004). A chamber was developed and constructed according to the guidelines OECD Toxicity Guide – 432 and ©ECVAM DB-ALM: INVITTOX N. 78. The chamber was built in stainless steel frame, with UVA lamps and dark area for negative control. The tests to qualify the chamber were performed with Sodium Lauryl Sulfate, recommended by the guides aforementioned, as negative control; and Bergamot oil (Givaudan-Roche), as positive control. Bergamot, *Citrus bergamia*, has, as major component, Bergapten responsible for its photosensitive activity. Both samples were diluted in Phosphate Buffered Saline with concentrations between 0.005 and 0.1 mg/mL, which were calculated by the dilution factor 1.47. These tests were performed over fibroblast BALB/c 3T3 culture and submitted to phototoxicity assay with MTS dye, under spectrophotometric reading, which allows determining the Photo Irritation Factor (PIF), what suggests that a substance with a PIF<2 predicts no phototoxicity; PIF>2 and <5 provides likely phototoxicity and PIF>5 provides phototoxicity. Sodium Lauryl Sulfate presented a PIF=1, being in accordance with the OECD. Bergamot oil has shown to be likely phototoxic with a PIF=2,475. These results provide that the chamber is qualified to be used to perform phototoxicity tests with assurance and security.

1. INTRODUCTION

The phototoxicity is defined by a toxic reaction, due to a chemical applied to the skin followed by exposure to light. This reaction may be caused or increased even with low doses of the substance, or irradiation skin induced after systemic administration of a chemical [1]. The basic mechanism of the phototoxicity assay could be described as the identification and the determination of the potential toxicity of a chemical induced by exposure to ultraviolet (UV) radiation and visible light, this way the toxic potential of a chemical or product is

measured by a reduction in the viability of cell exposed to this substance in the presence or absence of light. This assay predicts the probability of chemicals tested in cause phototoxicity *in vivo* [1; 2]. To perform the phototoxicity test it is necessary the utilization of a specific chamber, which one is marketed, however the guide [3], describes the requirements for its construction.

As to ionizing radiation, its definition is given by the radiation whose energy is higher than the electrons binding energy, being enough to eject electrons present in the atoms and molecules and convert them into electrically charged particles, called ions. There are two types of ionizing radiation. Electromagnetic radiations, which comprises radio waves, microwaves, visible light, ultraviolet light, x-rays and gamma rays. And the radiation from high energy particles that could be positively or negatively generated by machines such as positron and ion accelerator beams [4].

Regarding natural radiation, the sun emits several types that are filtered by the ozone layer, this energy, on a clear day without the presence of clouds, corresponds to electromagnetic radiation, which comprises only 45% of UV radiation, being 10% of sunlight energy, in other words, UVA-I from 340 to 400 nanometer (nm); UVA-II between 320-340 nm; UVB (290-320 nm); and UVC, germicidal spectrum, from 100 to 290 nm [5; 6]. Whereas the visible light is 5%, which can be seen to the naked eye, forming an essential component to photosynthesis, the infrared radiation (nonluminous) corresponds to 50%, between 400-800 nm and above, which are responsible for maintaining earth's heat. Now the human body reacts and detects the presence of these radiations in different ways, e.g., infrared rays by heating and dilation of blood vessels; visible rays detected by optical system; and UV radiation through photochemical reactions [7; 8].

The ultraviolet radiation is naturally stressful for most forms of life, the destruction of the ozone layer and global warming are the major causes of the increasing of radiation dose received by the man. When the defense mechanism is changed the radiation penetrates in the human skin, resulting from genetic mutations and abnormal cellular behaviors, due to their shorter wavelength [9].

According to Bouillon (2002) [10], the solar spectrum absorption percentage by the skin layers corresponds to: 0% of UVC rays, due to their low gamma energy and blockage of the ozone layer; 20% of UVB rays absorbed in the epidermal layer and 8% in the dermis, the remainder is blocked by the stratum corneum; as to UVA rays, 80% are absorbed in the epidermis and 20% in dermis; the visible light are absorbed in the dermis (70%), hypodermis (20%) and less than 10% by stratum corneum and epidermis; 65% of infrared rays are absorbed by dermis, 15% by hypodermis and less than 20% in the stratum corneum and epidermis.

