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3D bioprinted skin models to evaluate anti-pollution and antioxidant potential of compounds from marine origin

Araújo, Nayara F. T.[1]*, Fuzinaga, Thaís Y. T.[1], Debonsi, Hosana M.[1], Tonani, Ludmilla[1], Kress, Marcia RZ.[1]; Colepicolo, Pio[2]; Gaspar, Lorena R.[1].

[1] School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo
[2] Institute of Chemistry, University of São Paulo
nayara.tokashike@usp.br

1. Introduction

UV radiation is composed of UVA (400-320 nm), UVB (320-280 nm), and UVC (280-200 nm) rays and is one of the primary external factors causing damage to human skin [1]. The excess of UVA radiation can be harmful to skin health, as it penetrates the deepest dermal layer and is the main contributor to photoaging. This is due to its ability to increase the production of reactive oxygen species (ROS), which induce inflammatory reactions and DNA damage. Although UVB rays are essential for vitamin D synthesis, they are even more harmful in excess and can lead to sunburn [2]. In contrast, UVC radiation is completely absorbed by the ozone layer and does not reach the Earth's surface [3]. Therefore, UV radiation directly and indirectly destabilizes skin homeostasis, inducing cellular stresses such as oxidative stress in the endoplasmic reticulum and mitochondria [4, 5].

Environmental pollution has also become a major concern in terms of skin health. Pollutants such as particulate matter (PM), heavy metals, volatile organic compounds (VOCs), and polycyclic aromatic hydrocarbons (PAHs) are capable of interacting with the skin's surface, promoting oxidative stress and inflammation. Prolonged exposure to these pollutants can lead to an increase in skin diseases, as well as to accelerate the process of skin aging and cancer [6, 7, 8]. Particles with a diameter of 10 µm or less (PM10) can penetrate deeply into the lungs, but those with a diameter of 2.5 µm or less (PM2.5) are considered the most dangerous to health since PM2.5 can cross the pulmonary barrier and reach the bloodstream [7].

When combined, UV radiation and pollution potentiate oxidative damage [7, 8, 9], and the synergistic effect between these factors further aggravates the deleterious effects on the skin. Simultaneous exposure to UV radiation and PM results in higher ROS production and, consequently, higher damage to cellular DNA, reduction of skin elasticity and enhancement of hyperpigmented spots. These combined effects contribute to premature ageing and can increase the risk of the development of skin cancer. UV radiation acts as a catalyst, promoting the formation of free radicals and increasing the ability of pollutants to induce cell damage [8, 9].

Thus, the search for new active substances from natural origin that can contribute to protecting against these effects has become increasingly evident, especially concerning UV filters. Also, this research is very significant since the direct relationship between some UV filters, such as benzophenone-3, and coral bleaching is already known [10] and configures a risk for the marine environment survival. In this context, the seas and oceans stand out as a great source of biodiversity, covering around 70% of the Earth's surface [11]. The marine environment is characterized by providing a harsh environment, yielding a large diversity of natural products with bioactivities to be explored [12]. To withstand these conditions, microorganisms from Antarctica, for example, have different defense mechanisms to prevent radiation damage, such as the production of pigments capable of absorbing UV radiation [13] or antioxidants, which makes them highly potential for use in studies such as this one.

