UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Programa de Pós-Graduação em Fármacos e Medicamentos
Área de Produção e Controle Farmacêuticos

Antilipoperoxidative and anti-inflammatory efficacy of multifunctional sunscreens containing ferulic acid

Rafael Sauce Silva

Tese para obtenção do Título de DOUTOR
Orientador: Prof. Dr. André Rolim Baby

São Paulo
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Dedicatória

Gostaria de dedicar todo meu trabalho ao carinho, amor e suporte constante de minha família: meus pais ***Elaine*** *e* ***Eloi****, que me ensinaram a ter integridade e não desistir de suas batalhas; meu irmão* ***Bruno****, que me inspirou a ter paixão pela ciência e por conhecimento; meu filho* ***Vinicius****, que é meu maior orgulho e a razão de minha força; e minha gata* ***Mia****, que é uma companheira amável.*

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Epígrafe

"Em rio que tem piranha, jacaré nada de costas"

RESUMO

SAUCE, R.S. **Antilipoperoxidative and anti-inflammatory efficacy of multifunctional sunscreens containing ferulic acid. 2019.** Tese (Doutorado) - Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2020.

A exposição crônica desprotegida à radiação ultravioleta (UV) contribui para o desenvolvimento de câncer de pele e os filtros solares são relevantes para evitar tais efeitos prejudiciais, porém, os protetores solares tradicionais não geram proteção suficiente contra o estresse oxidativo cutâneo. Logo, espera-se o desenvolvimento de formulações fotoprotetoras multifuncionais, atuando não somente na absorção e/ou reflexão da radiação UV, mas, também, auxiliando na homeostase cutânea, com presença de agentes antioxidantes. No presente estudo foi utilizado o ácido ferúlico conjuntamente com dois filtros solares, o bemotrizinol e a triazona de octila, para determinação de métodos de segurança e eficácia, utilizando técnicas que melhor elucidem e comprovem os efeitos do ácido ferúlico. Foram realizados ensaios de permeação cutânea pela aplicação tópica de formulação contendo as três substâncias em voluntários, sendo o estrato córneo retirado pelo método de *tape stripping* (*ex vivo*) com subsequente quantificação por cromatografia líquida de alta eficiência (CLAE). O ensaio pôde avaliar a profundidade de penetração das substâncias, caracterizando-as. Ademais, a quantificação simultânea das três substâncias foi efetuada por método único e rápido, facilitando análise com aprimoramento da técnica. Em adição, foi realizado ensaios de TBARS (substâncias reativas ao ácido tiobarbitúrico) em estrato córneo removido por *tape stripping* (*ex vivo*), para avaliar o potencial de peroxidação lipídica cutânea, contendo ou não o ácido ferúlico. Até o presente momento, é a primeira vez que o método TBARS é utilizado para caracterização do estrato córneo (*ex vivo*) e quantificada por CLAE. O protocolo desenvolvido pode auxiliar na eficácia de agentes antioxidantes, em estudos que visam elucidar o nível de peroxidação lipídica causada por medicamentos e cosméticos e, até mesmo, na realização de estudos de base, caracterizando etnias e gêneros. Ademais, um ensaio anti-inflamatório *in vivo* com equipamento de fluxometria Laser Doppler foi utilizado para comparar a formulação fotoprotetora com ou sem ácido ferúlico. Os dados indicaram que o antioxidante reduziu o coeficiente angular das unidades de perfusão, mitigando os efeitos inflamatórios. Ainda, foi identificada diferença entre os gêneros, sugerindo reação inflamatória mais pronunciada em mulheres. O ácido ferúlico provou ser um recurso valioso, além de ser seguro e elevar o FPS dos fotoprotetores, também atenuando os efeitos da inflamação.

Palavras-chave: Filtro solar, tape stripping, CLAE, ácido ferúlico, TBARS, fluxometria Laser Doppler

ABSTRACT

SAUCE, R.S. **Antilipoperoxidative and anti-inflammatory efficacy of multifunctional sunscreens containing ferulic acid. 2019.** Tese (Doutorado) - Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2020.

Unprotected chronic exposure to ultraviolet radiation generates many harmful effects to human skin and UV filters are essential to health, however, traditional sunscreens do not provide enough protection against cutaneous oxidative stress, a process amplified by UV radiation. Therefore, is been proposed the development of multifunctional photoprotective formulations, acting in the absorption/reflection of UV radiation and assisting in cutaneous homeostasis. In the present study, ferulic acid is used in conjunction with two sunscreens, bemotrizinol and ethylhexyl triazone, for the determination of biosafety and efficacy methods, using techniques that better elucidate the effects of ferulic acid. Skin permeation assays were performed by applying a formulation containing the three substances in the *stratum corneum* of volunteers, which were removed by the tape stripping method (*ex vivo*) with follow quantification by high performance liquid chromatography (HPLC). The test was able to evaluate the penetration depth of the substances, characterizing them. In addition, the simultaneous quantification of the three substances was performed by a single and fast method, facilitating their analysis and improving the technique. Also, TBARS (thiobarbituric acid reactive substances) assays were performed in *stratum corneum* removed by tape stripping (*ex vivo*), evaluating the potential of cutaneous lipid peroxidation, with or without ferulic acid. To date, it is the first time that TBARS method is used to characterize the *stratum corneum* (*ex vivo*) and quantified by HPLC. The protocol developed may aid in the efficacy of antioxidant agents in studies aimed at elucidating the level of lipid peroxidation caused by drugs and cosmetics, and even in carrying out baseline studies characterizing different ethnicities and genders. As last, an anti-inflammatory *in vivo* assay with Laser Doppler flowmetry equipment was used to compare the sunscreen formulation with or without ferulic acid. Data indicated that the antioxidant reduced the angular coefficient of the perfusion units, mitigating the inflammatory effects. Furthermore, a significant difference was found between the genders, suggesting a more pronounced inflammatory reaction in women. Ferulic acid proved to be a valuable resource, besides being safe and raise the SPF of sunscreens, it also mitigates the effects of inflammation.

Keywords: UV filter, tape stripping, HPLC, ferulic acid, TBARS, Laser Doppler fluxometry

1. Introduction

The Sun has been shining Earth for more than 4 billion years. The evolution of life occurred throughout the properties of sunlight, and the life we know today is dependently of its rays. For humans, the most popular benefit is its ability to trigger the body's vitamin D supply by exposure to ultraviolet (UV) radiation B, which assists levels of phosphorous and serum calcium, supporting a wide range of metabolic functions as well as neuromuscular transmission, immune system and bone mineralization (MEAD, 2008; RAZZAQUE, 2011; BROWN; RAZZAQUE, 2016). However, overexposure to sunlight causes adverse effects on our skin, viz. UV radiation, being the development of cancer its most harmful effect, which is the most common type of cancer diagnosed worldwide (IARC, 2012; "AMERICAN CANCER SOCIETY", 2016; "WHO | World Health Organization", 2017).

UV radiation covers the wavelength range of 100 to 400 nm and is divided into UVC (100 to 290 nm), UVB (290 to 320 nm), UVA II (320 to 340 nm) and UVA I (340 to 400 nm) (DIFFEY et al., 2000). Since UVC rays are absorbed by the ozone layer in the atmosphere, it do not reach Earth's surface, while ~ 5% of UVB rays and ~ 95% of UVA rays surpass this layer, reaching human skin at different depths (LAUTENSCHLAGER; WULF; PITTELKOW, 2007; YOUNG, 2009; DE OLIVEIRA et al., 2015). UVB radiation promotes direct photochemical damage to skin DNA, causing genetic mutations, while UVA radiation has indirect effects on DNA, mainly through the generation of reactive oxygen species (LAUTENSCHLAGER; WULF; PITTELKOW, 2007). Both UV radiation can disrupt collagen fibers and vitamins, accelerate skin aging, and increase the risk skin cancer rate (MEAD, 2008). Despite this, the human body has endogenous mechanisms of protection against UV radiation, such as skin chromophores - molecules responsible for the conversion of energy absorbed into heat - such as melanin, tryptophan, tyrosine, nitrogen purine and pyrimidine bases (JANSEN et al., 2013). In addition, other mechanisms like inhibition of free radicals

by endogenous antioxidants, horny layer thickening, sweat, sebum and hair are also defenses that aid in preventing UV damage (CESTARI; OLIVEIRA; BOZA, 2012; JANSEN et al., 2013). However, such endogenous protection may not be sufficient for cutaneous homeostasis, in which additional measures for protection should be taken, such as wearing clothes, changing educational habits, and applying photoprotective formulations (CESTARI; OLIVEIRA; BOZA, 2012). Sunscreens present active substances that act on the surface of the skin, involving mainly two mechanisms: absorption (organic or chemical filters) or reflection (inorganic or physical filters) of the UV radiation (CESTARI; OLIVEIRA; BOZA, 2012).