When UV radiation is absorbed by a biological molecule, an electron is transferred to higher energy levels, resulting in free-radicals, which can cause death, chromosomal alterations and mutations in prokaryotic and eukaryotic cells [11]. In the literature it was demonstrated that this radiation can increase the expression of p53 tumor-suppressor encoded by the TP53 gene, induce skin pigmentation, metabolic alterations, besides being responsible for photo ageing. The cell damage caused by UVB radiation, result in the emergence of abnormal cells 24 hours after sunburn. These cells, called "sunburn cells" are keratinocytes which have begun a

process of cell death. However, the frequent and intense exposure can suppress the immune response of the skin and cause DNA damage expressed in the form of cancer [7; 12; 13; 14].

While UVB radiation is highly cytotoxic, UVA radiation is associated with phototoxic reactions that occur due to excessive free-radicals formation. Therefore, the choice of a suitable radiation source is utmost importance to the phototoxicity assays *in vitro* [1].

In accordance to the guide [3], the chamber to perform the phototoxicity test should provide a stable environment with about 80 cm high and 50 cm wide, to allow a good fit of 60 cm of the distance of exposure between the plate and the UVA light, to achieve the irradiance of 1.7 mW/cm^2 (= 1 J/cm^2 to 10 minutes of exposure), being that the protocol [1], provides that the dose of radiation over the chemical compound in contact with the cells must achieve 5 J/cm^2 [1; 3]. The solar simulation should be done in xenon arc lamp and with appropriate filters to allow only the passage of UVA rays [15]. Along with the guide [16], the test substance should be assessed prior to biological assay, with respect to its absorption spectrum in the UV / visible (UV-VIS) to indicate which wavelengths can cause photochemical degradation that depends on the total energy absorbed.

For validation and control of the radiation dose is necessary a physical device. It should determine the number of absorbed photons at a given light beam in a defined space. These are subdivided into two main types of detectors: the thermal (that converts radiant energy into heat) and photoelectric (that converts radiant energy into electrical current), which is used to detect visible and UV light, respectively namely: luximeter and spectral radiometer [15; 17].

The chamber for the phototoxicity assay is marketed, however it can be built following the guides [1, 3], which indicates the ideal conditions of this equipment for the phototoxicity tests. This study aimed to develop and qualify a suitable chamber to perform these assays.

2. MATERIAL AND METHODS

2.1 Construction of a Chamber for Phototoxicity Assay

According to the protocol [3], the chamber was designed in stainless steel frame, plate of 3 mm, matte painting in black ink, containing two UVA light bulbs (BL-T8, SYLVANIA). The distance between the lamp and the base that accommodates the plates should be 60 cm to achieve a proper adjustment of the radiation dose on the plate, of 1 J/cm^2 for 10 minutes and the total dose of 5 J/cm^2. The height of the chamber is at most 80 cm, to contain a dark area to accommodate the negative control (cytotoxicity). The width of the chamber is about 50 cm, enough space to accommodate six plates at the same time. For calibration of the radiation emitted by UVA light it was used radiometric measuring (UV Meter Basic, Hönle UV Technology), kindly provided by the company Chemyunion, from Sorocaba, São Paulo, Brazil.

2.1.1 Qualification of the Chamber and Validation of the Phototoxicity Method

The qualification of the chamber and method validation consisted of phototoxicity in two stages, according to the protocols [1; 3], namely:

The cells, fibroblasts Balb/c 3T3, were cultured in 96-well plates in recommended seeding density of 1.5 x 10^4 cells per well. The chosen reference substance, for qualifying the chamber and the method validation, was Sodium Lauryl Sulfate (SLS, CAS number

151-21-3), because it is a known non-phototoxic substance (negative control), recommended by the guide [1], and Bergamot Oil (Italy Orpue, number 001533, from ROCHE-GIVAUDAN), a known phototoxic substance, as positive control.

