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Study of the antioxidant capacity of fullerenes C_{60} in cosmetic formulations

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Abstract– Universities should prioritize equipping students with the ability to tackle real-world challenges during their professional training. This project aimed to inspire senior Nanotechnology Engineering students to develop creative solutions by using a problem-based learning approach. The focus of this project was to assess the use of Fullerenes (C_{60}) in commercial cosmetic formulations, and to verify their efficacy in achieving the advertised effects.

Nowadays, there is an increasing number of skin care cosmetics that claim to contain fullerenes (C_{60}) as antioxidant agents [1]. The properties of these products have been evaluated using human fibroblast and keratinocyte cell lines. However, there are no studies evaluating their antioxidant properties in vitro when incorporated into a cosmetic formulation. The present work evaluates the antioxidant capacity of fullerenes (C_{60}) in a cosmetic base formulation in a dermal fibroblast (HDFa) cell line. To modify its highly hydrophobic character, fullerenol $C_{60}(OH)n \cdot mH_2O$ was synthesised from pure C_{60} and incorporated into a cosmetic formulation as an active ingredient at concentrations of 0. 2 and 0.5 % w/w. Preliminary results show that the cell viability of HDFa fibroblasts was not affected by treatments at concentrations of 0.1 and 0.5 mg/ml.

With reference to its antioxidant properties, the DPPH inhibition test showed that there were no significant effects when fullerenol was incorporated into the cosmetic base formulation as an active ingredient at concentrations of 31.25, 62.5 and 125 µg/ml. However, in the cellular antioxidant capacity test, a tendency to reduce the production of reactive oxygen species was observed when fullerenol was incorporated as an active in the base formulation at both 0.2% and 0.5%, demonstrating its antioxidant potential.

Keywords— Fullerene, Fullerenol, antioxidant capacity, cytotoxicity, hydroxylation, higher education, educational innovation, professional education.

I. Introduction

The available scientific information related to the antioxidant properties of C_{60} fullerenes when applied to a cosmetic skin product is limited. Currently, the StatNano database of nanoengineered products reports that there are 10,765 commercial products, of which 993 are cosmetic products, but of these only 1 is reported to use C_{60} fullerenes in a topical cosmetic formulation claiming antioxidant activity. [1].

Regarding these molecules, it should be noted that $C60$ fullerenes are a unique class of molecules composed exclusively of carbon in the form of hollow spheres (Fig. 1) that have attracted considerable attention due to their reported antioxidant capacity. They are spherical molecules with a structure similar to that of a soccer ball that are composed of 60 carbon atoms, have an approximate diameter of 1 nm and are hydrophobic in nature [2].

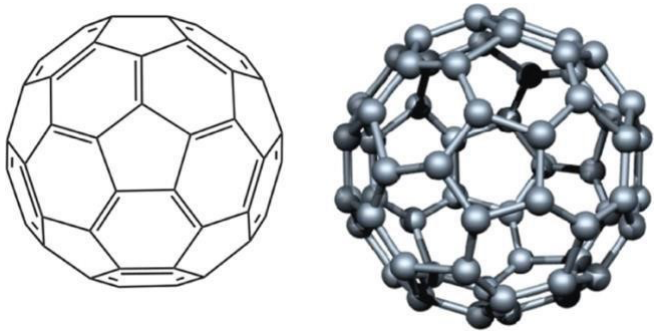

Fig 1. Schematic representation (2D and 3D) of the structure of C60 fullerene. Figure taken from [3].

$C60$ fullerenes possess free electrons on their surface, which allow them to interact directly with reactive species, neutralizing them and protecting cells from the harmful effects of oxidative stress [4]. Oxidative stress is caused by an

imbalance between the production of reactive species or free radicals of oxygen (ROS) and the body's antioxidant capacity.

ROS constitute a diverse category of highly reactive molecules (superoxides, hydrogen peroxide and hydroxyl radicals) that occur naturally as by-products of cellular metabolism, but their production can increase significantly in response to external factors such as UV exposure, pollution and stress [5]-[7]. Uncontrolled action can cause cellular damage and affect lipids, proteins, and nucleic acids, contributing to the progressive deterioration of cellular structures, including the skin. The skin is the first physical, chemical, and immunological protective barrier between the body and the external environment. Therefore, it is particularly susceptible to oxidative stress and the consequent conditions derived from this phenomenon, such as acne vulgaris, alopecia, and the acceleration of the aging process that results in wrinkle formation, loss of elasticity, and uneven pigmentation.