2. Materials and Methods

2.1. Fungus: growth, extraction, and fractionation

The marine microorganism originating from Antarctica was obtained on an expedition conducted in 2017 during PROANTAR's OPERANTAR XXXVI operation. The endophytic fungus was isolated from the red alga *Kallymenia antarctica*, collected on King George Island, in the Punta Demay (62º 13.141 S 058º 26.266 W) and Vauréal (62º 10.857 S 058º 17.850 W) regions. The fungus was isolated at the Laboratory of Organic Chemistry of the Marine Environment (NPPNS, FCFRP, USP) according to the methodology described by Teixeira et al (2019) [14]. Its identification was performed at the laboratory of Prof. Dr. Márcia R. V. Z. Kress (FCFRP, USP), through morphological analysis, molecular analysis by PCR, and sequencing of the Internal Transcribed Sequence 1 (ITS1) and ITS2 regions of ribosomal DNA. The fungus *Sarocladium* sp. was reactivated and cultivated for 28 days under upscaled conditions using a parboiled rice medium supplemented with laboratory-produced synthetic seawater SWBG-11 [14]. The mycelium was separated from the medium containing the released metabolites, which was submitted to a liquid-liquid extraction using ethyl acetate, avoiding chlorinated solvents to maintain the eco-friendly approach of the proposed project. The extracted material was concentrated in a rotary evaporator under reduced pressure and soft heat, yielding the crude extract. Subsequently, the dried extracts were subjected to Vacuum Liquid Chromatography (VLC) with gradient elution of *n*-hexane, methanol, and ethyl acetate, besides separations using reverse phase HPLC, aiming to isolate and purify the target compounds [14].

2.2. 3D bioprinted skin models

In order to standardize the 3D bioprinted skin, we used parameters already employed in our laboratory for *in-house* reconstructed human skin [15]. However, considering the use of the 3D bioprinter, it was necessary not only to adjust the number of cells used in the dermis and epidermis, but also to develop and improve a G-Code that allows control of printing parameters such as speed, height and pressure. The dermis was first prepared, containing collagen type I and human primary fibroblasts. The bioprinting was performed with a helical movement using the Genesis bioprinter (3DBS, Brazil), in 12-well plate size inserts. After 24 hours, the epidermis was bioprinted using human primary keratinocytes. The models were cultured in an air-liquid interface for 7 days to ensure the stratification and differentiation of the epidermis. The characterization of the tissues was performed by histological analysis using Hematoxylin and Eosin staining.

2.3. Anti-pollution and antioxidant potential

After full tissue differentiation, the models were incubated with the $DCFH_2$-DA (2',7'-dichlorodihydrofluorescein diacetate) (25 µM) probe for 45 minutes at 37°C. This probe is a non-fluorescent compound capable of permeating cell membranes. Intracellular esterases hydrolyze $DCFH_2$-DA, which is subsequently oxidized in the presence of ROS. Upon irradiation, this process generates the fluorescent compound 2'72-DCF [16].
Sequentially, they were exposed to nebulization of particulate matter (2.5 micrometers) using a Vitrocell Cloud™ System. To evaluate the antioxidant and anti-pollution potential, 40 µL of the studied fraction (1 µg/mL) was applied topically on the models and left in contact for 1 hour, then submitted to a total dose of 6 J/cm^2 UVA radiation. The skin models were frozen, cryosectioned and the diamidinophenylindole (DAPI) staining (0,3 µg/mL) was performed. The ROS production was measured through fluorescence analysis, calculated using the Image J software, in area/pixels, and expressed as a percentage of fluorescence intensity compared with the untreated model that was irradiated and exposed to pollution. Statistical analysis was performed using ANOVA followed by Tukey's post-hoc test with the RWizard software. *p*-values of ≤ 0.05 were considered statistically significant.

3. Results

3.1. Fungus: growth, extraction, and fractionation

After cultivation and extraction, the crude extract was fractionated and nine fractions were obtained in increasing order of polarity. The fraction used in this study (Fr. E) corresponded to 13% of extract amount.

Figure 1. *Sarocladium* sp. fungus reactivated in petri dishes using potato dextrose agar (PDA) medium.

3.2. 3D bioprinted skin models

After evaluating different strategies and conditions for the standardization of 3D bioprinted human skin, it was possible to obtain models with proper differentiation of the dermis, epidermis, and stratum corneum, as demonstrated by HE histology (Figure 2).