2.1.1.1 Phototoxicity Protocol

The substance-test should always be freshly prepared immediately before use, performing the assay in the dark to avoid polymerization and degradation of the substance prior to irradiation. For the test, two plates were prepared, being one for determination of cytotoxicity, UVA- (negative); and the other one for determining phototoxicity, UVA + (positive). The culture medium was added in peripheral wells of 96-well plate (= blank), and in the remaining wells was added cell suspension. The plates were incubated for 24 hours (37 ºC, CO_2 5%), period that allows cell adhesion and recovery.

On the second day after incubation, the culture medium was replaced by Phosphate Buffered Saline (PBS) containing the sample tested, in eight different concentrations between 0.005 and 0.1 mg/mL. The cells were incubated (37 ºC, CO_2 5%) per 1 hour. After this period, a plate (positive UVA) was subjected to exposure of UVA light being irradiated at room temperature for 75 minutes in the chamber built in accordance with item 2.1. The other plate was kept in the dark area of the chamber in the absence of light (UVA - negative control), standing at room temperature for the same period of UVA positive plate. The test-substance was removed, the plates were washed with PBS, the buffer was replaced by culture medium and the plates were re-incubated for 18-22 hours.

On the third day, it was proceed with the cell viability assay. For the interpretation of the results, the guide [1] recommends to calculate the Photo Irritation Factor (PIF), based on the formula:

$$PIF = \frac{IC_{50}(-Irr)}{IC_{50}(+Irr)}$$

Where, PIF (Photo Irritation Factor); IC_{50} (-Irr) is the dose corresponding to 50% of cell death of the non-irradiated control; IC_{50} (+Irr) is the dose corresponding to 50% of cell death in irradiated control. For substance with: PIF < 2, non-phototoxic; for PIF between 2 and 5, probable toxicity; for PIF > 5, phototoxic substance.

2.1.2 Protocol of Qualification of the Chamber and Validation of the Phototoxicity Method

Specifically for this stage, following the protocol aforementioned (2.1.1.1), using as reference substance the SLS, which one were placed on exposure to UVA light at nine 96-well plates containing 8 different concentrations of the sample, and each plate was equivalent to 1 Joule performed up to 9 Joules, in other words, the first plate received 1 J/cm^2, the second plate received 2 J/cm^2, and the third one received 3 J/cm^2, and so on, the ninth plate remained until 9 J/cm^2. A tenth plate in the same conditions remained in the dark as negative control (-UVA). In addition, it was also carried out the assay with bergamot oil, using just two plates, being one for positive control (+UVA), on the exact conditions described on item 2.1.1, until 5 J/ cm^2, and another as negative control (-UVA).
All data were compiled in the program Phototox® for analysis and interpretation of the results**.**

3. RESULTS AND DISCUSSION

3.1 Construction of a Chamber for Phototoxicity Assay

Outline of a specific chamber for phototoxicity assay, designed in accordance to the guide [3] (FIGURE 1). Chamber's Photography, evidencing its internal and external part, and components (FIGURE 2 e 3).

