New model of biological membrane (shed snakeskin) for studies of antioxidant activity in photoprotective formulation

Novo modelo de membrana biológica (ecdise de pele de cobra) para estudos de atividade antioxidante em formulação fotoprotetora

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ABSTRACT
Antioxidants of natural origin are used in medicines and cosmetics with several benefits, such as: photoprotective action, anti-aging, moisturizing and anti-pollutant. The human

epidermis has an important barrier effect and limited anti-oxidative capacity, so studies with the epidermis is essential. Shed snakeskin (*SS*) is composed of the stratum corneum and provide a barrier like human stratum corneum. This alternative does not show a tendency to microbiological degradation and can be considered ecologically correct. This study intends to present, in an innovative way, the Electron Paramagnetic Resonance spectroscopy (EPR) and The Forster Resonance Energy Transfer (FRET) were employed to evaluate the natural antioxidant substances (*Resveratrol/* RES 3.0 w/w and *Ferulic acid*/ FA 1.0 w/w) associated with organic sunscreens ingredients (*Ethylhexyl Methoxycinnamate/* EHMC 10.0%w/w and *Butyl Methoxydibenzoylmethano/* BMBM 5.0%w/w in a photoprotective emulsion (PB). Furthermore, the use of SS seedlings as a possible alternative to the use of human or animal skin *ex-vivo*. RES and FA can absorb the energy emitted by the EHMC in FRET, preventing the passage through the triplet state, favoring the photostability of this sunscreen, the same not ocorred with the BMBM. Antioxidant activity of the photoprotective formulations was evaluated *in vitro* by the percentual inhibition of the radical 2,2-diphenyl-1-picrihydrazyl (DPPH•). The antioxidant activity with RES, 97.0% inhibition of DPPH• in the PB, was higher than PB + FA (91.0%), however the concentration of RES in PB was higher than FA. The sample *SS* + PB + FA was the one with the lowest number of free radicals after irradiation, which corroborated the high percentage of radical inhibition *in vitro* and it was the better association with the photoprotective formulation.

Keywords: snakeskin, EPR, photoprotective formulation, Ferulic acid, resveratrol.

RESUMO

Os antioxidantes de origem natural são utilizados em medicamentos e cosméticos com diversos benefícios, tais como: ação fotoprotetora, antienvelhecimento, hidratação e antipoluição. A epiderme humana possui importante efeito de barreira e capacidade antioxidante limitada, portanto estudos com a epiderme são essenciais. A ecdise de pele de cobra (SS) é composta pelo estrato córneo e fornece uma barreira similar ao estrato córneo humano. Esta alternativa não apresenta tendência à degradação microbiológica e pode ser considerada ecologicamente correta. Este estudo pretende apresentar, de forma inovadora, a Espectroscopia de Ressonância Paramagnética Eletrônica (EPR) e a Transferência de Energia por Ressonância Forster (FRET) para avaliar as substâncias antioxidantes naturais (*Resveratrol*/ **RES** 3,0 p/p e *Ácido Ferúlico*/ **FA** 1,0 p/p) associado a ingredientes de filtros solares orgânicos (*Etilhexil Metoxicinamato*/ **EHMC** 10,0% p/p e *Butil Metoxidibenzoilmetano*/ **BMBM** 5,0% p/p em uma emulsão fotoprotetora (**PB**). Além disso, o uso de SS como uma possível alternativa ao uso de pele humana ou animal *ex-vivo*. **RES** e **FA** podem absorver a energia emitida pelo **EHMC** no FRET, impedindo a passagem pelo estado tripleto, favorecendo a fotoestabilidade deste filtro solar, o que não ocorre com o **BMBM**. A atividade antioxidante das formulações fotoprotetoras foram avaliadas *in vitro* pela inibição percentual do radical 2,2-difenil-1-picrihidrazil (DPPH•). A atividade antioxidante com **RES**, 97,0% de inibição do DPPH• na **PB**, foi superior a **PB** + **FA** (91,0%). Porém, a concentração de **RES** na **PB** foi maior do que no **FA**. A amostra SS + **PB + FA** foi a que apresentou o menor número de radicais livres após a irradiação, o que corroborou com o alto percentual de inibição dos radicais *in vitro* e foi a melhor associação com a formulação fotoprotetora.

Palavras-chaves: pele de cobra, EPR, formulação fotoprotetora, ácido ferúlico, resveratrol

1 INTRODUCTION

1.1. ANTIOXIDANT ACTIVITY AND THE SKIN

Ultraviolet B radiation favors the generation of free radicals in the epidermis, while ultraviolet A radiation decreases the levels of antioxidants, inactivates antioxidant enzymes and increases the detectable markers of lipid peroxidation in the skin (HERRLING *et al*., 2003; POPOV *et al.*, 2009). Reactive Oxygen Species (ROS) favor cellular oxidative stress, lipid peroxidation, release of membrane phospholipids, direct and indirect DNA damage associated with carcinogenesis and the formation of prostaglandins, linked to inflammatory processes. In addition, to the formation of radicals induced by external factors such as ultraviolet radiation, visible light, infrared and xenobiotics, there is the formation of endogenous radicals, naturally present in biochemical and physiological reactions, such as: radical oxygen species, superoxide (O_2^-), radical singlet oxygen ($^1O2$), hydroxyl (OH) and nitric oxide (NO), for example under normal conditions, the number of radicals is balanced by enzymes and antioxidants (DARIO *et al*., 2013; DARVIN *et al*., 2010; MARTINS *et al*., 2020; POPOV *et al.*, 2009; YADAV *et al*., 2019).