Topical application of antioxidants in cosmetic formulations is one way to fight oxidative stress in the skin. Antioxidants play an important role in preventing, reducing, and repairing damage caused by ROS through various mechanisms of action. Vitamin C, glutathione, and vitamin E are among the most used antioxidant compounds in cosmetic formulations [11]-[13].

On the other hand, given its recognized antioxidant capacity, fullerene C60 is a promising candidate for its application in cosmetic formulations as an active ingredient. Specifically, the antioxidant properties and cytotoxicity of fullerenes have been studied *in-vitro* mainly in human keratinocyte cell lines (HaCat) [14], [15]. However, the available scientific information related to its antioxidant capacity when incorporated into a cosmetic formulation is limited. Dermal fibroblasts are the main cell type of the dermis [16], [17]. They are responsible for synthesizing and remodeling extracellular matrix proteins and play a role in wound healing, inflammation, and cell proliferation [18], [19]. Due to these properties, fibroblasts are widely used as an invitro model in research and product development in the cosmetic industry, as a preliminary stage prior to in-vivo treatment.

The objective of this work was the evaluation of the capacity of C_{60} fullerenes as antioxidant agents in a cosmetic formulation in a cell line of adult human dermal fibroblasts, so the research question intended to be answered is:

Can fullerenes (C_{60}) or their derivatives exhibit antioxidant effects when tested on adult human dermal fibroblasts?

II. Methodology

A. C_{60} Functionalization

In this study, to improve its compatibility in polar environments, pure C_{60} fullerene was functionalized with hydroxyl groups and the functionalization was physicochemically characterized by Fourier transform infrared spectroscopy (FT-IR) and photoinduced force microscopy (PiFM). The resulting product was then incorporated into a cosmetic formulation to evaluate its antioxidant capacity and cytotoxicity in HDFa dermal fibroblasts.

A.1 Functionalization of Fullerene C60 to Fullerenol $C_{60}(OH)n \cdot mH_2O$

The synthesis of $C_{60}(OH)_n \cdot mH_2O$ was carried out following the methodology reported by Afreen et al [31]. To prepare the solutions, 200 mg of fullerene C_{60} was added to 20 mL of 30% H_2O_2 and stirred manually for a few seconds. The samples were prepared in quintuplicate. The solutions were then sonicated in a water bath for 2 hours while maintaining a temperature of 20-25 ºC to prevent overheating.

A.2 Characterization of Fullerenol

FT-IR was used to identify the formation and attachment of hydroxyl groups (-OH) to the $C60$ structure, using a PerkinElmer Frontier equipment (64 scans).

The particle thickness (width and height) of $C60(OH)n \cdot mH2O$ was determined using a PiFM Vista One device from Molecular Vista.

B. Preparation of the cosmetic formulation

The study used as active ingredient $C60(OH)n \cdot mH2O$ synthesized at concentrations of 0.2 and 0.5%. The cosmetic formulation contained carbopol as a rheological agent in the aqueous phase, cetyl alcohol, stearic acid and isopropyl myristate in the oil phase and additives and preservatives such as propylene glycol, methyl paraben, propyl paraben and potassium polysorbate 20 in an additional phase.
Triethanolamine was used as a pH adjuster.

C. Determination of cell viability in HDFa fibroblasts. VC method

HDFa cells (5×10^4 cells per well) were seeded in DMEM-F12 supplemented with 10% fetal bovine serum in a 96-well plate. The plate was then incubated at 37 °C and 5% CO_2 for 24 hours.

Dilutions of pure fullerene and fullerenol, as well as a base cosmetic formula without fullerenol, were prepared at concentrations of 1, 0.5, and 0.1 mg/mL. Additionally, 0.2% and 0.5% concentrations of the cosmetic formulation were prepared. Each sample was added to a well in triplicate, and the assay was performed. After incubation for 24 hours, 20 µL of the CellTiter 96® Aqueous One Solution Cell Proliferation Assay reagent (Promega, Madison, WI) was added to each well and incubated for 2 hours. Finally, the absorbance was measured using a microplate reader at 490 nm at room temperature to estimate cell viability.

D. Antioxidant activity measurement

D.1 Antioxidant activity CAA Method

HDFa cells (5×10^4 cells per well) were seeded in DMEM-F12 supplemented with 10% fetal bovine serum in a black 96-well plate.

The plate was then incubated at 37 °C and 5% CO_2 for 24 hours. After incubation, the medium was removed, and the cells were washed with Phosphate-buffered saline (PBS). Next, the cells were incubated with 100 µL of the prepared samples (diluted to 0.5 and 0.1 mg/mL) along with 60 µM of DCFH-DA (1:1, v/v) and 100 µL of DMEM-F12 for an additional 20 minutes in the same plate.