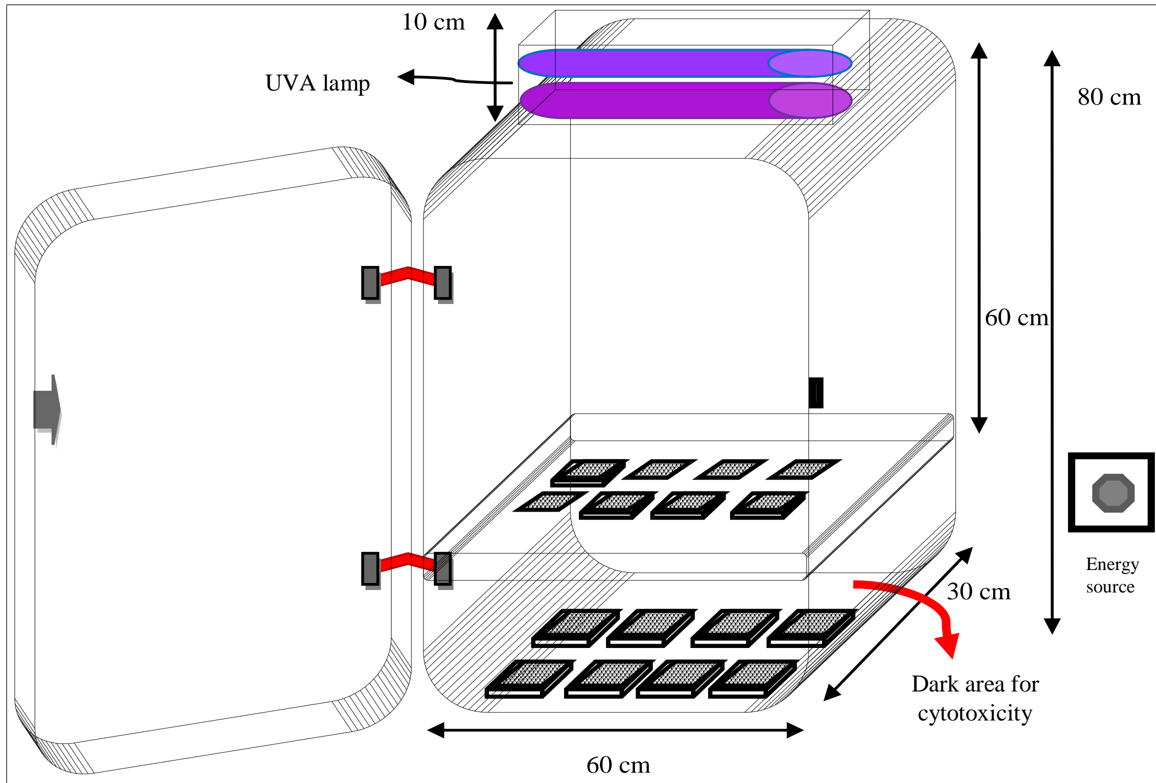


Figure 1: Outline of a phototoxicity chamber with total area of approximately 0.153 m^3 and desktop with about 153 cm^2.

This outline for the construction of the chamber allowed a preview of how this one should be, following the standards, set by the guide for the phototoxicity assay [3], facilitating its development therefore, from manufacturers.

Figure 2: Phototoxicity chamber's photography, with door closed, (A) before painting and (B) after painting.