The oxidative stress is an important factor that is amplified by UV radiation, being a biological condition responsible for several damages to the body, being caused by free radicals - highly unstable molecules, atoms or ions with unpaired electrons that can react with other molecules (CAROCHO; FERREIRA, 2013). Free radicals that can be harmful to our organism are mainly classified as reactive nitrogen species (RNS) and reactive oxygen species (ROS) (PHANIENDRA; JESTADI; PERIYASAMY, 2015). ROS/RNS can have a beneficial factor to organism; at moderate/low levels can be involved in immune function, mitogenic response, redox regulation and cellular signaling pathways (NORDBERG; ARNÉR, 2001; VALKO et al., 2007). In another hand, its excess can lead to disrupt the integrity of many biomolecules, like DNA, proteins and lipids (LÜ et al., 2010; CRAFT et al., 2012). The examples of ROS can include superoxide ($O_2^{\bullet -}$), oxygen radical ($O_2^{\bullet\bullet}$), hydroxyl ($OH^{\bullet}$) and peroxyl radical ($ROO^{\bullet}$); and, for NOS, nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$)(HALLIWELL, 2015).

Lipid peroxidation, one of the processes generated by oxidative stress, occurs when free radicals react with polyunsaturated fatty acids from the phospholipids of the cell membranes, thus disrupting them and allowing the penetration of radicals into the

intracellular structures (HALLIWELL; GUTTERIDGE, 1989). As a reaction result, there is formation of lipid peroxides, leading to the following deleterious actions in the cells: rupture of the cell membranes; mutations of DNA; oxidation of unsaturated lipids; formation of chemical residues and involvement of extracellular matrix components (proteoglycans, collagen and elastin), among others (HALLIWELL; GUTTERIDGE, 1989). The chemical reactions involving lipid peroxides can cause the production of RNS and/or ROS, which is associated with many serious diseases (RAHMAN, 2007; LOBO et al., 2010; LÜ et al., 2010; SINGH et al., 2010). Also, it can trigger inflammatory process, a natural response stimulated by strange pathogens, in which the body tries to neutralize it, or endogenous signals *e.g.* damaged cells, resulting in tissue repair, elimination of the cell, or sometimes pathology, when the response goes unchecked (“A current view on inflammation”, 2017; NETEA et al., 2017). It is a complex process mediated by activation of inflammatory or immune cells. Macrophages, part of the immune system, can overproduce pro-inflammatory cytokines and mediators (ROS, RNO and prostaglandin E2) generated by activated inducible cyclooxygenase and nitric oxide synthase (iNOS) (WALSH, 2003).

Although traditional UV filters are the main form of protection against UV radiation in formulations, they are not completely adequate as a single source of prevention against oxidative stress (ZHANG et al., 2010). Experiments conducted by Haywood et al., 2003 using electron spin spectroscopy to detect free radicals in human skin have shown that sunscreens reduced free radical formation by only 55% after exposure to artificial UV radiation. Consequently, there is a need for innovation and better elaboration of photoprotective formulations in order to contemplate a greater spectrum of protection from the damages caused by UV radiation.

Ferulic acid (4-hydroxy-3-methoxy cinnamic acid), a natural phenolic compound, belongs to the class of hydroxycinnamic acids, being found mainly in rice, citrus fruits, wheat,

corn, roasted coffee and several other vegetables (GRAF, 1992; SRINIVASAN et al., 2005; GERIN et al., 2016). It is known that phenolic compounds have benefits to human health, mainly because of their antioxidant potential (GERIN et al., 2016). In addition to its antioxidant properties, the specialized literature has demonstrated that ferulic acid also has anti-inflammatory, hepatoprotective, anticarcinogenic, antimutagenic and neuroprotective potential (GRAF, 1992; MURAKAMI et al., 2002; JIN et al., 2005; SRINIVASAN et al., 2005; BASKARAN et al., 2010). The antioxidant activity of ferulic acid occurs by the suppression of hydroxyl radicals, superoxide radicals, nitric oxide and peroxynitrite (GRAF, 1992). Ferulic acid has *cis-trans* isomerism, being the *trans* form the most commonly found in nature. The *cis* form is presented as a yellowish oil with maximum UV absorption of 316 nm, whereas the *trans* form is presented as crystal with two maximum absorption peaks in water (284 and 307 nm) (GRAF, 1992; ALMEIDA et al., 2018). Studies conducted by Peres et al., 2018 evaluated their photoprotection potential, amplifying the sun protection factor (SPF) by 37% and the UVA protection factor (FP-UVA) by 26% when in conjunction with UV filter in a formulation. Topical administration is a strategy to raise the concentration of antioxidants in the skin and prevent endogenous molecules degradation, which increases its half-life and, at the same time, protecting from negative effects caused by UV radiation (CHEN; HU; WANG, 2012). Therefore, adding this feature to its antioxidant property, ferulic acid is an excellent candidate for multifunction photoprotective formulations.

The surface of the human body is covered entirely by our biggest organ, the skin, acting on the defense against the external environment, and being directly exposed to UV solar radiation (DI DOMENICO et al., 2009). It is recommended that cosmetic products remain on the epidermis surface, preferably in the *stratum corneum* region, thus avoiding a possible blood absorption in the connective tissue. However, studies on the penetration and permeation of UV filters in the skin suggest that some of these can overcome the cutaneous

barrier, reaching the systemic circulation, which is not desirable (HAYDEN; ROBERTS; BENSON, 1997). Thus, in order to measure the protection of ferulic acid and the UV filters components of the photoprotective formulation, skin permeation assays were performed in this research work.

The thiobarbituric acid reactive species (TBARS) assay, a method to measure lipid peroxidation by the production of malondialdehyde (MDA), a product resulting from the peroxidation of polyunsaturated fatty acids, was used as an indicator of the oxidative stress (DEL RIO; STEWART; PELLEGRINI, 2005). However, until the present literature, there is no data on the measurement of lipid peroxidation by the TBARS method by HPLC in the *stratum corneum* of volunteers withdrawn by tape stripping (*ex vivo*) technique. This method allows the evaluation of the lipid peroxide species of the *stratum corneum* formed by artificial UV irradiation, in order to measure the antioxidant activity of the formulation with or without ferulic acid.

Considering the importance that sunscreens have for prevention against skin diseases from UV radiation, added to the need for better formulations for greater coverage of protective aspects to oxidative stress, this study is justified. Ferulic acid, due to its antioxidant and photoprotective properties, is a promising candidate for multifunctional formulations, besides having a history of cutaneous safety.

2. Objectives

The present study contemplated the efficacy of multifunctional sunscreens containing ethylhexyl triazone, bemotrizinol and ferulic acid (*trans*) by *ex vivo* and *in vivo* assays, namely:

- evaluation of ethylhexyl triazone, bemotrizinol and ferulic acid skin penetration by *ex vivo* tape stripping using high performance liquid chromatography (HPLC);
- determination by TBARS of ferulic acid antilipoperoxidant potential through analyses of treated epidermis by *ex vivo* test (tape stripping) in HPLC;
- and determination of *in vivo* anti-inflammatory activity of the photoprotective formulations by a method that associates the application of a vasodilator and perfusion by Laser Doppler.