According Silva, Michniak-Kohn, Leonardi (2017), antioxidants are compounds that inhibit or block the process of formation of free radicals. They must be oxidized first than the protected agent. Antioxidants of natural origin are used in medicines and cosmetics with diverse benefits, such topical photoprotective action, anti-aging, moisturizing and anti-pollutant (DARIO, BABY & VELASCO, 2015; MARTINS *et al*., 2020; MICHNIAK-KOHN & LEONARDI, 2017; SILVA, PERES *et al.*, 2018;). Recent studies have evaluated their action on the photostability of sunscreens such as the phenolic compounds that are known for their antioxidant action (PERES, 2015). The phenolic compounds demonstrated the ability to promote the enzymatic inhibition of specific proteinases such as collagenase and elastase. These proteinases promoting the degradation of important structural fibers, such as collagen and elastin, favoring skin aging. The studies with antioxidants of natural origin are trend in the cosmetics (SILVA, MICHNIAK-KOHN & LEONARDI, 2017).

According to Martins *et al.* (2020) the antioxidant mechanisms in the epidermis are more noticeable in relation to the dermis, due to the cornified envelope proteins and a high concentration of low molecular weight antioxidants that act together. These antioxidants act in several ways as chelation of metal ions or neutralizing free radicals directly. According Rinnerthaler *et al*. (2015), the human epidermis has an important

barrier effect and anti-oxidative capacity when young due, the cornified envelope itself has anti-oxidative capabilities, so studies with the epidermis is essential.

1.2 SCREENING OF ANTIOXIDANTS AS POTENTIAL PHOTOSTABILIZER

Chemical sunscreens are excited after absorption of ultraviolet radiation. After excitation, the molecule returns to the ground state and part of that energy is converted into heat, visible and/or fluorescence light. Some filters find it difficult to return quickly to the ground state and thus, continue to be able to absorb ultraviolet radiation. There are techniques used to predict whether a substance can assist these chemical filters in the absorption and dissipation of that energy. These substances are known as photostabilizers (KAWAKAMI *et al*. 2017, BONDA, 2008).

Recent studies mention antioxidants of plant origin with the additional function of photo-stabilization (MOROCHO-JÁCOME *et al.* 2020). The Forster resonance energy transfer (FRET) technique is an electrodynamic phenomenon, the most frequent in the inhibition of the singlet-singlet energy state. FRET occurs between a donor molecule in the excited state and an acceptor molecule in the fundamental state, so in this case, the filter would be the donor molecule and the antioxidant would be the acceptor molecule (LAKOWICZ, 2006). This technique can be used to choose the best antioxidant substance in photoprotective formulations, since to favor the photostability is necessary to inhibit the singlet or triplet energy state of photo-instable molecules (KAWAKAMI *et al.* 2017).

1.3 EVALUATION ANTIOXIDANT ACTIVITY IN THE SKIN AND BIOLOGICAL MEMBRANE ALTERNATIVE

Several *in vitro* methods are mentioned in the literature for detecting the antioxidant action of certain substances on the skin, such as the β-carotene method, DPPH radical inhibition, ABTS, Absorption Capacity of Oxygen Radicals (ORAC), using Fluorescein as a Fluorescent Marker, total Antioxidant Activity in Ferric Reducing Antioxidant Power (FRAP), CUPRAC assay and Electron Paramagnetic Resonance Spectroscopy (EPR). Currently, the description of the antioxidant potential of compounds using a greater variety of *in vitro* assays is recommended (MARTINS *et al*., 2020; SILVA, MICHNIAK-KOHN, LEONARDI, 2017).

Currently, the behavior of stratum corneum oxidative stress has been evaluated by *tape stripping* or skin model system and measured by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) or thiobarbituric acid reactive species (TBARS) methods. *Tape stripping*

technique was applied to evaluate *ex vivo* antioxidant activity of ferulic acid and rutin-loaded ethosomes by DPPH method (CANDIDO *et al.*, 2018; MARTINS *et al*., 2020).

Among these techniques we highlight Eletronic Paramagnetic Ressonance (EPR). Lohan *et al*. (2015) demonstrated by EPR that the radical production under irradiation with infrared and visible light in human skin was reduced with time due oral uptake and the topical application of antioxidants. Lund *et al*. (2007) described the development of a novel EPR assay that used the photogeneration of reactive melanin radical as a measure of UV light penetration to melanocytes *in situ* in human skin.

Studies with human skin bring results closer to the place where medicines and cosmetics will be applied, but ethical considerations, preservation care and availability difficult its use. In some cases, synthetic membranes or equivalent skin cultures are used (RIGG & BARRY, 1990; SCHMOOK, MEINGASSNER & BILLICH, 2001). Therefore, animal skin such as hairless rat, mice, guinea, ear pig, rabbit and shed snakeskin is generally used as an alternative (NGAWHIRUNPAT *et al*., 2006; OLIVEIRA *et al*., 2010).

According to Ribeiro (2019), the demand for ethically produced products, which defend the right of animals and seek a more sustainable life is moving the market in a new direction. It was estimated that 4% of the Brazilian population, about 7.6 million people are vegetarian, many of them, vegan. Given the above, the choice for shed snakeskin (SS) is the most convenient because it can be obtained abundantly without the death of the animal. The change of skin (ecdysis) occurs regularly in the adult animal, in general every 2 to 3 months, thus a single shed snakeskin can provide multiple samples and its use can be considered ecologically correct (BABY *et al*., 2008; BALOGH *et al.*, 2011; TAKAOKA *et al.*, 2010).

Shed snakeskin (SS) is composed of the stratum corneum and provide a barrier like human stratum corneum, including the thickness of the membrane, its proteic structure (keratin typo α and β) and lipidic composition. Shed snakeskin lacks hair follicles, so problems associated with the transfollicular penetration route, which can be significant in mammalian skins, can be avoided. The fact that it does not contain living tissues (viable epidermis) does not show a tendency to microbiological degradation, which makes its storage easier and its shelf life for use is prolonged (BABY *et al*., 2008; BALOGH *et al*., 2011; HAIGH, BEYSSAC, AIACH, 1998; RIGGY & BARRY, 1990; TAKAOKA *et al.,* 2010; WIDLER, SIGRIST, GAFNER, 2002). Abdel-Aal, Mansori, Zahouani (2019) in a comparative study of frictional response of SS and human skin,

concluded that shed snakeskin is highly compatible with human skin, despite differences in surface structure and topology. The human epidermis is similar the snake's epidermis layer.