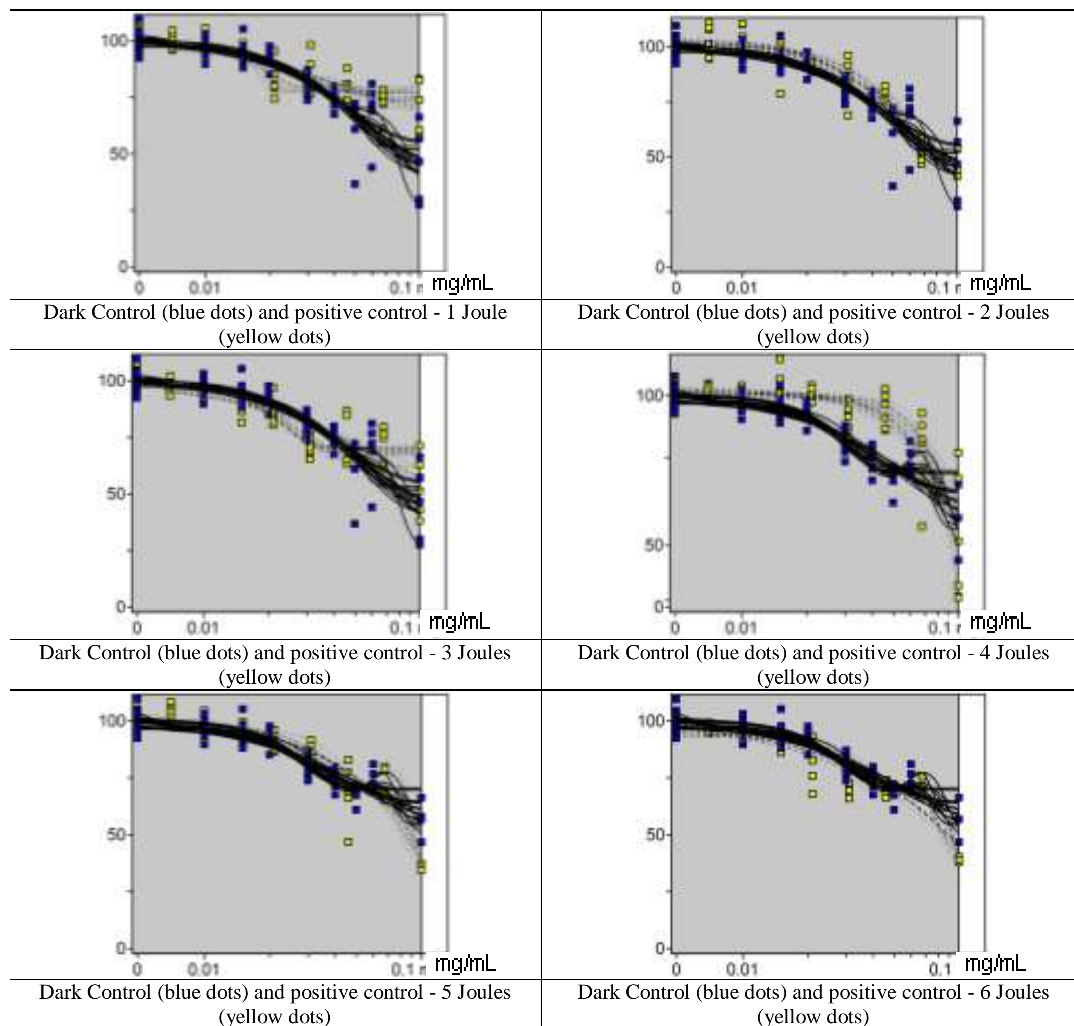
Figure 3: Phototoxicity chamber's photography, with door opened, (A) before painting, (B) after painting and (C) evidencing its components.

In figures 2 and 3, the stainless steel chamber, before and after painting. It is possible to observe, with the door open, the dark area for cytotoxicity (UVA-control, negative), the platform on which are placed the plates for exposure to UVA light emitted by the two UVA lamps, which are located in the upper inner area of the chamber. The intensity of the UV light

of the chamber was measured and calibrated with a radiometer equipped with UVA sensor, and spectral length between 320 and 400 nm.
The equipment used for the calibration of the radiance emitted by UVA light is indicated by the same guidelines that standardize the qualification and validation of the chamber. The results, obtained by the equipment, shown that the dose of 5 J/cm^2, was reached in 75 minutes of exposure, whereas the guides recommend a time of 50 minutes for achieving the same dose.

3.1.1 Qualification of the Chamber and Validation of the Phototoxicity Method

To validation of the phototoxicity test and qualification of the equipment, the SLS (negative control), reference substance chosen and recommended by the guide [1], was used for this assay in nine different positions (FIGURE 4), with eight different concentrations between 0.007 and 0.100 mg/mL. Bergamot oil (positive control) (FIGURE 5) was also used in the same eight different concentrations, with a plate being exposed to positive-UVA and another to negative-UVA.