3. Material and Methods

3.1) Chemical, reagents and instrumentation

Trans-ferulic acid was purchased from Sino Lion (USA), and ethylhexyl triazone and bis-ethylhexyloxyphenol methoxyphenyl triazine (bemotrizinol) were purchased from BASF (Brazil). Thiobarbituric acid, 1,1,3,3-tetramethoxypropane (TEP), tetrahydrofuran, methanol, H_2SO_4, H_3PO_4 and BHT were purchased from Sigma–Aldrich (St. Louis, MO, USA). N-butanol and 1,4-dioxan were purchased from Merck (Darmstadt, Germany). All reagents were of analytical grade and were used as received, without any further purification. Purified water was used for all experiments, using Merck Millipore® Mili-Q® Simplicity UV. Adhesive tapes were Tape Scotch® 3M Transparent 750 polypropylene; syringe filter was 0.22 µm, Millipore®, and the occlusive patch was Hill Top® Chamber occlusive patch (Hill Top® Research, Cincinnati, Ohio). HPLC was Shimadzu® Prominence (Kyoto, Japan) composed of SPD-M20A diode array spectrophotometric detector and CTO-20A column oven. The column C18 (250 × 4.6 mm, 5 mm particle size, Shimadzu, Japan) was preceded by a pre-column (10 × 4.6 mm). Laser Doppler flowmetry equipment was PeriFlux® System 5000 (Perimed®, Stockholm, Sweden). The artificial UV simulator chamber used was an Atlas Suntest® CPS+ with a xenon lamp (1500 W) and a filter responsible for simulating solar radiation, allowing the passage of wavelengths above 290 nm. Mechanical stirrer (IKA® RW 20n), analytical balance (Shimadzu® AUY 220), semi-analytical balances (Gehaka® BG 4000 and Ohaus® ARD 110), thermostatic bath (New Ethics® N480), ultrasonic bath (Unique® UltraCleaner 1600A), centrifuge (Hitachi® RX2) and single-channel micropipettes (Eppendorf® 100-1000 µL and 1.0-10.0 mL) were used.

3.2) Formulations

Emulsified oil-in-water (O/W) systems containing ethylhexyl triazone and bemotrizinol with or without ferulic acid were prepared. The qualitative and quantitative composition (% w/w) of the samples is described in

Table 1.

Table 1. Qualitative and quantitative composition (% w/w) of photoprotective formulations with or without ferulic acid (F1 and F2).

	Ingredients	Concentration (% w/w)	
		F1	F2
Oil phase	Ethylhexyl triazone	5.00	5.00
	Bis-ethylhexyloxyphenol methoxyphenyl triazine	10.00	10.00
	C12-C15 alkyl benzoate	9.00	9.00
	Butylene glycol cocoate	6.75	6.75
	Isopropyl myristate	6.75	6.75
	Cyclomethicone	1.75	1.75
	Cyclomethicone (and) dimethicone crosspolymer	1.25	1.25
	Hydroxyethyl acrylate (and) sodium acryloyldimethyl taurate copolymer (and) isohexadecane (and) polysorbate 60	4.00	4.00
Water phase	Glycerin	5.00	5.00
	Phenoxyethanol (and) methylparaben (and) ethylparaben (and) butylparaben (and) propylparaben (and) isobutylparaben	0.75	0.75
	Disodium EDTA	0.30	0.30
	Acrylates (and) C10–30 alkyl acrylate crosspolymer	0.10	0.10
	Ferulic acid	-	1.00
	Purified water	*	*

	Triethanolamine	**	**

∗ Sufficient to complete to 100%; ∗∗ Sufficient to adjust the pH value.

3.3) Ethical issue

The volunteers used in this research were provided with the necessary information and clarification regarding the trial. The consent was given by means of the signature through the responsible committee document, maintaining anonymity of the participant and guaranteeing his / her departure from the study, voluntarily, at any time. The project was approved by the Ethics Committee by the Faculty of Pharmaceutical Sciences of USP, number CAAE: 31583814.0.0000.0067, protocol number 735.493. Volunteers were maintained at a temperature of 21 ± 2 °C and relative humidity of 40-60%. Volunteers were admitted with healthy skin between age of 18-70, with skin type II-V, with no additional exclusion criteria. It was recommended not to apply cosmetic products for 24 hours before the experiment in the area to be tested.

3.4) Penetration

Chromatographic Conditions and Validation Method

A single method was used to quantify the three bioactive compounds by HPLC, modified from Modi; Vukkum, 2014. The mobile phase was composed in isocratic mode of sodium acetate buffer pH 4.2: 1,4-dioxane (20%: 80%) filtered through syringe filter, with a flow rate of 1.5 $mL.min^{-1}$ for 25 minutes at 50°C, with 10 µL of sample injection amount. The diode detector was set at 311 nm for sample detection.

Linearity

Analytical curves were prepared for ferulic acid, ethylhexyl triazone and bemotrizinol by diluting 1.0 mg of compound to 1.0 mL of a THF: 1,4-dioxane solution (50%:50%).

Through this solution, concentrations of each active were prepared, as shown in **Erro! Fonte de referência não encontrada.**. Finally, it was filtered by a syringe filter and added to vials, in triplicate.

FERULIZ ACID	ETHYLHEXYL TRIAZONE	BEMOTRIZINOL		
Conc ug/mL			Conc ug/mL	Conc ug/mL
2.0	10.0	20.0		
4.0	20.0	40.0		
6.0	30.0	60.0		
8.0	40.0	80.0		
10.0	50.0	100.0		
12.0	60.0	120.0		
16.0	80.0	160.0		
20.0	100.0	200.0		
24.0	120.0	240.0		
28.0	140.0	280.0		

Selectivity

Samples without ferulic acid, ethylhexyl triazone and bemotrizinol, as well as adhesive tapes with *stratum corneum* were extracted with sodium acetate buffer pH 4.2: 1,4-dioxane (20%:80%), followed by ultrasonic bath for 15 minutes. Therefore, they were individually inserted into vials after syringe filtration. HPLC readings were performed as already cited.

Limit of Quantification

The limit of quantification was calculated according to parameters of the analytical curve, like seen on Equation 1.

Equation 1.

$$LQ = \frac{10\ x\ \sigma}{IC}$$

LQ = limit of quantification

σ = standard deviation calculated from the residual standard deviation of the regression line

IC = slope of the calibration curve

Limit of Detection

The limit of detection was calculated according to parameters of the analytical curve, like seen on Equation 2.

Equation 2.

$$LD = \frac{3.3\ x\ \sigma}{IC}$$

LD = limit of detection

σ = standard deviation calculated from the residual standard deviation of the regression line

IC = slope of the calibration curve

Ex vivo

The volunteer`s volar forearm was clean once with purified water, followed by application of the formulation (2.0 mg.cm^{-2}) containing ferulic acid (FA), bemotrizinol (BT) and ethylhexyl triazone (ETZ). After 2 hours, the *stratum corneum* was removed by the tape stripping technique, with 20 adhesive tapes (BENFELDT et al., 2007; ALONSO et al., 2009; DE OLIVEIRA et al., 2015; PERES, 2015).

Adhesive tapes extracted were divided in three regions: 1, 2 and 3. *Region 1:* composed by the first tape extracted, which represents the uppermost layer of the *stratum corneum*; *Region 2:* composed by tapes 2-10, representing intermediate layers of *stratum*

corneum; and *Region 3:* composed by tapes 11 - 20, which represents bottom layers of the *stratum corneum*.

The *stratum corneum* was transferred to a glass beaker and extracted by the addition of 10 mL sodium acetate buffer pH 4.2:1,4-dioxane (20:80%), with ultrasonic bath for 15 minutes (ALONSO et al., 2009; PERES, 2015). After mixed in vortex and filtrated in syringe filter, the samples were quantified by HPLC.

3.5) Determination of lipid peroxidation by TBARS assay

Chromatographic Conditions and Validation Method

HPLC analysis were performed in isocratic mode with a mobile phase composed of 35% methanol and 65% phosphate buffer (50 mM, pH 7.0) filtered through syringe filter at a flow rate of 1 $mL.min^{-1}$ for 10 minutes at 30°C, with 40 µL of sample injection. The diode detector was set at 532 nm to detect the MDA-TBA adduct formed by the reaction.

Preparation of MDA stock solution

The method was adapted from Bastos et al., 2012. A solution was prepared with the addition of 22.0 µL of 1,1,3,3-tetramethoxypropane (TEP) to 10.0 mL of H_2SO_4 (1%) and maintained at room temperature for 2 hours protected from light, generating MDA by the acid hydrolysis of TEP. Then, 10.0 µL of this solution was added to 3.0 mL of H_2SO_4 (1%), forming the stock solution of MDA. Its concentration was determined by reading the absorbance through spectrophotometer at 245 nm (ε_{245nm} = 13700 $L.mol^{-1}.cm^{-1}$).