1.4 EFFICACY STUDIES OF ACTIVE INGREDIENTS WITH BIOLOGICAL MEMBRANE (SHED SNAKESKIN)

Several studies have evaluated the penetration, permeation and transdermal absorption of drugs and cosmetic ingredients in shed snakeskin as Ngawhirunpat *et al*. (2006), that evaluated the *in vitro* transdermal permeation of hydrophilic and lipophilic drugs using SS of *Elaphae obsoleta* and human skin. They concluded the permeability coefficient of lipophilic drugs was the same range for both biological membranes. The thickness and lipid content of SS and human skin were significantly equal, suggesting the use of ecdysis shed snake as barrier membrane percutaneous absorption studies *in vitro* of lipophilic compounds.

Kouchak & Handali (2014), evaluated *in vitro* the aminophylline transdermal absorption through SS using Franz diffusion cells and investigated the influence of various enhancers in the transepidermal permeation of drug. They concluded that type and concentration of enhancers can influence the process. Oliveira *et al*. (2010) evaluated the effect of copaiba oil as permeation enhancer of kojic acid in Franz diffusion cells with shed snakeskin membrane of *Crotalus durissus terrificus*. They concluded that copaiba oil at 25 and 50% had the capacity to promote penetration of kojic acid.

Baby *et al*. (2008) evaluated *in vitro* cutaneous stability and penetration of rutin in a cosmetic emulsion, using an alternative model with *Crotalus durissus* biomembrane. According to the results, the emulsion did not favor the cutaneous penetration of rutin, but only its retention in the stratum corneum of *Crotalus durissus*.

Praça, Medina, Bentle (2015) evaluated of the brazilian snakeskin (*Crotalus durissus terrificus*) as a substitute model of biological membrane for *in vitro* skin permeation drug trials and found many similarities in the permeation rates between the human stratum corneum and the snakeskin. Itoh *et al*. (1990) evaluated the use of shed snakeskin as a model membrane for *in vitro* percutaneous penetration studies in comparison with human skin. The permeability of various compounds and the contribution of several functional groups to the permeability were also found to be similar between shed snakeskin and human skin. Moreover, the permeability of compounds

through shed snakeskin was increased by azone, one of the most extensively studied transdermal penetration enhancers.

Kuramoto *et al*. (1996) evaluated the characteristics of SS permeability to indomethacin and fatty alcohols. The flux of fatty alcohols in the shed snakeskin was greater than that reported in the human skin and SS had similar esterase activity as the human skin. Takahashi *et al*. (2001) evaluated the influence of pH on the permeability of *p*-toluidine and aminopyrine through shed snakeskin as a model membrane. From these results, the ionized species of *p*-toluidine were transported through the SS, but the ionized species of aminopyrine did not.

Harada *et al.* (1993) evaluated *in* vitro permeability of salicylic acid in human, rodent and shed snakeskin. They discovered that the SS and hairless rat skin had similar permeability to human breast and thigh skin. Riggy & Barry *et al*. (1990) evaluated the SS and hairless mouse skin as model membranes during permeation studies as substitute of human skin. Considering the similarities between the shed snakeskin and human skin, ease of storage and handling, and low cost, the SS may offer a good model membrane for transdermal studies.

Some studies have evaluated the moisturizing and sunless tanning of cosmetic ingredients in shed snakeskin as Takaoka *et al.* (2010), that evaluated the interaction of active moisturizing compounds in *Crotalus durissus* biomembrane by means of differential scanning calorimetry and RAMAN spectroscopy. They suggested that moisturizing ingredients have the necessary security, as they have not markedly altered the structure of the stratum corneum. The organic silicon solution and the hydrophilic gel with urea showed better hydrating power. Balogh *et al.* (2011) they studied of sunless tanning formulas using molted shed snakeskin as an alternative membrane model.

This study intends to present, in an innovative way, the EPR technique as screening of antioxidant substances associated with organic sunscreen ingredients in a topic formulation using shed snake seedlings as a possible alternative to the use of human or animal skin *ex-vivo*.

2 MATERIALS

2.1 REAGENTS AND ANTIOXIDANTS OF NATURAL ORIGIN

3-(Carboxy)-2,2,5,5- tetramethyl-1-pyrrolidinyloxy (PCA) (Sighma-aldrich, purity 99.94%) and 2,2-diphenyl-1-picrihydrazyl (DPPH$^{\bullet}$) (Sighma-aldrich, purity 97%) and Ferulic Acid **(**FA**)** 1.0% w/w in photoprotection formulation (GAMMA, purity

98,6%) and Resveratrol (RES) 3.0% w/w in photoprotection formulation (Active Pharmaceutica, purity 99,8%).

2.2 PHOTOPROTECTIVE FORMULATION UVB/UVA

I.N.C.I. (International Nomenclature of Cosmetics Ingredients)

Ethylhexyl Methoxycinnamate 10.0% w/w (Fagron, content 98,6%), and *Butyl Methoxydibenzoylmethano* 5.0% w/w (Fagron, purity 98,2%), *ammoniumacryloyl-dimethyltaurate/ VPcopolymer, propylene glycol, acqua, cetearylalcohol (and) dicetylphosphate (and) ceteth-10 phosphate, caprylic/caprictriglyceride, phenoxyethanol (and) methylparaben (and) ethylparaben (and) butylparaben (and) propylparaben (and) isobutylparaben* (PINTO, 2014).