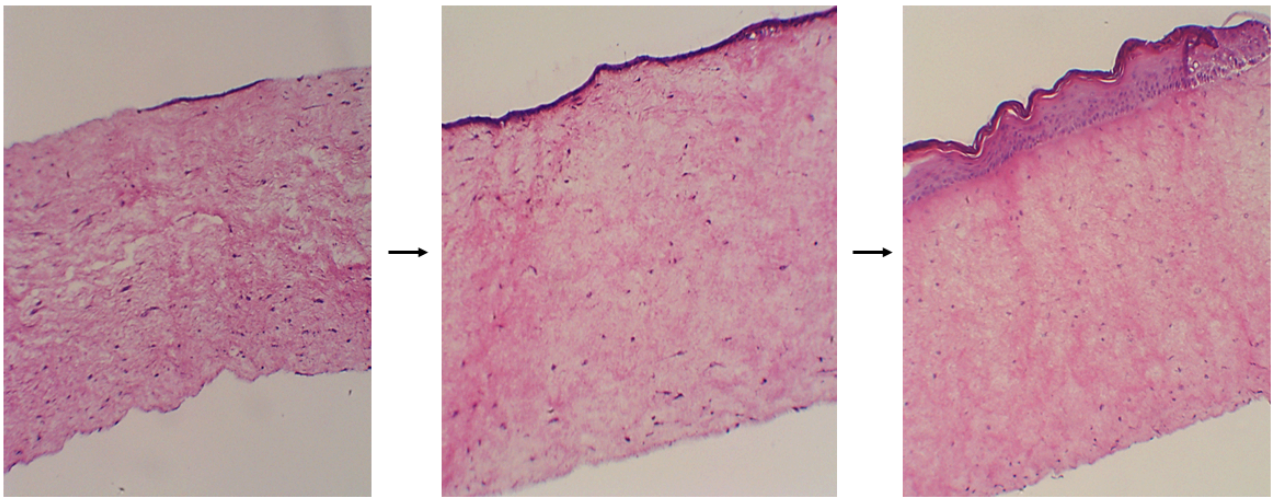

Figure 2. Characterization of the 3D bioprinted skin models by histological characterization by Hematoxylin and Eosin staining.

3.3. Antipollution and antioxidant potential

Images obtained from fluorescence electron microscopy show that fluorescence, which is correlated with ROS production by UV radiation and pollution exposure, was most evident in the epidermis, with keratinocyte nuclei evident when stained with DAPI (Figure 3). When applied before exposure to UVA radiation, Fr. E (1 µg/mL) induced a significant reduction of ROS generation (44%) ($p < 0.05$), when compared to the polluted and irradiated control (PM 2.5 + 6UV) (Figures 4 and 5).

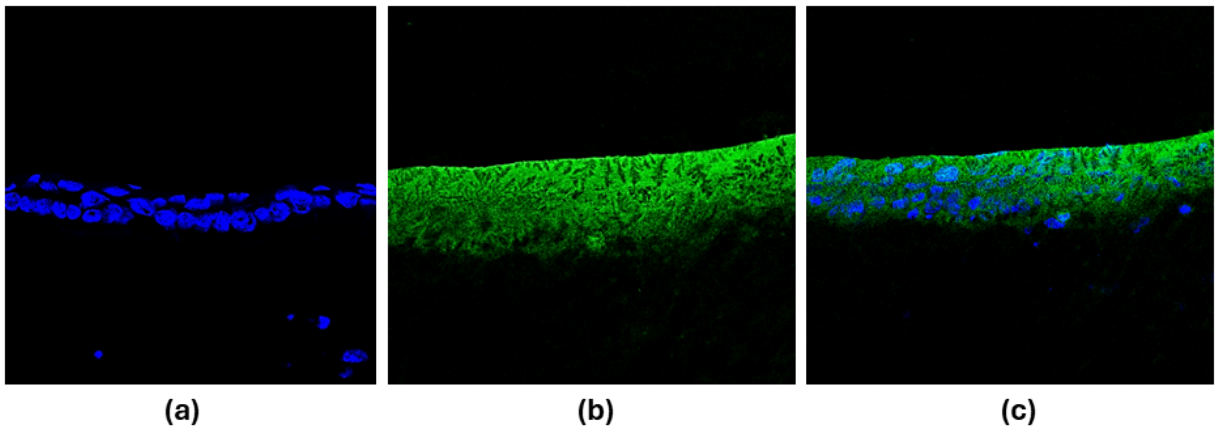

Figure 3. Images obtained from fluorescence electron microscopy of the control PM 2.5 + 6UV with **(a)** DAPI ; **(b)** $DCFH_2$-DA; **(c)** merged.