Preparation of the reaction

The method of Hong et al., 2000 with modifications was used. 1.0 mL aliquots of the stock solution/*stratum corneum* sample was added to 144 µL of 0.2% BHT (in methanol) and 400 µL of H_3PO_4 (0.44 M) in a glass tube, capped. Ttubes were vortexed for 10 minutes and kept at room temperature. Then, 600 µL of 0.6% TBA (in 0.44 M H_3PO_4) was added to the tubes, with subsequent mixing with vortex. The tubes were heated in a thermostated bath for 45 minutes at 90°C. So, 600 µL of n-butanol was added, with vortex mixing for 1 minute and centrifugation at 3000g, for 10 minutes. After syringe filtration, the supernatant was analyzed by HPLC.

Linearity

The analytical curve was prepared by diluting the known concentrations of MDA with PBS, with subsequent reaction of the method. Increasing concentrations were used (0.001, 0.05, 0.2, 0.4, 0.6, 0.8 µM).

Selectivity

All reagents that was used in TBARS experiment, as well as the photoprotective formulation with ferulic acid (irradiated or not, to simulate possible degradation processes of the formulation) and the adhesive tape used in the *ex vivo* assay were examined individually. The formulations and the tapes were first extracted with 10 mL of methanol with 15 minutes of ultrasonic bath, followed by the TBARS method. Therefore, the supernatant of the reaction and all reagents were individually inserted into vials after syringe filtration, with HPLC readings through the same chromatographic conditions as the TBARS assay.

***Stratum corneum* sample**

The *ex vivo* tape stripping method was applied in 12 volunteers. Four regions were defined in the volar forearm of the volunteers: two control regions, one with and other without consecutive artificial irradiation, and regions applied with photoprotective formulations with and without ferulic acid. After 2 hours of application, 10 adhesive tapes per region were used for *stratum corneum* removal (BENFELDT et al., 2007; ALONSO et al., 2009; DE OLIVEIRA et al., 2015; PERES, 2015). Tapes were irradiated (except for a control region) in a solar simulator chamber, at a dose of 5506 $KJ.m^{-2}$. Extraction of the *stratum corneum* from the tapes were performed by adding 5 mL of methanol followed by ultrasonic bath for 15 minutes (ALONSO et al., 2009; PERES, 2015). The adduct quantification was performed according to reaction method already cited.

3.6) Anti-inflammatory assay

Anti-inflammatory studies with Laser Doppler flowmetry were performed in 13 volunteers. Three areas of 2.25 cm^2 each were used in the volar forearm of each of the volunteers. The areas were composed of the control region (purified water) and regions treated with the photoprotective formulation with or without ferulic acid, being applied through an occlusive patch, for 2 hours. Later, the patches were removed and its excess on the skin removed through soft paper, with consequent 30 minutes rest until the beginning of the measurement with Laser Doppler flowmetry. Filter paper soaked in 0.5% methyl nicotinate solution was placed in the regions to be tested for 60 seconds, followed by measurement of the equipment for 15 minutes. The determination of which forearm (left or right) and the order of the regions were given in a random manner (GALLIN; OGNIBENE; JOHNSON, 2017).

The perfusion unit values, maximum perfusion unit value, area under the curve, angular coefficient and start of infusion unit increase were measured using the Laser Doppler

flowmetry equipment and analyzed through PeriSoft® software, version 2.5.5. The results were statistically evaluated using t-test followed by Wilcoxon's test to make comparisons between the different parameters with the help of GraphPad Prism® software version 5.0 (GraphPad® Software, Inc.).

4. Results and Discussion

4.1) Penetration

Linearity

The linearity of a method is the analytical responses directly proportional to the concentration of an analyte in a sample with a linear connection over the range established, with at least 5 different concentrations in triplicate, assuming that there is a linear link between input (x) and output (y), which $y = f(x)$ (ARAUJO, 2009; ANVISA, 2017). The linearity of an analytical curve can be verified by inspecting the coefficient of correlation (r) and the coefficient of determination (r^2) (ARAUJO, 2009).

The correlation coefficient is the degree of relationship between two variables, x and y, being between -1 and 1; 1 indicates that the two variables are moving in unison, perfect correlation, while -1 means that the two variables are in perfect opposites. If they are not correlated, then the correlation value can still be calculated, which would be 0. Correlation can be justly explained for simple linear regression - since you have only one variable x and one y. For multiple linear regression, the square r (r^2) is used since it has several variables. The coefficient of determination (r^2) shows percentage variation in which y is explained by all variables x together. It is always between 0 and 1, and cannot be negative (it is a square value). The closer to 1, the more reliable it is, which must be above 0.990 to be accepted (ICH, 2005; ANVISA, 2017). The linearity of all the active compounds were obtained in

triplicate with a r^2 of 0.99, proving their linearity, as illustrated in Figure 1, Figure 2 and Figure 3.

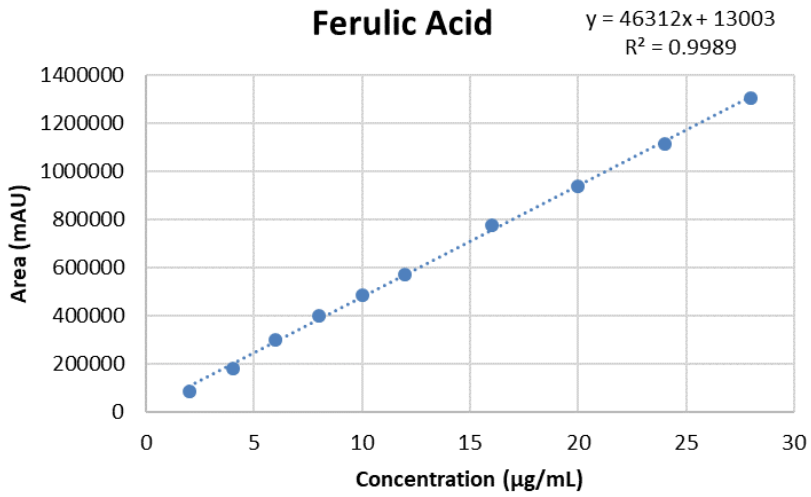

Figure 1. Analytical curve of ferulic acid in the range of 2 to 28 µM, with line equation y = 46312x + 13003.
Legend: y is area (mAU), x is concentration (µg/mL).

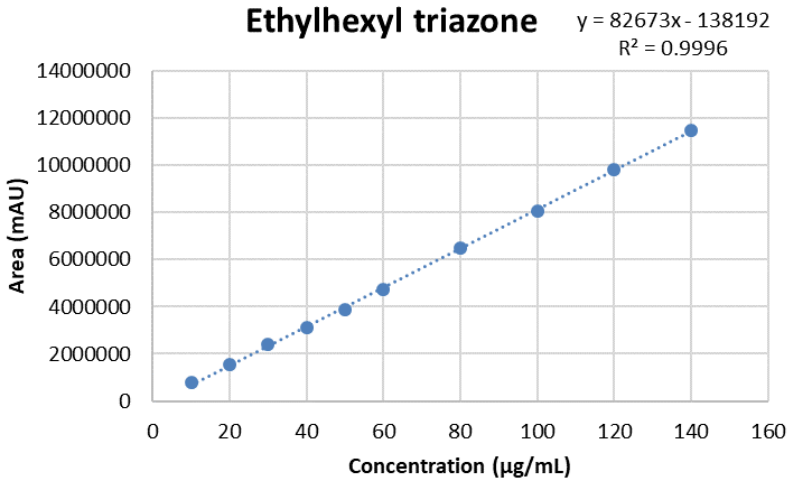

Figure 2. Analytical curve of ethylhexyl triazone in the range of 10 to 140 µM, with line equation y = 82673x + 138192.
Legend: y is area (mAU), x is concentration (µg/mL).

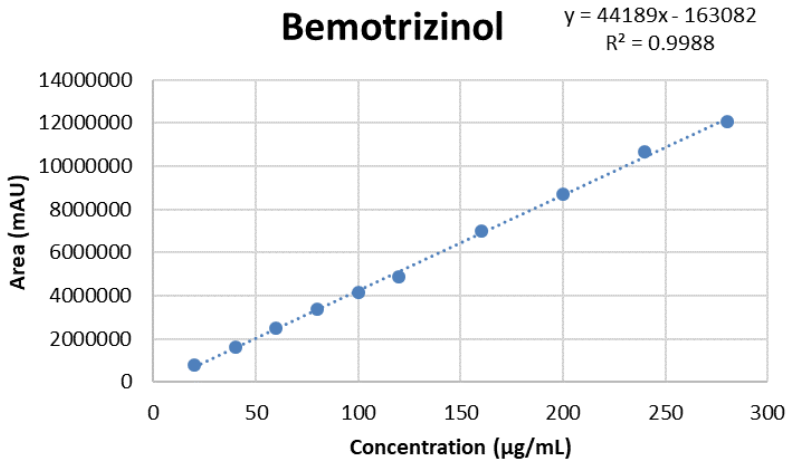

Figure 3. Analytical curve of bemotrizinol in the range of 20 to 280 µM, with line equation y = 44189x + 163082.
Legend: y is area (mAU), x is concentration (µg/mL).