3 METHODS

3.1 SCREENING OF ANTIOXIDANTS AS POTENTIAL PHOTOSTABILIZER BY FORSTER RESONANCE ENERGY TRANSFER (FRET)

Fluorescence quenching experiments was obtained in Spectrofluorimeter Shimadzu RF-5301PC, software RFPC, using solutions of **BMBM** and **EHMC** in ethyl acetate; 100 µL of **EHMC** and 100 µL of **BMBM** stock solution (0.83mg/mL) were transferred, each one, to a 1.0 cm quartz cuvette of 3 mL, then was transferred 1.8 mL of ethyl acetate. The excitation wavelength of **EHMC** was fixed at 310 nm and the excitation wavelength of **BMBM** was fixed at 360nm. The emission spectra were then recorded in a spectral range of 220 to 700 nm.

The absorption experiment was obtained in Espectrophotometer UV Vis – Thermo®, model Evolution 600 UV-Vis. 1mL of stock solution of **RES** (0.95mg/mL) was transferred to volumetric flask of 10 mL, then 750 µL of this solution was transferred to volumetric flask of 10 mL and to a 1.0 cm quartz cuvette of 3mL. 1mL of stock solution of **FA** (0.95mg/mL) was transferred to volumetric flask of 10 mL, then 500 µL of this solution was transferred to volumetric flask of 5 mL and to a 1.0 cm quartz cuvette of 3mL. The absorption spectra were recorded in a spectral range of 200 to 400 nm. All measurements were performed at 25.0±2.0 °C. The spectrum was obtained by overlapping between the absorption and emission spectrum of donor and acceptor, respectively.

3.2 ANTI-RADICAL ACTIVITY

The method used to evaluate the antiradical activity of the formulations was the reduction of the radical 2,2-diphenyl-1-picrihydrazyl (DPPH•) in methanol solution. The solution with this radical has a purple color with maximum absorption between 515 - 528 nm, which was altered by reduction of the radical and allows spectrophotometric monitoring with Espectrophotometer UV Vis – Thermo®, model Evolution 600 UV-Vis.

The formulations were solubilized in methanol with a concentration of 0.1g mL^{-1}, centrifuged at 5000 rpm for 15 min. An aliquot, 0.5 mL, of the supernatant will be added in 2.5 mL of $DPPH^{\bullet}$ (100µM) and homogenized, it kept at room temperature (20.0 ± 2.0 ºC). After this period, the reduction of the free radical $DPPH^{\bullet}$ will be measured by reading the absorbance in a UV-VIS spectrophotometer at 517 nm. The negative control will consist of $DPPH^{\bullet}$ (100 µM) in methanol (blank). The results will be expressed as the percentage of $DPPH^{\bullet}$ radical inhibition according to the following Equation (BALOGH, 2011; BRAND-WILLIAMS, BONDET, BERSET, 1997; DARIO *et al*., 2013; SÁNCHEZ-MORENO, LARRAURI, SAURA-CALIXTO, 1998).

$$\%DPPH^{\bullet}\ \text{inhibition} = (AA - AS)/AA\ x100$$

Legend: ***AA***: Absorbance of negative control; ***AS*****:** Absorbance of the sample*;* ***DPPH:*** 2,2-diphenyl-1-picrihydrazyl

3.3 ELECTRONIC PARAMAGNETIC RESONANCE (EPR) *EX VIVO*

A relevant observation of this technique is the degree of hydration of the material, that can difficult the EPR measurements as the water molecules absorb microwaves, interfering with the reading of EPR (GÓMEZ *et al.*, 2011). In this context, it is one important limitation of this method applied to biological studies, being important the choice of the skin type, as the shed snakeskin that has lower water content in relation to the pig ear or other types, being considered an ideal biological membrane for the EPR technique (PONGJANYAKUL *et al*., 2002).

3.3.1 Preparation of shed snakeskin (SS)

The ecdysis of the shed snakeskin of the Crotalus durissus Rattle snake species was donated by the Herptology Laboratory of the Butantã Institute, with use acknowledged by the Ethics Committee on the Use of Animals with letter No. 034.2020. It was washed twice with distilled water in order to remove mucus and dirt, after they were added on absorbent paper to dry (BABY, 2007; BALOGH *et al*., 2011). Then,

standardized fragments of the ventral region SS were cut. **Figure 1** illustrates the ventral region of SS.

Figure 1. Shed snakeskin used in the experiment.

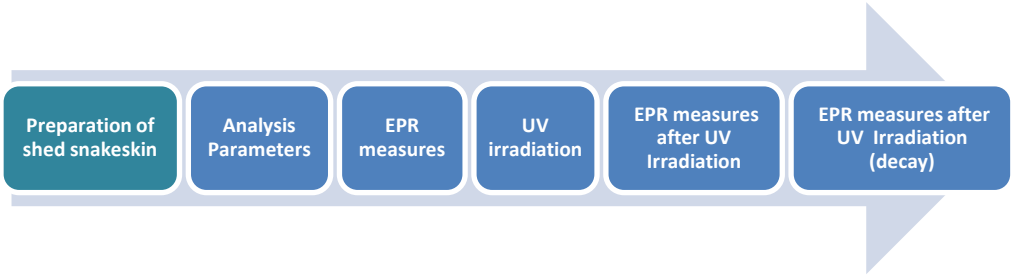
To allow the penetration of the rotating EPR trap (3- (Carboxy) -2,2,5,5-tetramethyl-1-pyrrolidinyloxy - PCA), tape stripping with adhesive tape (Scotch®) was performed one by biopsy to facilitate the penetration of PCA (DARVIN *et al*., 2010). PCA is a free radical of the nitroxide type that is reduced to the corresponding hydroxylamine when it reacts with an ROS (ALBRECHT *et al*., 2016). Biopsies of shed snakeskin approximately 1.00 cm^2 in area and 0.080 mm thick were incubated in PCA solution (0.1 mM in 50/50 water/ethanol (v/v) solution) for 20 min in a capped glass beaker. The formulations were applied at a rate of 2 $mg.cm^{-2}$ in the biopsies of SS spreading them as evenly as possible.