After the incubation period, the medium was removed, and two washes with PBS were performed. Then, 100 µL of 100 mM AAPH was added to all wells except for the negative control and the blank to generate oxidative stress.

The plate was incubated for 90 minutes at 37 °C, and finally, the fluorescence emitted at 538 nm with excitation at 485 nm was measured.

The positive control cells were cultured with DCFH-DA (no sample) and stimulated with AAPH.

D.2 Chemical measurement of compounds with antioxidant activity by DPPH free radical inhibition method

A solution of 60 µM DPPH was prepared and serially diluted to 125, 62.5, and 31.25 µg/mL in 80% methanol.

Next, 100 µL of each sample (Am) was added to a 96well plate, with 80% methanol as the blank ($Ablc$). The µM DPPH solution (1:1 v/v) was added to each sample; the test was performed in triplicate for each sample. The plate was allowed to rest at room temperature (20-22 ºC) for 30 minutes. After the rest period, the absorbance was measured using a microplate reader at 517 nm to determine the inhibition of DPPH.

III. Results & Discussions

III A. Synthesis of $C_{60}(OH)_n \cdot mH_2O$

Figure 2A confirms that the fullerene C_{60} is initially immiscible with H_2O_2, resulting in a heterogeneous mixture, which after 1 hour of sonication became a partially miscible light brown solution (Figure 2B). After 2 hours of sonication, dark brown solutions were obtained, indicating the hydroxylation of C_{60} fullerene (Figure 2C).

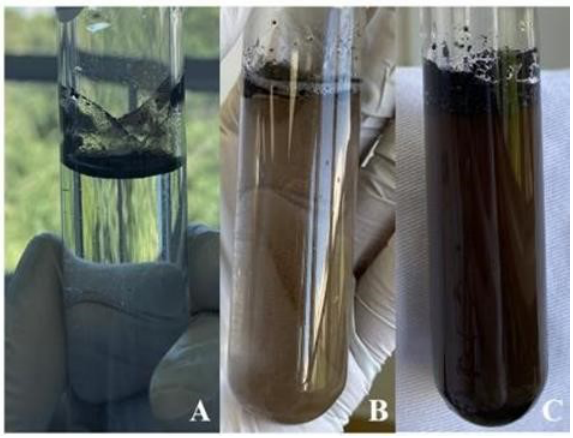

Fig 2. Synthesis of $C_{60}(OH)n \cdot mH_2O$. (a) C_{60} (dark gray solid) /H_2O_2 (colorless liquid) before sonication (b) C_{60} / H_2O_2, 1 hour in sonication. (c) C_{60}/$C_{60}(OH)n \cdot mH_2O$ 2 hours in sonication.

After adding toluene to the solution for the first wash, the solution separated into two layers, as shown in Fig. 3.

The lower layer is dark brown and contains $C_{60}(OH)n \cdot mH_2O$ in H_2O_2, while the upper layer is dark violet (Fig. 3A), which is a characteristic color due to the dissolution of pure C_{60} particles in toluene.

In the last wash, the upper layer turns translucent purple (Fig. 3B), indicating the removal of pure, unreacted $C60$ from the lower layer.

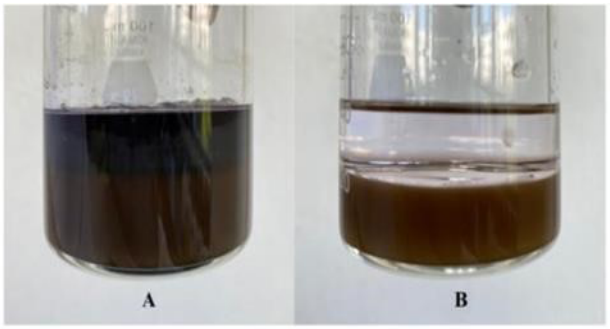

Fig 3. Separation of C_{60}: A) Wash 1 resulted in an upper layer containing C_{60}/toluene in a dark violet color and a lower layer in a dark brown color. B) Wash 12 resulted in an upper layer containing C_{60}/toluene in a translucent purple color and a lower brown layer containing $C_{60}(OH)\ n \cdot mH_2O$ /H_2O_2.

After drying and purification in a rotary evaporator, a dark brown solid was obtained as shown in Figure 4, identified as hydroxylated fullerene ($C_{60}(OH)n \cdot mH_2O$).