Dark Control (blue dots) and positive control - 1 Joule (yellow dots)

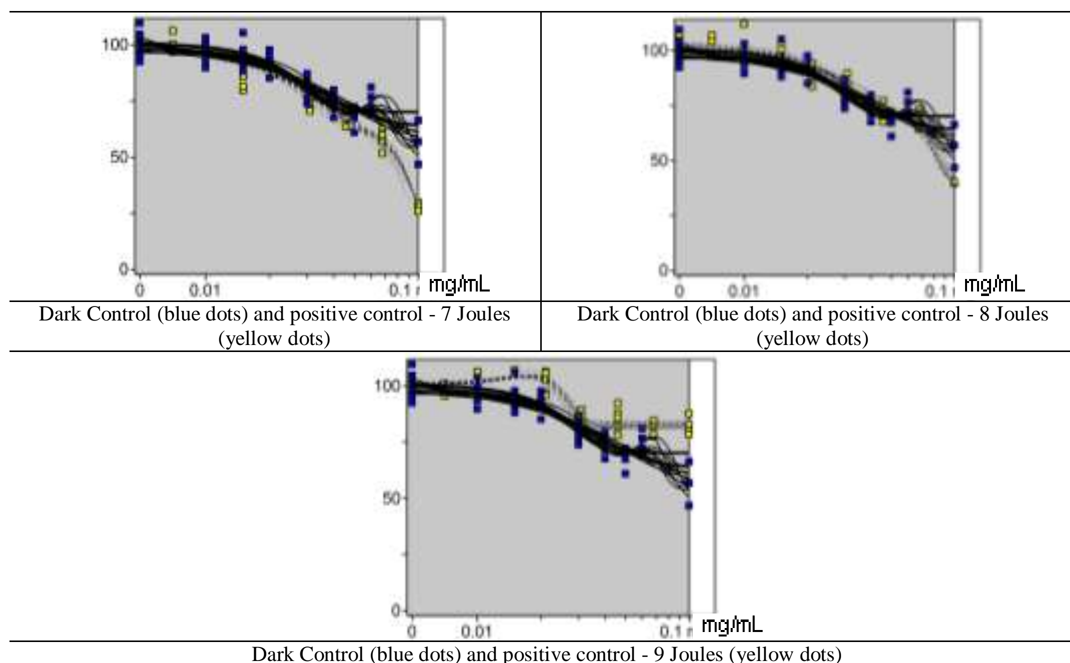
Dark Control (blue dots) and positive control - 2 Joules (yellow dots)

Dark Control (blue dots) and positive control - 3 Joules (yellow dots)

Dark Control (blue dots) and positive control - 4 Joules (yellow dots)

Dark Control (blue dots) and positive control - 5 Joules (yellow dots)

Dark Control (blue dots) and positive control - 6 Joules (yellow dots)



Dark Control (blue dots) and positive control - 7 Joules (yellow dots)

Dark Control (blue dots) and positive control - 8 Joules (yellow dots)

Dark Control (blue dots) and positive control - 9 Joules (yellow dots)

Figure 4: Graphic created by Phototox® program, with readings of phototoxicity of Sodium Lauryl Sulfate (SLS).

The results of the exposure of the 9 plates under UVA light (FIGURE 4), each one having remained under these conditions until the last one reached 9 J/cm^2, and a plate remained in the dark under the same conditions as negative control. The 9 plates, exposed to light, had their results compared with the negative control plate and the values for PIF (Photo Irritation Factor) (see item 2.1.1.1) of SLS ranged between 1 and 1.288, in order words, non-phototoxic substance, according to the classification of the guide [1], being established within this.

In Figure 5, it is possible to observe the graph obtained by reading the absorbance of the phototoxicity of the Bergamot Oil in fibroblasts (Balb/c 3T3).

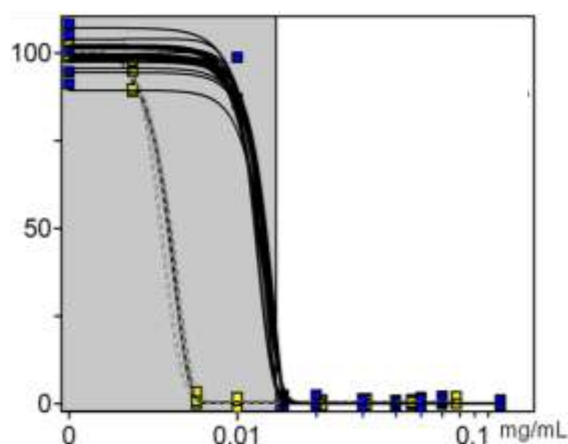


Figure 5: Graphic created by Phototox® program, with readings of the phototoxicity of the Bergamot Oil.