3.3.2 EPR Parameters

The biopsies were accommodated without folds on the bottom of quartz tube to EPR with 10mm in diameter. The EPR spectra were measured in the X-band, Bruker's EMX PLUS equipment, 9.64 ms conversion time, 4 G Modulation amplitude (0.4 mT), field central of 3470 G (347 mT), scanning window of 1000 G (100 mT) and 10 numbers of scans with an ambient temperature of 20.0 ± 2.0 ºC. The peak-to-peak value of the spectrum was used to evaluation of the signal intensity. The signal intensity is proportional to the number of free radicals present in the sample.

3.3.3 Flowchart of the experiment

This is a flowchart of carrying out experiments to determine the EPR measures.



Legend: ***EPR*** - Electron Paramagnetic Resonance spectroscopy; ***UV*** – Ultraviolet radiation.

3.3.4 EPR reading before and after UV irradiation

The initial EPR signal was measured before and after UV irradiation step, both at an ambient temperature of 20.0 ± 2.0 ºC (DARIO, BABY, VELASCO, 2015). To ensure homogeneity of the irradiated dose the samples were irradiated between two quartz plates. The total radiation dose was 1.378 KJ/m^2 over 30 min, irradiated in the Suntest® CPS$^+$ solar simulator. The time interval was recorded to assess the possible signal decay, especially after the UV Irradiation (**Table 1**).

Table 1. Details of measures: shed snakeskin (alone), with base cream and with photoprotective formulation UVB/UVA (*Ethylhexyl Methoxycinnamate* 10.0% w/w + *Butyl Methoxydi-benzoylmethano* 5.0% w/w) additives or not with *Ferulic Acid* (1.0% w/w) or Resveratrol (3.0% w/w), before and after irradiation.

Time (day)	Duration of the measures (between the analysis/ min)	Measure (samples)
1	48	1ª - Not Irradiated (NIR)
	50	2ª - Irradiated (IR) by UV
	35	3ª - Irradiated (IR) by UV (signal decay)
	20	4ª - Irradiated (IR) by UV (signal decay)
3	45	5ª - Irradiated (IR) by UV (signal decay)

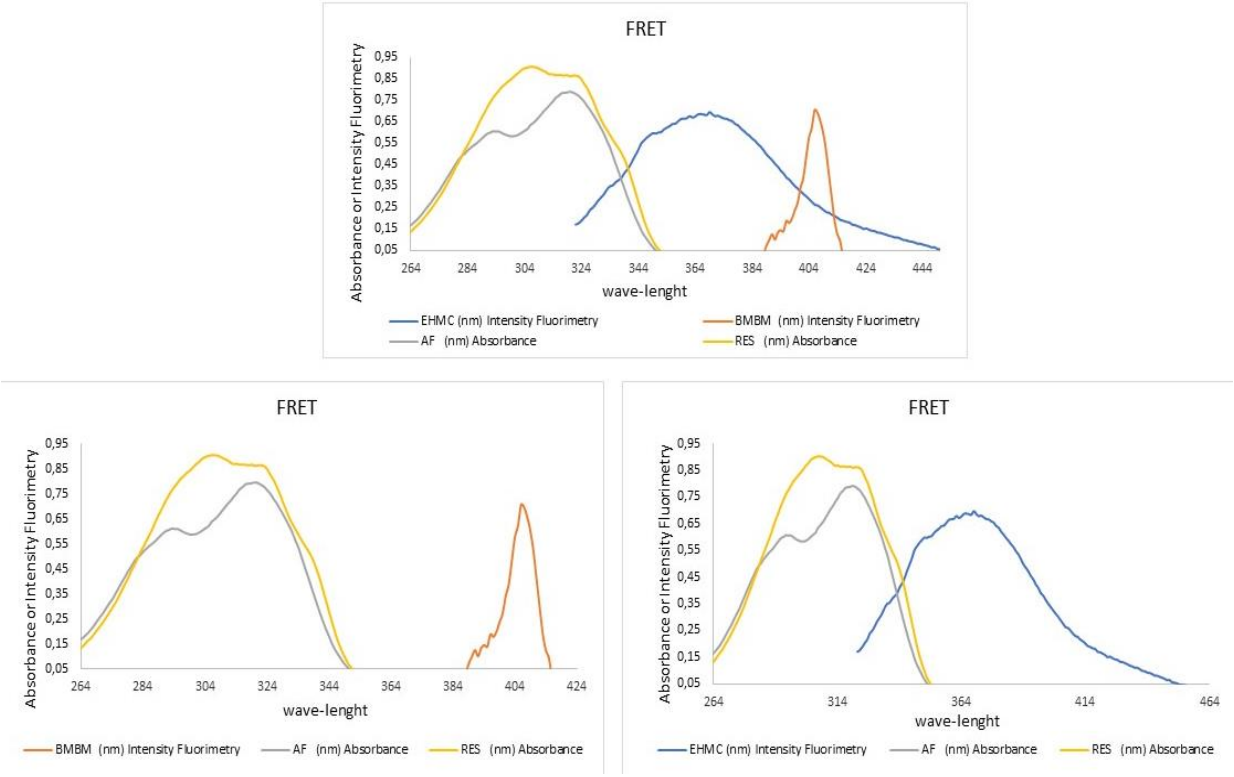
Legend: ***NIR****:* Not Irradiated; ***IR****:* Irradiated; ***UV:*** Utraviolet radiation

4 RESULTS

4.1 SCREENING OF ANTIOXIDANTS AS POTENTIAL PHOTOSTABILIZER BY FORSTER RESONANCE ENERGY TRANSFER (FRET)

The theories for energy transfer developed by Förster (FRET) relate the energy transfer rates from the donor in the singlet excited state to the recipient through an integral spectral overlay. The spectral magnitude and the overlap integral, in turn, is related to the probability that the donor and the receptor are energetically compatible (BONDA, 2008), as present in the **Figure 2.**

Figure 2. Overlapping of the absorbance and fluorometric intensity curves of the ethyl acetate solutions with **BMBM**, **EHMC**, **FA** and **RES**.