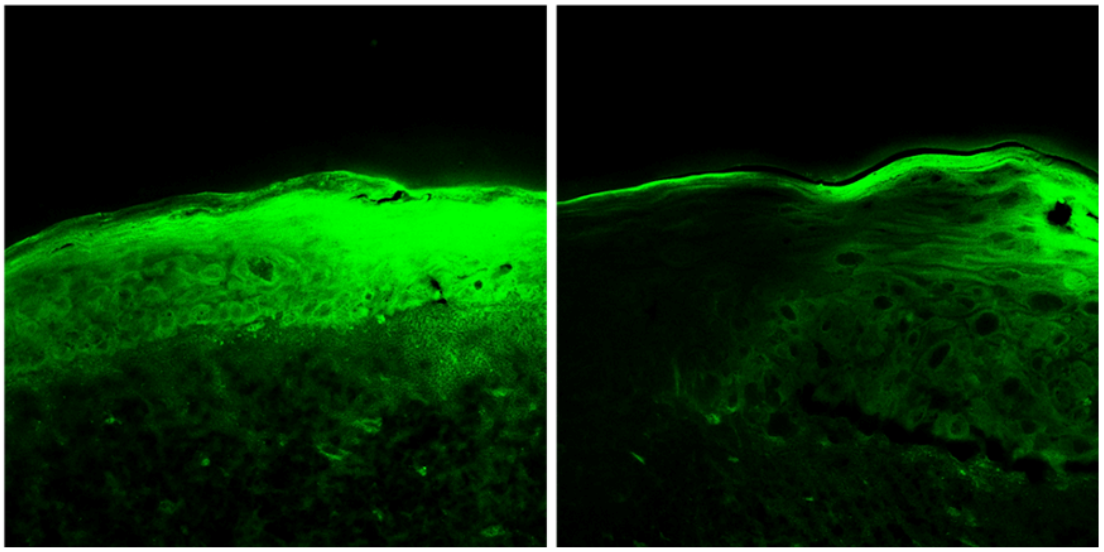

Figure 4. Images obtained from fluorescence electron microscopy with the application of the studied fraction (Fr. E) **(a)** PM 2.5 + 6UV ; **(b)** PM 2.5 + Fr. E + 6UV.