The PIF obtained from Bergamot Oil was equal to 2.475, in order words, according to the recommended by the Guidelines [1; 16], this substance is probable phototoxic.

These results (FIGURE 4 and FIGURE 5) indicate, therefore, that the chamber can be regarded as qualified.

The chamber was developed (FIGURE 1) and constructed attending to the standards described in the guide [3], in stainless steel frame, 3 mm (FIGURE 2), it has the accessories needed for the execution of the test (FIGURE 3). The calibration, performed with a radiometer equipped with UVA sensor (Hönle America), showed that in 75 minutes of exposure it is possible to achieve 5 J/cm^2, as standardized [3].
The chamber qualification was performed by the same phototoxicity assay recommended by the guide [1], which indicates the use of one of the substance standardized by the same as negative reference (non-phototoxic substance) and another one, as positive reference (substance with probable phototoxicity or phototoxic) [3]. The phototoxicity protocol also indicates the use of Phosphate Buffered Saline (PBS) containing 0.1% (w/v) of Ca^{2+} and 0.13% (w/v) of Mg^{2+} (PBS+), because these ionic salts present in the buffer solution maintain the cells attached on the surface of the well during the wash, and it is also used when the cells remain outside the incubator for a long period of time [18]. Knowing that the cells remain outside the CO_2 incubator for a period of approximately 75 minutes, the protocol also indicates that after irradiation of the cells, they must return to the incubator (37 °C, 5% CO_2), remaining to 18-22 hours, for then, to be submitted to the MTS dye test, this period is considered enough for cell recovery [1; 3]. For the implementation of the phototoxicity test, as negative reference substance, was chosen Sodium Lauryl Sulfate (SLS, PIF=<2), recommended by the guide [1], applied in eight different concentrations between 0.007 e 0.100 mg/mL. The positive reference substance chosen was the Bergamot oil, due to appear in the literature as probable substance with photosensitive activity, being considered likely phototoxic oil [19]. The values for PIF, Photo Irritation Factor, of Sodium Lauryl Sulfate (negative substance) in the nine positions tested ranged from 1 to 1.288 (FIGURE. 4). As to Bergamot Oil (FIGURE 5), the PIF achieved with Balb/c 3T3 cells was 2.475, in order words, a substance with likely phototoxicity. These results indicate that the chamber is able to carry out the phototoxicity assay, in any position.

4. CONCLUSION

The chamber proposed to the realization of the phototoxicity test was developed meeting the standards required by guides ©ECVAM DB-ALM: *INVITTOX* protocol: **3T3 NRU Phototoxicity Assay.** ***INVITTOX* nº 78**, and OECD Toxicity Guide – 432, being qualified, the method validated, and therefore, considered suitable to perform phototoxicity assay.

ACKNOWLEDGMENTS

To IPEN/CNEN-SP by infrastructure; to CAPES by scholarship; and FAPESP by financial support given through the project number: 2010/05824-3.

REFERENCES

1. **______**. Guidelines for the Testing of Chemicals. **Test No. 432: *In vitro* e 3T3 NRU phototoxicity test**, 2004.

2. SPIELMANN, H.; BALLS, M.; DUPUIS, J.; PAPE, W.J.W.; PECHOVITCH, G.; 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.; BRANTOM, P. The international EU/COLIPA phototoxicity validation study: results of phase II (blind trial), part 1: the 3T3 NRU phototoxicity test. **Toxicology *In Vitro***, v. 12, p. 305-327, 1998.