Selectivity

It is the ability of the method to accurately measure a compound in the presence of other components, such as impurities, degradation products and matrix components (ANVISA, 2017). There were no interferents in the method, proving its effectiveness.

Limits of detection and quantification

Limit of detection is the detection of the smallest amount value of the analyte in a sample, but not necessarily quantified, whereas the limit of quantification is the quantification of this smallest amount value, all under the established experimental conditions (ANVISA, 2017). The values are shown in Table 2.

Table 2. Limit of detection and quantification of ferulic acid, ethylhexyl triazone and bemotrizinol (µM)

	Ferulic acid	Ethylhexyl triazone	Bemotrizinol
Limit of Detection (µM)	0.60	1.71	6.11
Limit of Quantification (µM)	1.83	5.18	18.51

Ex vivo

Understanding the penetration of substances topically applied over the skin is important to optimize the safety and efficacy of cosmetics, especially for sunscreen products (WEIGMANN et al., 2005; ROUSSEL et al., 2015). An ideal sunscreen should have substantivity without penetrating the viable epidermis, dermis, hair follicle or systemic circulation, only protecting the upper layers of the tissue (TAMPUCCI et al., 2017). In contrast, a good topical antioxidant should penetrate deeper layers of the *stratum corneum*, where the UVA radiation generates its oxidative damage, thus increasing its effectiveness.

Several HPLC studies on the specialized literature were already performed for simultaneous analysis of UV filters on sunscreens, but these were applied only in formulations and not in biological samples, like this present investigation (DURAND et al., 2009; WHARTON et al., 2011; CHANG; YOO; LEE, 2015; DAVIES et al., 2017). Percutaneous studies can be divided into *in vivo*, *in vitro* and *ex vivo*. *In vivo* percutaneous absorption studies are performed using animal skin (such as rat with/without hair, or guinea pig) (MONTI et al., 2011). The most popular *in vitro* technique is the vertical diffusion cell method, using human skin from surgery or autopsy, pig skin or artificial membranes as a model barrier. Pig ear skin is useful for its availability and ease of preparation, as well as

morphologically and functionally resembling human skin, with similar percutaneous permeation rates (BENECH-KIEFFER et al., 2000; KLINUBOL; ASAWANONDA; WANICHWECHARUNGRUANG, 2008). However, difficulties in obtaining human skin samples, as well as the increasing ban on animal testing around the world, have led researchers to develop new models for the evaluation of skin permeation, such as the tape stripping technique.

The tape stripping technique can be used to quantify UV filters in the skin layers by analytical methods such as HPLC, UV absorption spectroscopy, gas chromatography and mass spectrometry (KLINUBOL; ASAWANONDA; WANICHWECHARUNGRUANG, 2008). This technique allows the use of human skin, increasing the precision of the experiment without causing harm to the volunteers. The technique is considered *ex vivo*, as it uses the topical interaction of the sample with the volunteer's skin, with subsequent removal of the *stratum corneum* by adhesive tapes, generating flexibility for several types of tests on the removed part of the tissue (*e.g.* irradiation or chemical reactions) (ALONSO et al., 2009).

The physicochemical properties of sunscreens are fundamental in the process of penetration and permeation through the skin (TAMPUCCI et al., 2017). The value of partition coefficient (log P) of the molecules is a relevant element to evaluate its permeation ability through the skin (SOUZA; MAIA CAMPOS, 2017; TAMPUCCI et al., 2017). It is known that log P values around 5.0 may allow passive diffusion of the molecule into the skin; lower partition coefficients mean that the molecule is so soluble in water that they are not able to penetrate across the *stratum corneum*. However, if log P is too high, the molecule may be able to accumulate and form reservoirs within the lipid phases of the *stratum corneum* (DURAND et al., 2009; TAMPUCCI et al., 2017). Molecules that exhibit both hydrophilic and lipophilic properties would be better candidates to permeate the skin (TAMPUCCI et al.,

2017). The log P values of the UV filters used in the present study are considered high, therefore ideal to accumulate in the *stratum corneum* (Table 3).

Table 3. Physicochemical parameters of bemotrizinol, ethylhexyl triazone and ferulic acid (ZHANG et al., 2010; LIFENG, 2014)

Name	Molecular weight (Da)	log P
Bemotrizinol	627.81	12.93
Ethylhexyl triazone	823.07	16.13
Ferulic acid	194.18	1.67

Another strategy to reduce the cutaneous absorption of sunscreens is the use of UV filters of high molecular weight, like bemotrizinol or ethylhexyl triazone (Table 3). It is alleged that most chemical compounds with molecular weight above 500 Da does not penetrate the skin, being an important factor for this type of study (ESSENDOUBI et al., 2016). Thus, since both filters used had molecular weights greater than 500 Da, it was expected that their penetration into the skin would be irrelevant.

Studies such as Souza; Maia Campos, 2017 analyzed the penetration potential of bemotrizinol (BT) and ethylhexyl triazone (ETZ), using porcine ear skin in a vertical diffusion cell, with analysis of the receptor fluid by HPLC after 24 hours. They used 16 adhesive tapes (tape stripping technique) in order to quantify retained samples in the epidermis. As a result, none of the UV filters could be detected in the recipient fluid, being considered retained in the skin, in which more than 90% were restricted to the *stratum corneum* (SOUZA; MAIA CAMPOS, 2017). Potard et al., 2000, applying a formulation with ETZ on excised human skin in diffusion cell, removed the *stratum corneum* by tape stripping after 30 minutes and

16 hours of exposure, analyzing by HPLC. As a result, 96% of ETZ was retained in the *stratum corneum*, where only the remainder penetrated into the viable epidermis without permeation to the dermis (receptor fluid of diffusion cell) (POTARD et al., 2000). Durand et al., 2009 also performed penetration experiments of BT (aerosol formulation), using excised human skin in a vertical diffusion cell, which it was later not found in the receiving fluid. Thus, the present work highlights its important, since it is the first time to date that a penetration of UV filters was tested in epidermis removed by *ex vivo* assays and analyzed by HPLC, further increasing the safety and reliability of the results considering the use of bemotrizinol or ethylhexyl triazone.

It is known that ferulic acid (FA) has solubility in hot water, being insoluble in oil (SOUTO et al., 2005). Zhang et al., 2010, through *in vitro* permeation tests in Franz cells (pig skin), observed low FA deposition on the skin, possibly being insufficient to induce its antioxidant activity. Peres, 2015, by tape stripping technique, analyzed the antioxidant activity of the corneous layer of epidermis, not observing differences among the adhesive tapes obtained (skin layers), justified by the low degree of FA skin penetration, responsible for keeping it only on the *stratum corneum* surface. Some of FA physicochemical characteristics can be found in Table 3.

In the present work, tape stripping was used, an efficient method to investigate the penetration of cosmetic actives (LADEMANN et al., 2009). In this method, *stratum corneum* cell layers are successively removed from the same skin area after topical application of the formulations using adhesive films (LADEMANN et al., 2009). The adhesive tapes contain amounts of corneocytes and corresponding amounts of the penetrated formulation, which can be quantified by analytical methods, such as HPLC (LADEMANN et al., 2009). The *stratum corneum* thickness of the human skin is given between 10-30 µm, having at least 16 layers of cell with a diameter of 1 µm each (YA-XIAN; SUETAKE; TAGAMI, 1999;

BÖHLING et al., 2014). Lademann et al., 2009 measured the amount of *stratum corneum* removed by the tape stripping technique; 5 adhesive tapes could remove about 3 µm of *stratum corneum*. The first adhesives tapes removed contain a high concentration of corneocytes, whereas when increasing the consequential number of tapes, its concentration decreases significantly. So, 20 adhesives tapes, like used in the present work, should not exceed 10 µm of thickness. The quantification of the active compounds in the *ex vivo* assay through HPLC is shown in Table 4.