Legend: **BMBM** = *Butyl methoxydibenzoylmethane* 5,0% w/w; **EHMC**: *Ethylhexyl methoxycinnamate* 10,0% w/w; **RES**: Resveratrol 3,0% w/w; **FA**: Ferulic acid 3,0% w/w; $\mathbf{t_0}$ = 48h of preparation of formulation. Results: mean 3 determinations ± standard deviation.

The curves of the antioxidants **RES** and **FA** intercepted the curve of the **EHMC**, which means that these antioxidants can absorb the energy emitted by the **EHMC**, preventing the passage through the triplet state, favoring the photostability of this sunscreen. Regarding **BMBM**, there was no interception, which indicated that these antioxidants are not able to absorb the energy emitted by this sunscreen by this mechanism.

4.2 ANTIOXIDANT ACTIVITY (% Inhibition Of DPPH•)

The anti-radical activity of the formulations without radiation exposure was assessed from the test with the free radical DPPH• (**Table 2**).

Table 2. Antioxidant activity of *Photoprotective Base* (**PB**) with or not the antioxidants *Ferulic Acid* ***(FA)*** and Resveratrol ***(RES)*** by the DPPH• radical (%) inhibition.

Formulation	% inhibition of DPPH• ± SD
PB	20.5 ± 2.6^{A}
PB+FA	91.0 ± 0.0^{B}
PB+ RES	97.0 ± 0.4^{C}

Legend: ***PB*** - Oil in Water (O/W) emulsion with *Ethylhexyl Methoxycinnamate* 10.0% w/w + *Butyl Methoxydi-benzoylmethano* 5.0% w/w; ***FA****: Ferulic Acid* 1.0% w/w*;* ***RES****:* Resveratrol 3.0% w/w; ***SD*** =Standard deviation. Means that do not share a letter are significantly different, with p-value ≤ 0.05.

From the data presented in **Table 2**, the addition of **FA** and **RES** in the photoprotective formulations influenced the results statistically significatively. The **PB (BMBM+EHMC)** showed low values of inhibition of the radical DPPH• (average 20.5%), an expected result since there are no antioxidant ingredients in this formulation. The addition of Ferulic Acid (**FA**) 1.0% w/w in the **PB (BMBM + EHMC)** inhibited the DPPH radical by 91.0%. This behavior was expected, since one of the main benefits of adding phenolic compounds in topical formulations is its antioxidant action. The addiction of the antioxidants is benefic to formulation and the skin, protection the chemical filters as consequence its efficacy (MARTINS *et al*., 2020; SILVA, MICHNIAK-KOHN, LEONARDI, 2017). Silva, Michniak-kohn, Leonardi (2017) mentioned in their review a work with photoprotective property with addition of resveratrol in the formulation on a panel of 15 volunteers.

According to Kikuzaki *et al*. (2002), 20µM solutions of **FA** inhibited 27.3% of the DPPH radical• a 100 µM (being considered a potent antioxidant in comparison to ferulates and *p*-cumaric acid. According to Maurya & Devasagayam (2010), the antioxidant activity of **FA** was noted by inhibition of DPPH radical assay due to the presence of the -CH = CH-COOH group, which was complex with DPPH•.

According to Bandoniene et al. (2002), the percentage of DPPH radical inhibition of polyphenols was 72.0% to Ferulic Acid at 0,5 mol antioxidant/1mol DPPH•. The antioxidant activity of **PB** with addition of **FA** was 91.0% and it was higher than the cientific literature, corroborating the results of high antioxidant action of the Ferulic Acid. In this situation, there was a possibility of synergism of FA with the sunscreens *Ethylhexyl Methoxycinnamate* and *Butyl Methoxydibenzoylmethano*, since the photoprotective base without the antioxidant inhibited 20.5% of radical DPPH•.

With the addition of 3.0% w/w Resveratrol (**RES**), the inhibition of the radical was higher than with the **FA** added in the photoprotective base (average of 97.0%). These

values were statistically different because the means did not share the same letter in the test ANOVA On Way followed by Tukey.

According to Gulçin (2010), **RES** has *in vitro* antioxidant activity and radical scavenging activity, obtained by different methodologies, including DPPH• activity. IC50 (concentration of an inhibitor where the response is reduced by half) was 17.8% at 30µg/mL indicating the potential antioxidant activity of this substance.

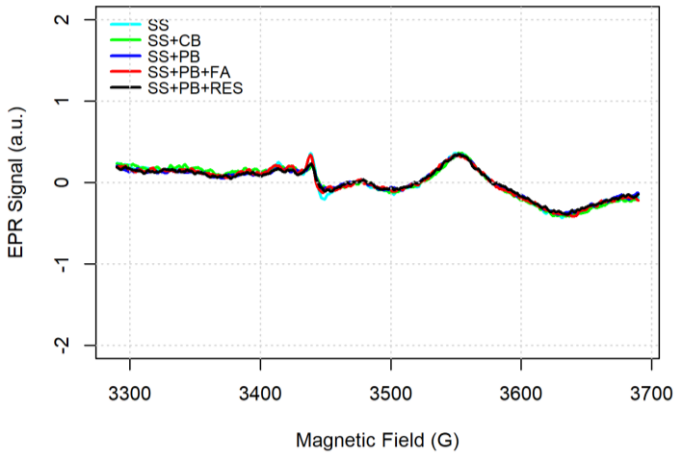
Based on the literature and the pharmacy practice, the usual concentration of **AF** and **RES** in topical formulations is 1.0% and 3.0 w/w, respectively as it was demonstrated in this research.