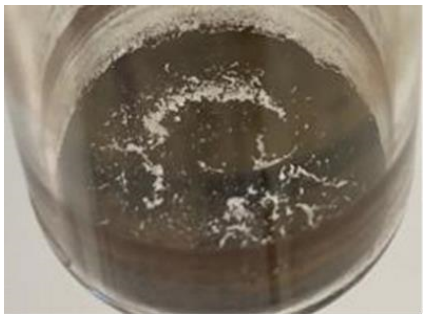

Fig 4. The solid brown is identified as $C60(OH)n \cdot mH2O$.

III B. Results of Cosmetic Product Formulation

The resulting cosmetic formulation prepared with the active ingredient $C_{60}(OH)n \cdot mH_2O$ at a concentration of 0.2 and 0.5% (Figs. 5A and 5B) has a brown color. The color is darker in the formulation with the higher concentration. The color of the cosmetic base formula was white before the addition of the active ingredient.

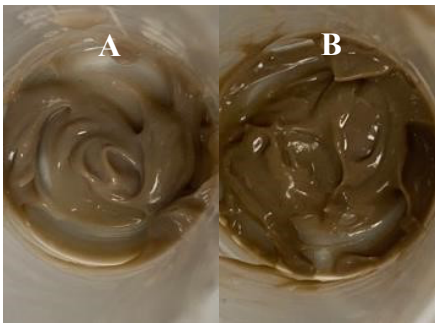

Fig 5. Gel-cream type cosmetic formulation with $C60(OH)n \cdot mH2O$ as active ingredient at A) 0.2 and B) 0.5 in %w/w.

III C. Characterization by Identification of Hydroxyl Groups (OH)

To identify the functional groups, FT-IR analysis was performed on the dry $C_{60}(OH)n \cdot mH_2O$ (Fig. 6). A signal was observed at 3386 cm^{-1} in Fig. 6B, which is within the range of 3400 to 3300 cm^{-1} and is the characteristic peak of O-H stretching. This indicates the presence of the hydroxyl group (OH) in the sample, confirming the hydroxylation of fullerene. However, the signal obtained is not as intense as that reported in previous studies. Therefore, it would seem that the degree of hydroxylation of the compound is low [31].

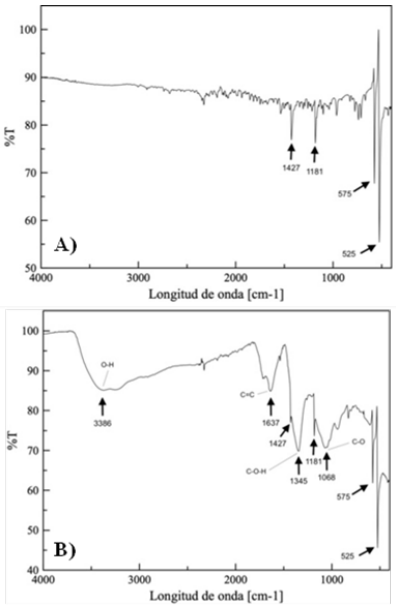

Fig 6. The experimental FTIR spectrum shows A) pure C_{60} and B) $C_{60}(OH)n.mH_2O$.

Likewise, the study observed signals at 1068, 1345, and 1637 cm^{-1}. Based on previous studies [32]-[34], the absorption in the region of 1100-1000 cm^{-1} is associated with C-O bonds, the region of 1400-1300 cm^{-1} with C-O-H bonds, and the region of 1660-1600 cm^{-1} with C=C bonds, indicating the presence of carbon-carbon double bonds in the fullerene structure.The spectra in Fig. 6A and 6B both show additional peaks at 525, 575, 1181, and 1427 cm^{-1}, which are characteristic of pure C_{60} [35]. This suggests that the hydroxylated fullerene may still contain unreacted pure C_{60} residues.

III D. Particle size measurement by Photo-induced force microscopy (PiFM)

Previous studies have reported that the aggregative nature of fullerenol particles $C_{60}(OH)n \cdot mH_2O$ decreases as the number of -OH groups decreases [36]–[39]. A PiFM scan was conducted to observe the topography, size, and agglomerations of the $C_{60}(OH)n \cdot mH_2O$ in the sample.

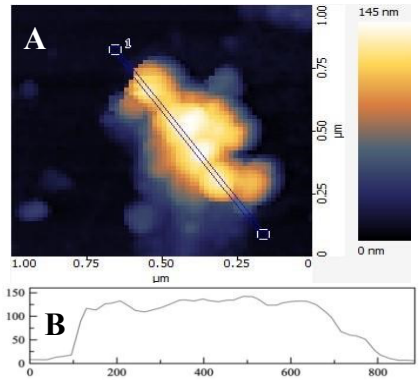

Figura 7. A) Topography of agglomerate of $C60(OH)n \cdot mH2O$ observed in the sample and B) topography vs distance graph for thickness measurement.