Table 4. Penetration test performed by removal of *stratum corneum ex vivo*. Expected ferulic acid concentration in the skin = 20 µg/mL, expected bemotrizinol concentration in the skin = 200 µg/mL, expected octyl triazone concentration in the skin = 100 µg/mL

FERULIC ACID	CONC (µg/mL)	RECOVERY (%)	TOTAL RECOVERY (%)
Region 1	3.43	17.17	
Region 2	6.34	31.69	55.74
Region 3	1.38	6.88	
ETHYLHEXYL TRIAZONE	**CONC (µg/mL)**	**RECOVERY (%)**	**TOTAL RECOVERY (%)**
Region 1	27.49	27.49	
Region 2	20.61	20.61	56.34
Region 3	8.24	8.24	
BEMOTRIZINOL	**CONC (µg/mL)**	**RECOVERY (%)**	**TOTAL RECOVERY (%)**
Region 1	49.75	24.88	
Region 2	29.57	14.79	46.84
Region 3	14.35	7.17	

The formulation applied on the volar forearm of the volunteers had a concentration of 20 µg/mL for ferulic acid, 100 µg/mL for ethylhexyl triazone and 200 µg/mL for bemotrizinol. Ferulic acid showed to be more concentrated on region 2, where its recuperation was 31.69%, suggesting that it can penetrate easier than the UV filters, wherein ethylhexyl triazone and bemotrizinol were more concentrated on region 1, with recuperation of 27.49% and 24.88% respectively. However, only 55.74% of ferulic acid, 56.34% of ethylhexyl triazone and 46.84% of bemotrizinol was found in the removed skin of the *ex vivo* assay. Considering that the *stratum corneum* can have up to 30 µm thickness and the adhesive

tapes used should not exceed 10 µm of removal, it is suggested that the quantities not found of the active compounds should be in the bottom layers of *stratum corneum*.

4.2) Determination of *stratum corneum* lipid peroxidation by TBARS assay

When using HPLC, an analytical method that allows the identification and quantification of different substances in a sample variety, it is fundamental to prove that it produces reliable and appropriate results for the intended purpose (ICH, 2005). Thus, they must present adequate linearity, specificity, precision, sensitivity, quantification limit and accuracy (ANVISA, 2003; ICH, 2005).

Linearity

The linearity of all points was obtained in triplicate with r^2 equal to 0.99, as illustrated in Figure 4.

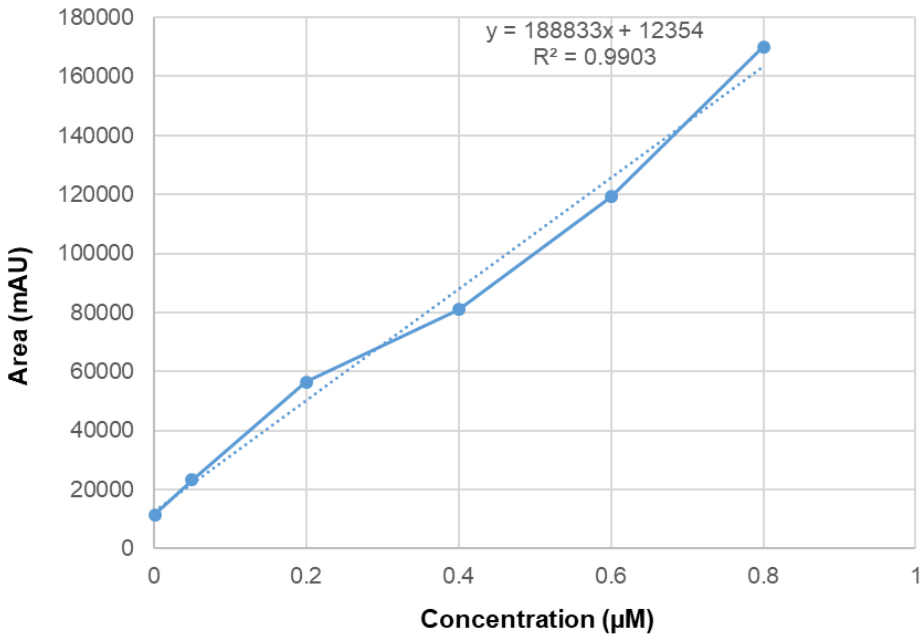

Figure 4. Analytical curve of the TBARS assay in the range of 0.001 to 0.8 µM, with line equation y = 188833x + 12354.

Selectivity

It was necessary to know if there was any interfering molecule at the same retention time as the TBARS adduct read at 532 nm. The method proved to have no interferences in the TBARS assay.

TBARS

Lipoperoxidation is the oxidative deterioration of lipids, often affecting polyunsaturated fatty acids for they multiple carbon double bounds which contains reactive hydrogen atoms in the methylene bridges (-CH2-). Subsequently, lipoperoxidation disturbs the biophysical properties of membranes, like membrane fluidity and electrical resistance, allowing this natural barrier to be corrupted and affecting the organelles inside (DEVASAGAYAM; BOLOOR; RAMASARMA, 2003). Malondialdehyde (MDA) is the most study end-products formed by the lipid peroxidation, being considered an increase indicator of oxidative stress (DEL RIO; STEWART; PELLEGRINI, 2005). Among many methods in the specialized literature for detection and determination of MDA in biological matrices, the main method is the TBARS assay, using the thiobarbituric acid (TBA), first used by Yagi et al., 1968 (YAGI et al., 1968). TBA can react with a variety of oxidized lipids like aldehydes, urea and sucrose, being able to form various chromogens, referred as TBA-reactive substances (TBARS) (BUTTKUS; BOSE, 1972; KOSUGI; KIKUGAWA, 1985; ALESSIO, 2000). However, the main indicator of lipoperoxidation is the reaction of two molecules of thiobarbituric acid (TBA) with MDA based upon heating of the sample under acidic conditions, generating the adduct MDA-TBA_2 (**Erro! Fonte de referência não encontrada.Erro! Fonte de referência não encontrada.**), a pink pigment detected by colorimetry at 532 nm or fluorimetry (excitation at 532 nm and emission at 553 nm) and mass ion at 323 amu (BERNHEIM, F.; BERHEIM, M. L.; WILBUR, ; YAGI, 1976; KNIGHT;

ROBERT; MCCLELLAN, 1988; DIB et al., 2002; JARDINE et al., 2002; PANJAMURTHY; MANOHARAN; RAMACHANDRAN, 2005; MOSELHY et al., 2013).

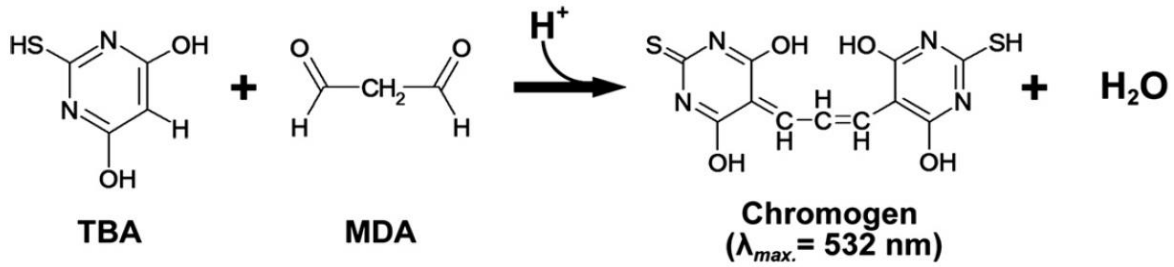

Figure 5. Formation of the adduct MDA–TBA_2 (BASTOS et al., 2012).

The TBARS assay have been analyzed by different kinds of techniques. However, since TBA can react with other molecules like saturated and unsaturated aldehydes, a more specific method was desired for specificity in the evaluation of MDA (KNIGHT; ROBERT; MCCLELLAN, 1988; ABUJA; ALBERTINI, 2001). The HPLC method can improve the specificity of MDA-TBA_2 detection, being a reliable method for lipoperoxidation evaluation, confirmed and utilized by some of the recent specialized literature (BASTOS et al., 2012; MOSELHY et al., 2013; AL-RIMAWI, 2015). Still, unfortunately, due to its extended process timing and high cost, the simple spectrometric method is still a majority use. Many studies have used the TBARS method to analyze the lipid peroxidation in different fields, especially in food and medical area, but this is the first time up to date that an *ex vivo* method with TBARS quantification through HPLC was used, being a real innovation in the field.