With these values, we obtained the satisfactory antioxidant response and **RES** (97.0%) in the photoprotection base (**PB**) was higher than **PB+FA**. The addition of sunscreens enhanced the antio-xidant activity of **AF** and **RES**, corroboring with the literature. Hubner et al. (2019) mentioned the possible synergism with grape marc extract *Vitis vinifera* L. with UV filters and it has high *in vitro* antioxidant activity using the DPPH method, due to the presence of phenolic substances.

4.3 EPR MEASURES

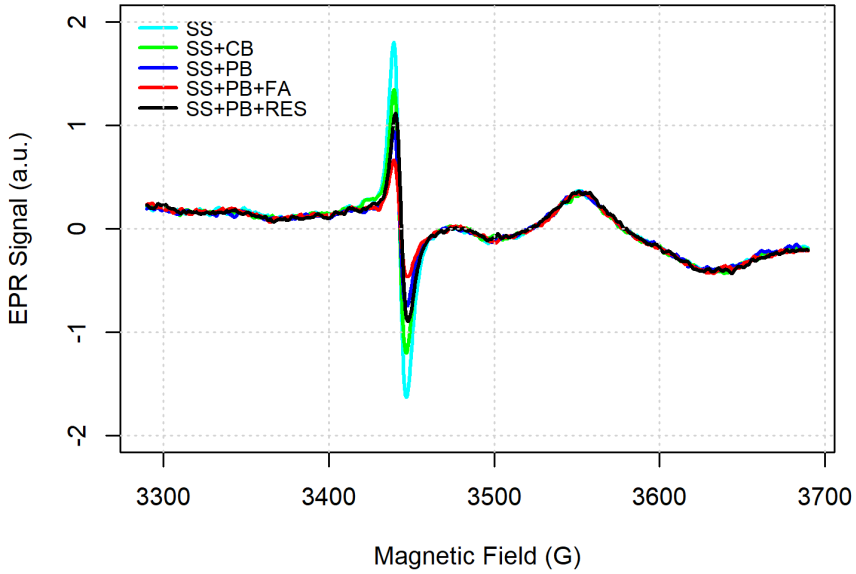
All shed snakeskins had been immersed in the PCA solution. Depending on the formation of free radicals that interacted with the samples the signal decreased. In the **Figure 3**, the samples before being irradiated had the same radical profile, being only *SS* and **SS+ PB + FA** with a slightly greater amplitude in relation to the other samples. In this work there was always a decay with irradiation and without irradiation.

Figure 3. Electronic Paramagnetic Resonance (EPR) measures of Shed snakeskin (**SS**), Shed snakeskin with Cream Base (**SS+CB**), Shed snakeskin with Photoprotective Base (**PB**) UVB/ UVA (*Ethylhexyl Methoxycinnamate* 10.0% w/w+ *Butyl Methoxydibenzoylmethano* 5.0% w/w) additives or not of (1.0% w/w) *Ferulic Acid* (**SS+PB+FA**) or (3.0% w/w) Resveratrol (**SS+PB+RES**), before irradiation.



After the irradiation, the signs of all samples increased in amplitude and that the smallest sign in relation to the SS sample with the PCA was the *SS* + **PB** + **FA** (**Figure 4**).

Figure 4. Electronic Paramagnetic Resonance (EPR) measures of Shed snakeskin (SS), Shed snakeskin with Cream Base (**SS+CB**), Shed snakeskin with Photoprotective Base (**SS+PB**) UVB/ UVA (*Ethylhexyl Methoxycinnamate* 10.0% w/w+ *Butyl Methoxydibenzoylmethano* 5.0% w/w) additives or not of (1.0% w/w) *Ferulic Acid* (**SS+PB+FA**) or (3.0% w/w) Resveratrol (**SS+PB+RES**), after irradiation.



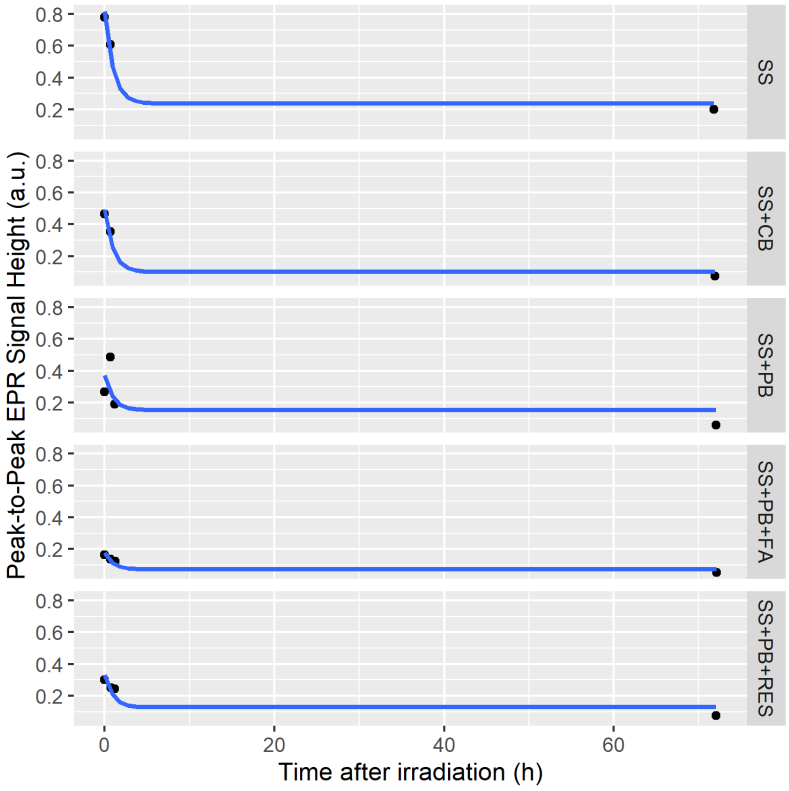
Highlighting that the spectra with antioxidants **AF** and **RES** had their signals attenuated. Approximately 72 hours later, the sample with only snakeskin and PCA, increased slightly its amplitude in relation to the other samples (**Table 3**, **Figure 5**). This means that after UV radiation exposure the snakeskin continued with presence of free radicals, the radiation UVB modify entire inflammatory cascade and produced reactive oxygen species (ROS). Over time these radicals rebalance. The fact that the ingredients in the formulations moisturize and nourish, probably protected the stratum corneum (MARTINS *et al*., 2020).