Figure 7A indicates a high degree of aggregation of the $C_{60}(OH)n \cdot mH_2O$ particles, where the width and height of the agglomerate were 1000 nm and 100-45 nm, respectively.

In contrast, Figure 8A shows a lower degree of aggregation, with a particle width of 100 nm and height of 1525 nm. Mapping was conducted in multiple areas of the sample, revealing a high number of agglomerates. This suggests that $C_{60}(OH)n \cdot mH_2O$ has fewer -OH groups attached to its structure, which leads to a higher degree of particle aggregation. Additionally, the degree of agglomeration can be affected by sample preparation. In this instance, the sample was dissolved in a drop of ethanol 24 hours before analysis.

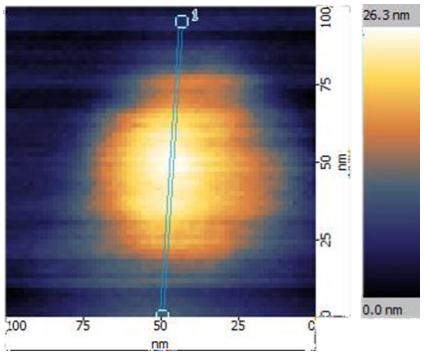

Fig 8. A) Particle topography of $C60(OH)n \cdot mH2O$ and B) topography vs distance graph for thickness measurement.

However, the color of the solid obtained reaffirms the low degree of hydroxylation. Previous studies have shown that highly hydroxylated fullerenes tend to be pale yellow or milky white [36], while those with low hydroxylation tend to be dark brown (Fig. 4).

III E. Biological assays in HDFa cells E.1 Determination of cell viability (CV)

Cell viability was evaluated in HDFa fibroblasts at concentrations of 0.1, 0.5 and 1 mg/mL for samples of pure fullerenes, fullerenol, basic cosmetic formulation (without antioxidant) and cosmetic formulations containing 0.2 and 0.5% fullerenol. The results are presented in Figure 9.

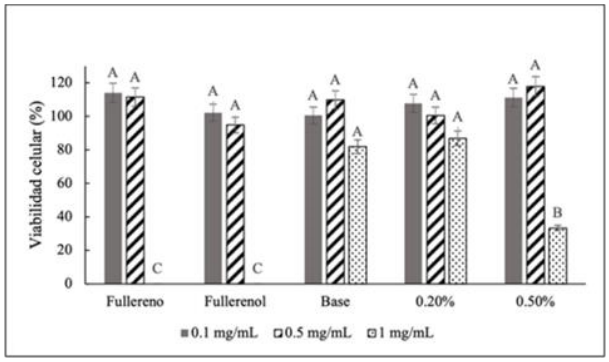

Fig 9. Cell viability (CV) of samples of fullerene, fullerenol, base formulation and cosmetic formulations at 0.2% and 0.5% by weight of fullerenol in HDFa cells at concentrations of 0.1, 0.5 and 1 mg/mL. A, B, C Indicate statistical differences between the treatments at each of the concentrations evaluated (Tukey test, <0.05)

Figure 9 shows that at a concentration of 0.1 mg/mL, all cells remained viable without significant differences between treatments. Additionally, Figure 10 demonstrates that fibroblasts experienced minimal cellular stress. It is worth noting that in the fullerene and fullerenol treatments, there are remnants that are not fully dissolved (black dots in Fig. 10 A and B). This is due to the low solubility of fullerene in the solvent used, which was isopropanol (only in that treatment) with 0.0021mg/mL [41]. Despite increasing its miscibility in water, undissolved residues can also be observed in the fullerenol treatment.

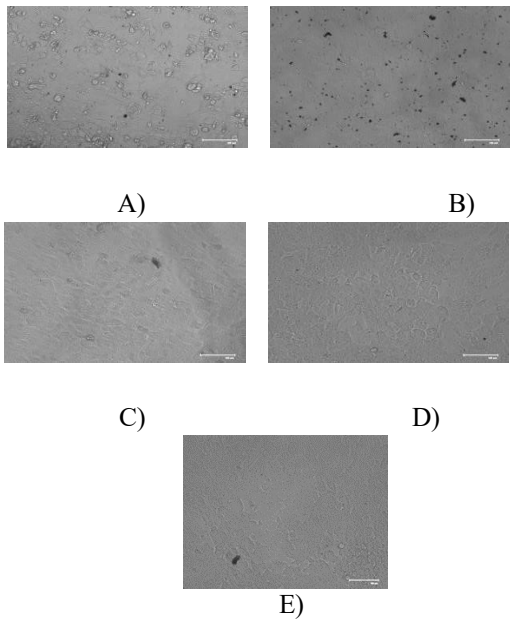

Fig 10. Bright field micrographs in an inverted microscope of HDFa fibroblasts 24 h after being treated with a concentration of 0.1 mg/mL of A) Fullerene, B) Fullerenol, C) Base formulation, D) 0.2% fullerenol formulation and E) 0.5 formulation % fullerenol, at a scale of 100 µm.