Ex vivo

Previously to the TBARS experiment, it was tested if the irradiation dosage was significant to raise lipoperoxidation induction, quantified by the MDA molecule. It was used a screening test of four volunteers with two different UV irradiation intensities, 2753 $KJ.m^{-2}$

and 5506 KJ.m^{-2}, using two sample sites, the control skin and the irradiated one (same parameters already described). A raise of 34.72% was observed in lipoperoxidation on the intensity of 2753 KJ.m^{-2}, whereas 5506 KJ.m^{-2} had a raise of 110,88%, proving its efficacy and being chosen for use.

The *ex vivo* results of the 12 volunteers is illustrated in Figure 6. To minimize the effect of inter-individual variability, the results were analyzed as the ratio between the values obtained at each sample site and the control values for all subjects on the parameters analyzed (GRAZIOLA et al., 2016; OLIVEIRA et al., 2016; PERES et al., 2016).

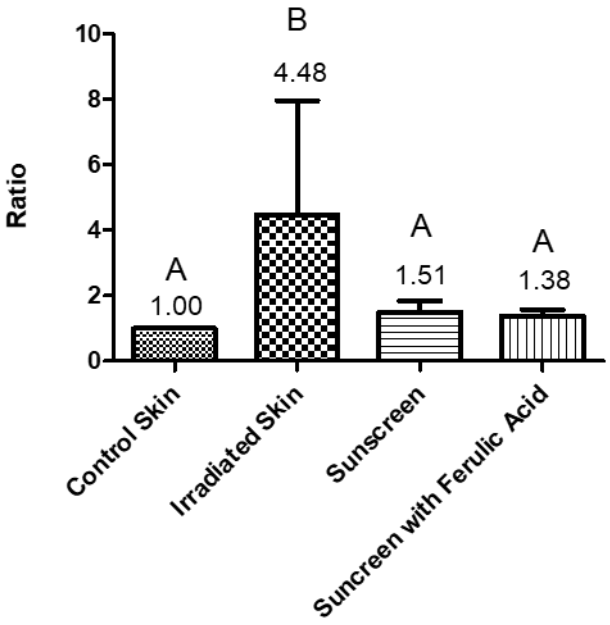

Figure 6. Ratio between values obtained at each sample site of the *ex vivo* TBARS assay
Different letters for the same parameter indicate statistically significant differences between samples (one-way ANOVA : * $p < 0.05$; n=10).

The results showed that the irradiated skin had a significant increase on the lipoperoxidation by MDA production compared to the control skin and both sunscreens (with or without ferulic acid). This was an interesting outcome result, since the formulations were in the same protection level as the control skin, the only one that not suffered irradiation.

This suggested that, for all samples, the lipoperoxidation was avoided by the photoprotection provided by bemotrizinol and ethylhexyl triazone, not allowing the initiation of the oxidative stress cascade phenomenon. It was expected that ferulic acid, as an antioxidant, could act in neutralizing lipid peroxidation, therefore decreasing its value compared to the sunscreen without it. However, ferulic acid probably did not act at that level since the UV filters already avoided the occurrence of oxidation by photoprotection. Both UV filters are photostable and have high efficacy. Bemotrizinol acts as broad spectrum absorption (UVB and UVA) with elevated molar absorptivity at 310 and 343 nm and ethylhexyl triazone acts in the UVB region, with high molar absorptivity at 314 nm (TUCHINDA et al., 2006; PERES et al., 2018). So, for further studies, its suggested that the ferulic acid was in a formulation without UV filters, testing its antilipoperoxidative potential.

4.3) Anti-inflammatory assay

Laser Doppler equipment is a noninvasive instrument used to measure tissue perfusion (VERTUANI et al., 2003). Laser Doppler flowmetry have been used to assess skin blood flow in clinical and experimental studies, offering an excellent temporal resolution of local skin blood flow changes caused by diverse variables (PETERSEN, 2013). Its functioning is through the emitted light (formed by Helion-Neon laser), in which it is dispersed and partially absorbed by the moving blood cells, suffering then changes in the wavelength (Doppler shift), while the light that reaches the static objects is not altered (FULLERTON et al., 2002). The magnitude and frequency distribution of these changes in wavelength are directly related to the number and velocity of blood cells in the sample volume. The information is captured by a feedback fiber, converted into an electronic signal and analyzed by PeriSoft® software, where blood perfusion is shown in real time throughout the procedure, expressed in arbitrary perfusion units (PU). Inflammatory mediators can be measured by

quantitative blood flow instruments, like Laser Doppler flowmetry, and have been used by clinical and experimental studies on inflammatory reactions (CRACOWSKI et al., 2006; HENRICSON et al., 2007). The main advantage is an equipment that can quantified inflammatory reaction through *in vivo* experiment, being a noninvasive, painless and fast method.

In the present study, three occlusive regions were analyzed on the forearm of volunteers: sunscreens with or without ferulic acid, as well as the control region (purified water). After, 0.5% of methyl nicotinate solution was applied to the regions to stimulate inflammatory reaction, so the measurement could be performed. The topical application of methyl nicotinate stimulates a fast vasodilatation of peripheral blood capillaries of the connective tissue bellow the epidermis (ELSNER; MAIBACH, 1991; JUMBELIC; LIEBEL; SOUTHALL, 2006).

It was measured five parameters: perfusion unit values, maximum perfusion unit value, area under the curve, angular coefficient and start time of perfusion unit increase. The comparison among the three regions (Table 5) was statistically analyzed by one-way ANOVA with GraphPad Prism® software version 5.0 (GraphPad® Software, Inc.).

Table 5. Laser Doppler flowmetry analysis of control, sunscreen and sunscreen with ferulic acid regions

Parameters	Control (water)	Sunscreen	Sunscreen with ferulic acid
Perfusion unit values (PU)	$94.45^{A} \pm 48.72$	$73.75^{A} \pm 23.44$	$76.84^{A} \pm 23.47$
Maximum perfusion unit value (PU)	$176.2^{B} \pm 68.11$	$138.5^{B} \pm 28.38$	$139.3^{B} \pm 34.42$
Area under the curve (unit.seconds)	$84490^{C} \pm 43340$	$66280^{C} \pm 21060$	$68650^{C} \pm 21220$
Angular coefficient	$0.5670^{D} \pm 0.3003$	$0.3567^{E} \pm 0.1177$	$0.2861^{E} \pm 0.09646$
Start time of perfusion unit increase (unit.seconds^{-1})	$116.0^{F} \pm 155.0$	$136.3^{F} \pm 143.3$	$123.3^{F} \pm 145.6$

All parameters were statistically analyzed by comparing the regions control and photoprotective formulations with and without ferulic acid by one-way ANOVA (n=13). Different letters in each row mean statistical difference found. Only the parameter "Angular Coefficient" resulted in statistical differences (*$p<0.05$).

The angular coefficient of both formulations had significant decrease value compared to the control region. It is known that water is a common penetration enhancer to the skin, raising its hydration and being used in occlusive clinical assays to potentiate drugs locally applied to cutaneous skin (MERWE; ACKERMANN, 1987; BOND; BARRY, 1988; LUNDBORG et al., 2018). So, probably, the high angular coefficient of the control (purified water) region could be explained by its enhancement of permeation, whereas both sunscreens have not had such action considering its physicochemical characteristics. In other words, the inflammatory effect of methyl nicotinate solution applied on the skin after the occlusion time were amplified, resulting in a higher value on the angular coefficient.

In addition, another evaluation was performed, this time only with both formulations, which the only variable between then was ferulic acid. Hence, if a difference were detected between then, it would be related to ferulic acid presence. The evaluation showed significative differences in the angular coefficient among the formulations, as seen in Figure 7.