3. ECVAM DB-ALM: *INVITTOX* protocol: **3T3 NRU Phototoxicity Assay. *INVITTOX* nº 78**, 21p., 2008

4. DAHLAN, K.Z.M. Radiation Sciences. In: PHILIPS, G.O.; NATHER, A. ***Advances in Tissue Banking v. 5 The Scientific Basis if Tissues Transplantation*** World Scientific, 2001.

5. VELASCO, M.V.R.; RIBEIRO, M.E.; BENDIN, V.; OKUBO, F.R.; STEINER, D. Rejuvenescimento da pele por "peeling" químico: enfoque no "peeling" de fenol. ***Anais Brasileiros de Dermatologia,*** v. 79, n.1, p. 71-79, 2004.

6. BALOGH, T.S.; PEDRIALI, C.A.; BABY, A.R.; KANEKO, T.M.; VELASCO, M.V.R. Proteção à radiação ultravioleta: recursos disponíveis na atualidade em fotoproteção. **Anais Brasileiros de Dermatologia**, v. 86, n. 4, p. 732-742, 2011.

7. FLOR, J.; DAVOLOS, M.R.; CORREA, M.A. Protetores solares. **Química Nova**, v. 30, n. 1, p. 153-158, 2007.

8. SVOBODOVA, A.; WALTEROVA, D.; VOSTALOVA, J. Ultraviolet light induced alteration to the skin. **Biomedical Papers of the Medical Faculty of the University Palacký, Olomouc, Czechoslovakia,** v. 150, n. 1, p. 25-38, 2006.

9. MAHMOUD, U.M.; MEKKAWY, I.A.A.; SAYED, A.E.D.H. Ultraviolet radiation-A (366 nm) induced morphological and histological malformations during embryogenesis of Clarias gariepinus (Burchell, 1822). **Journal of Photochemistry and Photobiology, B: Biology**, v. 95, p. 117-128, 2009.

10. BOUILLON, C. **Skin a living envelope**. Paris: Gallimard, 2002. 127p. (Découvertes Gallimard).

11. WORLD HEALTH ORGANIZATION OF THE UNITED NATIONS. **Health and environmental effects of ultraviolet radiation**: A scientific summary of environmental health criteria 160: Ultraviolet radiation (WHO/EHG/95.16). Geneva: WHO, 1994.

12. SEITÉ, S.; MOYAL, D.; VERDIER, M.-P.; HOURSEAU, C.; FOURTANIER, A. Accumulated p53 protein and UVA protection level of sunscreens. **Photodermatolgy, Photoimmunology & Photomedicine**, v. 16, p. 3-9, 2000.

13. PALM, M.D.; O'DONOGHUE, M.N. Update on photoprotection. **Dermatologic Therapy**, v. 20, p. 360-376, 2007.

14. MATTS, P.J. Solar ultraviolet radiation: definitions and terminology. **Dermatologic Clinics**, v. 24, p. 1-8, 2006.

15. AZEVEDO-FILHO, C.A.; ***Construção de uma câmara de fotoestabilidade, estudo da fotodegradação da vitamina A e da ação antioxidante do polifosfato de sódio em misturas de nutrição parenteral***. 2008. Dissertação (Mestrado) – Universidade Federal do Pernambuco, Recife, PE.

16. ______. Guideline for Testing of Chemicals. **Guidance Document 101: "UV-VIS Absorption Spectra" (Spectrophotometric Method)**, 1981.

17. KUHN, H.J.; BRASLAVSKY, S.E.; SCHIMIDT, R. **Chemical actinometry**. Pure Appl Chem., v. 76, n. 12, p. 2105 – 2146, 2004.

18. DULBECCO, R.; VOGT, M. Plaque formation and isolation of pure lines with poliomyelitis viruses. ***Journal of Experimental Medicine***, v. 99, p. 167-182, 1954.

19. KANĎÁROVÁ, H. ***Evaluation and validation of reconstructed human skin models as alternatives to animal tests in regulatory toxicology***. 2006. Tese (Doutorado). Department of Biology, Chemistry and Pharmacy of Freie Universität Berlin, Berlin.