When the concentration of the treatments was increased to 0.5 mg/mL, a slight decrease in VC was observed in cells treated with fullerene, fullerenol, and 0.2% formulation, while

maintaining high VC values of 111.45%, 94.78%, and 100.49%, respectively. In contrast, a slight increase was observed in treatments with the base formulation and at 0.5%, resulting in values of 109.74% and 117.84%, respectively. However, no significant differences were found between treatments at concentrations of 0.1 and 0.5 mg/mL.

III.F Measurement of compounds with antioxidant activity by inhibition of the DPPH free radical

To evaluate the antioxidant properties of fullerene C60 in cosmetic formulations, we measured the inhibition capacity of the DPPH free radical (%) in various samples. These included pure fullerene, fullerenol, a base cosmetic formulation without an antioxidant, and cosmetic formulations containing 0.2% and 0.5% fullerenol at concentrations of 31.25, 62.5, and 125 µg/mL. The results are presented in Fig. 11.

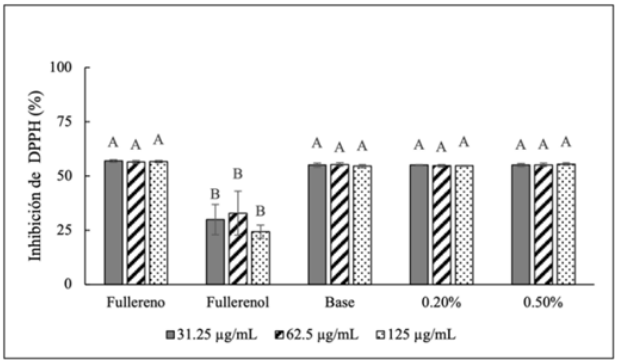

Fig 11. Inhibition of the DPPH free radical at concentrations of 31.25, 62.5 and 125 µg/mL. A, B They indicate statistical differences between the treatments at each of the concentrations evaluated (Tukey test, <0.05)

Figure 11 shows that at a concentration of 31.25 µg/mL, both the fullerene and base formulations with 0.2% and 0.5% fullerenol exhibited DPPH inhibition values of 56.95% and 55.05%, respectively. However, these values were not statistically different from the DPPH inhibition of fullerenol, which inhibited 29.88% and had the lowest inhibition value at this concentration. In addition to the characteristics mentioned above, it was observed that the inclusion of fullerenol as an ingredient in the cosmetic formulations at 0.2% and 0.5% did not have any effect on the percentage of DPPH inhibition compared to the base formulation.

At a concentration of 62.5 µg/mL, the trend observed is like that at 31.25 µg/mL. Only the fullerenol sample differs significantly from the other four samples analyzed, exhibiting a DPPH inhibition value of 32.89%. The fullerene samples and the base formulations, 0.2% and 0.5%, respectively, presented values of 56.47%, 55.37%, 54.73%, and 55.21%. Finally, at an applied concentration of 125 µg/mL, fullerenol showed an inhibition of 24.34%, while the fullerene samples and the base formulations at 0.2% and 0.5% presented an inhibition of 56.63%, 54.57%, 54.73%, and 55.52%, respectively.

It should be noted that the DPPH inhibition of fullerenol did not follow a regular pattern of increase or decrease depending on the applied concentrations. This variation in DPPH inhibition may be related to the structure and agglomeration of the synthesized fullerenol and could be attributed to the selective nature of DPPH reactivity towards free radicals and hydrogen atom donors [27]. At higher concentrations, fullerenol tends to agglomerate, which may reduce the availability of reactive sites for interaction with DPPH. This is due to the limited space around its divalent nitrogen, which sterically inhibits the addition of bulky groups in that region [40]. This phenomenon could explain the observed decrease in inhibition at the highest concentration applied (125 µg/mL).