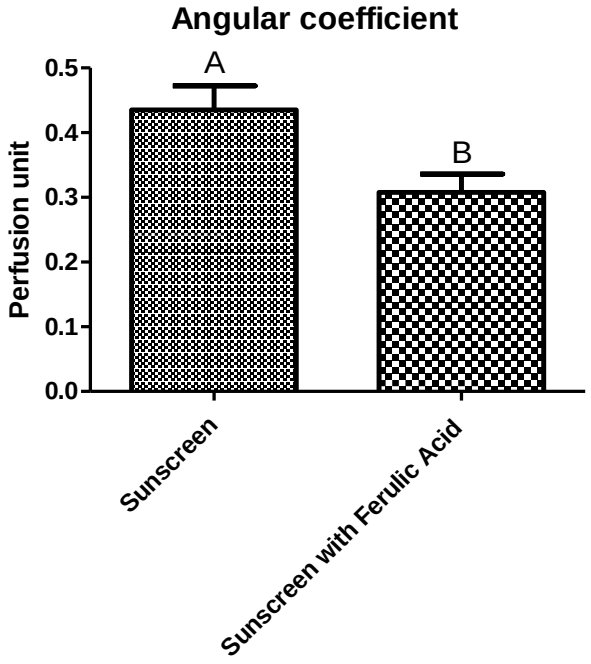

Figure 7. Bar graph containing the angular coefficient of sunscreens with or without ferulic acid, analyzed by the Laser Doppler flowmetry equipment. The parameters were statistically examined by Mann-Whitney U test (*p<0.05, n=13). Different letters in each row mean statistical difference found.

Therefore, it was concluded that ferulic acid was responsible for the decrease of the angular coefficient, mitigating the effects of inflammatory activity. This is a new result in the literature, being the first time reported the influence of ferulic acid with Laser Doppler flowmetry. It is well established that methyl nicotinate raises prostaglandin synthesis in skin cells, acting in the peripheral blood capillaries to cause vasodilatation (WILKIN et al., 1985; KATZMAN et al., 2003). The prostaglandin synthesis is a subproduct of COX enzymes in our body, which is responsible for the formation of important biological mediators called

prostanoids (like prostaglandins, prostacyclin and thromboxane) (RESLER et al., 2014). Studies have indicated that the ferulic acid could inhibit the activity of COX-2 enzyme, which triggers a pro-inflammatory activity (HOSODA et al., 2002; BOLLEDDULA JAYAPRAKASAM et al., 2006; NILE et al., 2016). Like seen in Figure 8, if the COX pathway is blocked, it would affect prostaglandins synthesis, consequently affecting inflammatory responses. So, it could be hypothesized that the formulation with ferulic acid had an anti-inflammatory effect detected by the Laser Doppler flowmetry assay because of its potential to decrease the COX-2 enzyme.

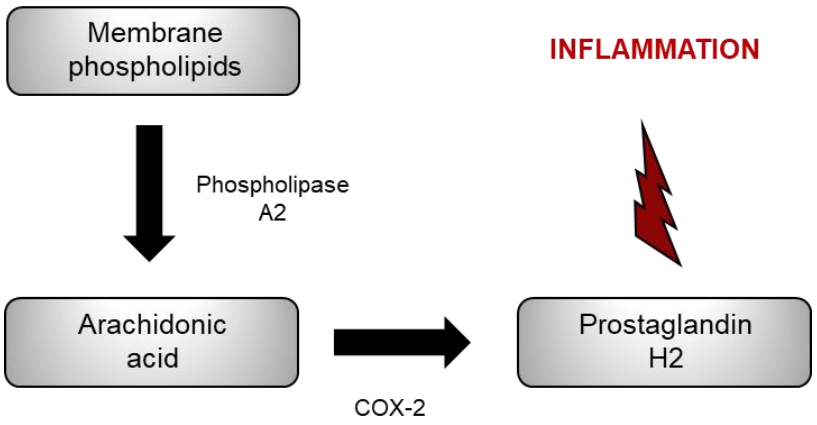

Figure 8. Inflammatory pathway (adapted from MARTEL-PELLETIER et al., 2003; RESLER et al., 2014)

Peres et al., 2018, in earlier studies of our research group, found that ferulic acid, in a sunscreen formulation, amplified the SPF *in vivo* in about 37% and UVA-PF in 26%. The results from this present work contribute with the explanation why this phenomenon occurred. The SPF evaluation *in vivo* is measured by the UVB energy required to produce a MED on protected skin of volunteers, divided by the UVB energy required to produce a MED on unprotected skin. In other words, substances that delay the formation of erythema on the skin could significantly support the UV photoprotection of sunscreens, like anti-inflammatory or antioxidant molecules. Indeed, the ferulic acid proved to have anti-

inflammatory activity, clarifying the SPF *in vivo* amplification, being an excellent molecule to be added into formulations.

Inflammatory differences in gender

In the inflammatory assay through Laser Doppler flowmetry equipment, it was measured parameters in both genders. Despite the objective of this work was not focused on differences in genders, the analysis of the results generated interesting information. Comparison between gender in the control region (purified water) was performed for all the parameters cited before, as seen on Table 6.

Table 6. Laser Doppler flowmetry analysis of control region between male and female

Parameters of control (water) region	Male	Female
Perfusion unit values (PU)	$54.33^{A} \pm 11.50$	$140.4^{B} \pm 33.99$
Maximum perfusion unit value (PU)	$114.5^{A} \pm 34.84$	$228.6^{B} \pm 64.50$
Area under the curve (unit.seconds)	$48893^{A} \pm 10441$	$125060^{B} \pm 30341$
Angular coefficient	$0.2191^{A} \pm 0.1229$	$0.7715^{B} \pm 0.2348$
Start time of perfusion unit increase (unit.seconds^{-1})	$109.0^{A} \pm 62.39$	$63.00^{A} \pm 30.33$

The parameters were statistically examined by Mann-Whitney U test (*$p<0.05$, $n=5$). Different letters in each row mean statistical difference found.

It was observed a difference between gender on almost all the inflammatory parameters, except for start time of perfusion unit increase. The female group had a higher value compared to the male group, suggesting that female has a more predisposition to

inflammatory activity. It is well established that sex differences have a major impact on inflammation (BERKLEY; ZALCMAN; SIMON, 2006). One of the main factors in the sexual dimorphism is the hormones, which affect different mechanism on cell signaling cascade (CANDORE et al., 2010; CASIMIR et al., 2010). The female hormone estrogen is able to recruit specific related cells like macrophages, neutrophils, monocytes and T cells, modulating the inflammatory responses in the body (CANDORE et al., 2010; CASIMIR et al., 2010). Leukotrienes is a mediator linked to inflammation, being its biosynthesis superior in female on cells responsible for its production, like neutrophils and monocytes (PACE et al., 2017). Also, androgens, driven mainly by males, could affect inhibitory effects on leukotrienes formation in human innate immune cells, consequentially decreasing its concentration compared to females (PERGOLA et al., 2011). However, the comprehension between genders is still not completely elucidated, wherein controversial information on the specialized literature is found. Nonetheless, considering its complexity, it could be justified, since inflammatory responses can be related to many other biological factors than sexual hormones.

In the present work, the result comparing inflammatory responses between male and female was clear. Females presented elevated perfusion unit values and maximum perfusion unit values, which meant more blood flow during the assay, a direct effect of inflammation; elevated area under the curve value in 15 minutes of assay, meaning more reactivity with methyl nicotinate; and elevated angular coefficient, which indicated that the inflammatory reaction occurred in a more intense way. This result could open path for more studies regarding gender differences, contributing to biological literature.

5. Conclusion

The evaluation of skin penetration of ferulic acid, ethylhexyl triazone and bemotrizinol by an *ex vivo* tape stripping method was performed with an unique HPLC method. Almost 50% of all compounds were found in the adhesive tapes quantified, suggesting that the remaining amounts penetrated deeper on the *stratum corneum*.

An exclusive protocol to measure lipid peroxidation assay through TBARS by *ex vivo* method, using HPLC, was developed. By testing sunscreens with and without ferulic acid, it was not observed differences between *stratum corneum* lipid peroxidation, whereas both formulations had a significative decrease in lipoperoxidative damage compared to the skin without any formulation (control). The protocol developed may be usefull in the efficacy of antioxidant agents in studies that aim to elucidate the level of lipid peroxidation caused by drugs and cosmetics, and even in carrying out baseline studies characterizing different ethnicities and genders. In addition, on the Laser Doppler flowmetry analysis, ferulic acid acted as an anti-inflammatory substance, mitigating the effects of inflammatory activity compared to the sunscreen formulation without this compound. This is a remarkable result, as its anti-inflammatory activity could explain the reason why the ferulic acid elevates the SPF *in vivo* of sunscreens, which is measured by the amount of generated erythema. Thus, difference in gender was found, where females had more inflammatory response compare to men, aiding in the specialized literature about inflammation gender.

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