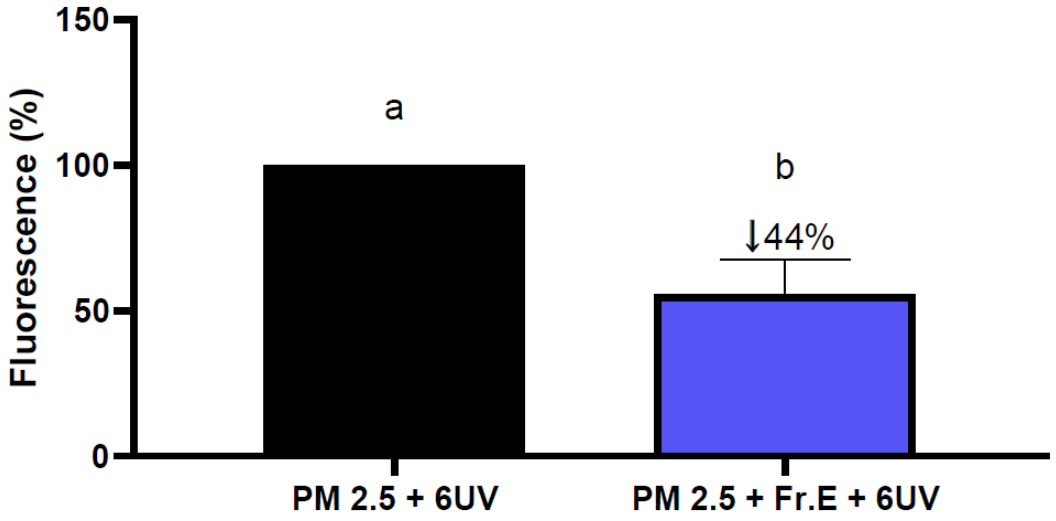

Figure 5. UVA-induced intracellular ROS production in 3D bioprinted skin model exposed to pollution and UV radiation (PM 2.5 + 6UV) and exposed to pollution, UV and pretreated with the studied fraction (PM 2.5 + Fr. E + 6UV). Different letters indicate statistically different means ($p < 0.05$), according to one-way ANOVA followed by Tukey post-hoc tests (n=3, independent experiments).

4. Discussion

In vitro cell cultures of human tissues are widely used and well established for preclinical testing, as alternative methods to animal testing; they are used for the initial assessment of the efficacy of a new active ingredient, allowing the assessment of toxicological risks of a vast range of substances [17]. Monolayer models have several disadvantages, including changes in cell shape, function and response, as well as a lack of interactions between cells

[18, 19]. In these 2D systems, the cells have a higher contact surface with the culture medium and the plastic of the bottles or plates in which they are grown, rather than cell-cell contact, which can lead to changes in cell morphology and behavior, promoting an organization that does not necessarily reflect the physiological conditions of the tissues [20, 21].

The use of 3D *in vitro* systems has been a promising alternative to these issues, although these models cannot completely recreate the complexity and functionality of the tissue. The use of 3D systems mimicking bioprinted tissues and organs, as well as microfluidic systems, has shown great relevance and predictive capacity for evaluating the safety and efficacy of new drug candidates and cosmetics, increasing the chances of successful clinical studies. Reconstructed skin models are considered physiologically relevant to replace animal testing, and the use of bioprinters has shown better results in terms of tissue organization and reproducibility between models.

In this context, the development of more complex and efficient systems, combined with the progression of assays to scientifically prove the efficacy and safety of products, has been a tendency in the pharmaceutical and cosmetic areas. As a result, these systems became a strategy adopted by leading companies in the market to increase the added value of the product and its competitiveness, especially for UV filters [22].

Proper differentiation of the dermis, epidermis, and stratum corneum enables the obtained 3D bioprinted model to be used as a reliable platform for evaluating safety and efficacy parameters of new substances. The characterization of the models showed that the methodology was adequate to self-organizing the cells in a similar pattern to natural tissues. Quantitative analysis of UVA-induced intracellular ROS production confirmed the physiological responsiveness of the developed 3D bioprinted models to UV radiation and environmental pollutants, establishing their potential to be applied as a tool for multifactorial toxicological assays.

The Fraction E studied was obtained from Antarctic endophytic fungi *Sarocladium* sp.. For the biological potential analyses, 1 µg/mL concentration sample was used. The results demonstrated that Fr. E exhibits significant antioxidant and anti-pollution activity when applied to the models before UVA radiation exposure, demonstrating the potential of Antarctic organisms to prevent radiation damage. The findings are consistent with the concept that pre-treatment allows antioxidants to be readily available to scavenge free radicals as soon as they are formed, preventing the following oxidative stress in the skin [23].

The standardization of bioprinted skin models adds relevance to the evaluation of the safety and efficacy of new cosmetic ingredients. The study of the fungal fraction analyzed provides important information for the development of topical materials designed to protect the skin against UV-induced damage and pollution.

5. Conclusion

The 3D bioprinted human skin model exhibited proper cellular organization throughout the dermis and epidermis. This model was employed as a tool to evaluate the harmful effects of pollution and UV radiation. The studied fraction (Fr. E) obtained from *Sarocladium* sp. reduced 44% of ROS generation in the polluted bioprinted skin models when applied before exposure to UV radiation. These findings indicate the potential of the developed model as a promising tool for assessing the effects of solar radiation and pollution on human skin. The

studied fraction presents high antipollution and antioxidant potential; however, other clinical studies will be performed to confirm its safety and efficacy.

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7. Conflict of Interest Statement

NONE.

6. References

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