III.*G Determination of compounds with antioxidant activity by cellular antioxidant activity (CAA)*

The study of cellular antioxidant capacity of samples of pure fullerene, fullerenol, pure cosmetic formulation (without antioxidant) and cosmetic formulations with 0.2 and 0.5% fullerenol was performed in HDFa fibroblasts. HDFa fibroblasts were used for the test. To ensure accurate results, the cellular antioxidant activity test was carried out at concentrations of 0.1 and 0.5 mg/mL, as these concentrations showed viability values above 94.78%. The results are
presented in Figure 12

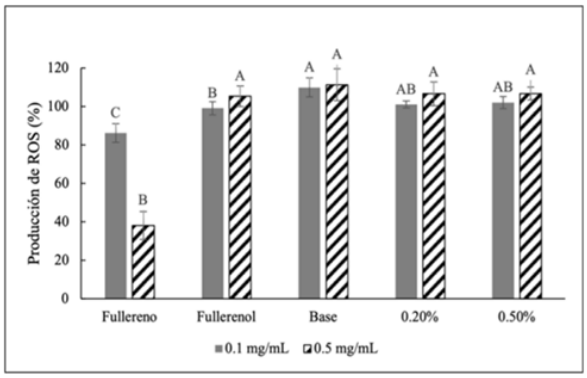

Fig 12. Cellular antioxidant activity assay of samples of fullerene, fullerenol, base formulation and cosmetic formulations at 0.2% and 0.5% by weight of fullerenol in HDFa cells at concentrations of 0.1 and 0.5 mg/mL. A, B, C Indicate statistical differences between the treatments at each of the concentrations evaluated (Tukey test, <0.05)

Figure 12 shows a significant decrease in ROS production at a concentration of 0.1 mg/mL for both fullerene and fullerenol treatments, with values of 86.26% and 99.15%, respectively. The fullerene treatment resulted in a significant reduction in ROS production compared to the fullerenol treatment. Similarly, a significant difference was observed in the ROS production between the base formulation (without any antioxidant agent) with a value of 109.97% and fullerenol, which demonstrated its antioxidant potential, with a value of 99.15%. However, the fullerenol formulations with concentrations of 0.2% and 0.5% showed values of 101% and 102%, respectively. Although these values are lower than that of

fullerenol as an active ingredient, there is still a noticeable tendency to reduce the production of ROS. It is important to note that this reduction was not statistically significant.

Saitoh et al. [43] conducted a similar study to this one to determine the cellular antioxidant capacity of three types of polyhydroxylated fullerenes in HaCat epidermal keratinocytes, but instead of adding APPH to induce oxidative stress, they irradiated the cells with UV. Fullerenol with a lower degree of polyhydroxylation $C_{60}(OH)_{6-12}$ showed poor neutralization of the hydroxyl radicals generated by UV irradiation, which led to a non-significant result at a low degree of hydroxylation, which agrees with the carried-out research.

In comparison, the micrographs obtained from the treatments after the addition of the DCFH-DA reagent (Fig. 13) show the fluorescence observed after the treatment with DCFH-DA.

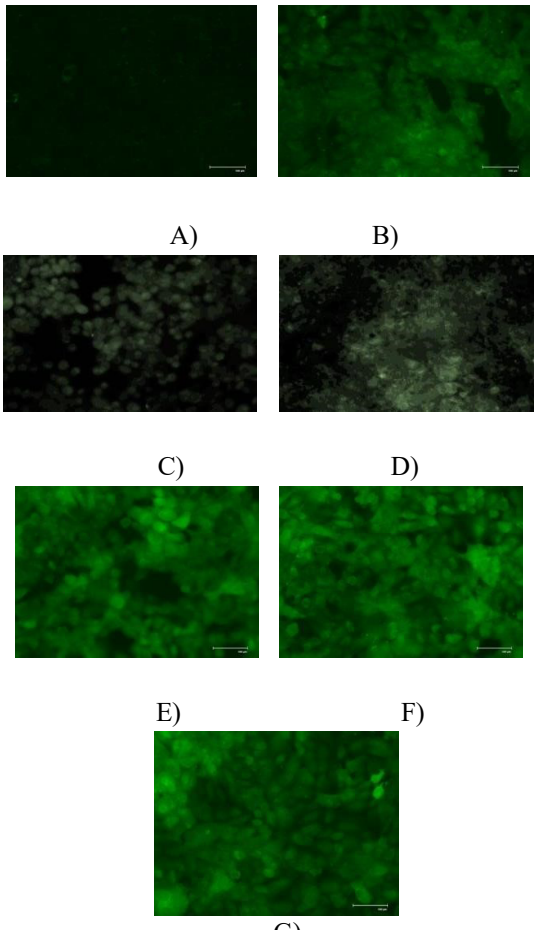

Fig 13. Fluorescence micrographs related to reactive oxygen species (ROS) production of HDFa fibroblasts treated with A) Negative control B) Positive control C) Fullerene, D) Fullerenol, E) Base formulation, F) 0.2% fullerenol formulation and G) Formulation 0.5% fullerenol, after addition of DFCH reagent (except for the negative control) at a scale of 100 µm.