Table 3. Decay of Electronic Paramagnetic Resonance **(**EPR) measures *before* (No irradiated – **NIR**) and *after* irradiation (day 1 and 3).

SS+**PB+FA**			
time (day)	**time (h:min:sec)**	**time(h)**	**Signal Peak-to-Peak**
1	07: 45: 22	NIR	0.75
1	13: 17: 01	0.00	1.13
1	13: 58: 40	0.68	0.93
1	14: 30: 15	1.22	0.94
3	13: 24: 19	72.12	0.79
SS+**PB+RES**			
time (day)	**time (h:min:sec)**	**time(h)**	**Signal Peak-to-Peak**
1	08: 00: 52	NIR	0.74
1	13: 27: 27	0.00	2.01
1	14: 11: 05	0.72	1.68
1	14: 39: 19	1.18	1.64
3	13: 30: 57	72.05	0.82
SS+**PB**			
time (day)	**time (h:min:sec)**	**time(h)**	**Signal Peak-to-Peak**
1	07: 29: 45	NIR	0.74
1	13: 09: 22	0.00	1.69
1	13: 52: 09	0.70	3.05
1	14: 21: 22	1.20	1.20
3	13: 12: 46	72.05	0.79
SS+**CB**			
time (day)	**time (h:min:sec)**	**time(h)**	**Signal Peak-to-Peak**
1	07: 23: 16	NIR	0.78
1	13: 02: 08	0.00	2.55
1	13: 43: 17	0.68	1.94
3	12: 57: 52	71.92	0.78
SS			
time (day)	**Time (h:min:sec)**	**time(h)**	**Signal Peak-to-Peak**
1	07: 13: 26	NIR	0.79
1	12: 55: 11	0.00	3.43
1	13: 36: 41	0.68	2.67
3	12: 43: 58	71.82	0.92

*Legend***: *SS* -** Shed snakeskin, ***SS+CB*** - Shed snake skin with Cream Base, ***SS+PB*** - Shed snakeskin with Photoprotective Base UVB/UVA (*Ethylhexyl Methoxycinnamate* 10.0% w/w+ *Butyl Methoxy dibenzoylmethano* 5.0% w/w) additives or not of (1.0% w/w) *Ferulic Acid* (***SS+PB+FA***) or (3.0% w/w) Resveratrol (***SS+PB+RES).***

Figure 5. Decay of Electronic Paramagnetic Resonance **(**EPR) measures after three days of irradiation.



*Legend***: SS** - Shed snakeskin, ***SS+CB*** - Shed snakeskin with Cream Base, ***SS+PB*** - Shed snakeskin with Photoprotective Base UVB/UVA (*Ethylhexyl Methoxycinnamate* 10.0% w/w+ *Butyl Methoxydibenzoylmethano* 5.0% w/w) additives or not of (1.0% w/w) *Ferulic Acid* (***SS+PB+FA***) or (3.0% w/w) Resveratrol ***(SS+PB+RES***).

The post-irradiation signal increased in amplitude and over the hours and days decreased, approaching the amplitude value before irradiating. In the *SS* + **PB** sample the signal increased after the first reading of the irradiated sample, probably due the instability of the mixture *Butyl Methoxydibenzoylmethano* (avobenzone) with *Ethylhexyl Methoxycinnamate*. The signal profile of avobenzone form enol is close to that found by Oguchi-Fujiyama *et al*., (2012). Among all samples, *SS*+**PB+FA** was the least sign after UV irradiation. According to Martins *et al*. 2020, the nature provides an abundant source of antioxidant substances and natural phenolic compounds, which demonstrate a topical photoprotective action, as well as antioxidant potential, anti-aging, moisturizing, anti-pollutant, which is beneficial to the skin.

According to Jia *et al*. (2008), the resveratrol decreased the formation of superoxide anions radicals (potassium superoxide) in anhydrous solution and xanthine oxidase/ xanthine system evaluated by enzymatic spectrophotometric method. These radicals are present in several patho-logical oxidative processes, and with the addition of resveratrol there was a decrease in the signal of the radical stabilizer 5-

(Diethoxyphosphoryl) -5-methylpyrroline-N-oxid (DEPMPO). Over the course of the hours, it was still noticed the presence of free radicals, but the signal decayed returning to the profile before irradiating, which explains the cumulative effect of UV radiation on the skin.

5 CONCLUSIONS

The choice of shed snakeskin was appropriate to EPR measures due to be dehydrate membrane, similarly the human stratum corneum and it is ecologically correct because it came from the spontaneous release of the snake and it would be discarded.

The antioxidants RES and FA was adequate analysed by FRET, due their photoestabilization of the filter EHMC in the photoprotection formulation.

From the point of view of the free radicals found in the samples, considering the appropriate normalizations (parametric, equipment performance and the shed snakeskin mass of each sample), the sample *SS* **+ PB + FA** was the one with the lowest number of free radicals after irradiation, which corroborated the high percentage of radical inhibition *in vitro* and it was the better association with the photoprotective formulation.

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