IV. CONCLUSION

The FT-IR and PiFM results indicate that the fullerene $C_{60}(OH)n \cdot mH_2O$, functionalized using the methodology [31], has a moderate or low degree of hydroxylation and a significant degree of agglomeration. This agglomeration can impact its antioxidant properties as it may hinder its ability to interact with other molecules in the environment.

No significant effects were observed in the DPPH inhibition test when fullerenol was incorporated as an active ingredient in the base cosmetic formulation at concentrations of 31.25, 62.5, and 125 µg/mL. This lack of effect can be attributed to three main factors: the low hydroxylation of the synthesized fullerenol, the reactivity selectivity of DPPH towards bulky groups, and the limited range of concentrations evaluated.

Therefore, this method is not recommended for this analysis. However, the CAA test showed a tendency to reduce ROS production when fullerenol was incorporated as an active ingredient in the base formulation, at both 0.2% and 0.5%, demonstrating its potential as an antioxidant.

Regarding cell viability, the synthesized fullerenol demonstrated cytotoxicity in HDFa cells at a concentration of 1 mg/mL. However, at concentrations of 0.1 and 0.5 mg/mL, the samples did not exhibit cytotoxicity in HDFa cells. This suggests that incorporating synthesized fullerenol as an antioxidant active ingredient in cosmetic formulations at these concentrations is safe.

V. CONTRIBUTION OF THE RESEARCH

The physicochemical characteristics of functionalized fullerene C60(OH)n·mH2O were evaluated.

The degree of hydroxylation and tendency to agglomeration were notable.

This information is crucial for understanding the antioxidant properties, development, and potential application of the fullerenes in cosmetic formulations.

The physicochemical characteristics of functionalized fullerene $C_{60}(OH)n \cdot mH_2O$ were evaluated. The degree of hydroxylation and tendency to agglomeration were notable. This information is crucial for understanding the antioxidant properties, development, and potential application of the fullerenes in cosmetic formulations.

In terms of cell viability evaluation, it was found that the synthesized fullerenol did not exhibit cytotoxicity in HDFa cells at concentrations of 0.1 and 0.5 mg/mL. This information supports the safety of incorporating fullerenol as an antioxidant active ingredient in cosmetic formulations at specific levels.

These contributions reinforce the understanding of the properties of functionalized fullerene. They highlight its antioxidant potential at specific concentrations, despite challenges associated with its hydroxylation and agglomeration. Additionally, the cell viability results obtained suggest its viability as an active ingredient in cosmetic formulations. These results offer valuable insights for future

research and development of cosmetic formulations that integrate functionalized fullerenes.

VI. FUTURE WORKS

Proposals for future research on the antioxidant capacity of fullerenes in the cosmetic industry should evaluate various functionalization methods, both in synthesis and in the addition of different functional groups to pure fullerene.

This will determine the type of fullerenol that produces the best response in a biological context, in terms of antioxidant activity and cell viability. and consider that prior to carrying out a biological evaluation it is important to estimate the degree of agglmeration of the functionalized nanoparticles, both in solid state and in suspension because their antioxidant potential is affected by the nature of aggregation of the nanoparticle.

Likewise, a further area of opportunity is to improve the hydroxylation process and obtain a product with a higher degree of hydroxylation of the C60 fullerene based on the methodology used in the present work. This is crucial for enhancing its properties, particularly in the context of its application in cosmetic formulations and other research fields.

Furthermore, a more precise synthesis process or the use of different dispersing agents could help achieve better dispersion and homogeneity of particles, reducing the tendency to form agglomerates and thereby enhancing its effectiveness as an antioxidant agent.

To obtain a complete profile of its physicochemical characteristics, additional and complementary characterization techniques to those used in this work must be included; including dynamic light scattering (DLS) for evaluation in sample suspension, transmission electron microscopy (TEM) for morphology and agglomeration evaluation, and finally, Xray photoelectron spectroscopy (XPS) and elemental analysis to estimate the degree of functionalization.

Finally, to provide a broader biological context of study, additional cell lines, both dermal and epidermal, can be used to have a broader view of their antioxidant activity in the skin and include permeability assays to observe the effects of the incorporation of fullerenes. functionalized as active ingredients in cosmetic formulations that could eventually be marketed.

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VII. REFERENCES

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