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Ex vivo lipid peroxidation profile of the subjects' stratum corneum by the HPLC-TBARS-EVSC protocol

Perfil *ex vivo* da peroxidação lipídica do estrato córneo de participantes de pesquisa por meio do protocolo HPLC-TBARS-EVSC

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Comissão Julgadora da Dissertação para Obtenção do Título de MESTRE

Prof. Dr. André Rolim Baby

Orientador/Presidente

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1º Examinador

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2º Examinador

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3º Examinador

São Paulo, ___ de_____ de 2023.

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ABSTRACT

MARQUES, G. A. *Ex vivo* lipid peroxidation profile of the subjects' stratum corneum by the HPLC-TBARS-EVSC protocol. 2023. 49f. Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2023.

Background: Considering the need to improve safety and efficacy protocols to evaluate the human stratum corneum (CS) and its interaction with topical and cosmetic formulations by minimally or non-invasive methodologies, the objective of our research work was to improve the performance of the HPLC-TBARS-EVSC methodology (high performance liquid chromatography - thiobarbituric acid reactive substances - stratum corneum ex vivo), exploring the results of a group of subjects.

Methods: The study included 18 healthy subjects aged between 19-57 years old (9 females and 9 males) with phototypes from II to V. Two sites in the forearm of each volunteer were randomly delimited and the SC was collected by the tape stripping technique. High performance liquid chromatography (HPLC) was used to quantify the MDA-TBA_2 adduct from the tape-stripped SC, irradiated and not by an ultraviolet simulator chamber.

Conclusions: Observing the findings of our present investigation, and the statistical approach applied, the use of the ratio between the treatment site and control would be an adequate strategy to better discriminate and evaluate the results. Additionally, an optimal selection of the volunteers to respond specifically to the purpose of the *ex vivo* assay also can be considered advantageous. It seems that in future studies focusing on the impact of lipid peroxidation induced by UV irradiation, the most suitable subjects are females aged less than 35 years old, with phototype II.

Keywords: stratum corneum; lipid peroxidation; HPLC; phototype; TBARS

RESUMO

MARQUES, G. A. Perfil *ex vivo* da peroxidação lipídica do estrato córneo de participantes de pesquisa por meio do protocolo HPLC-TBARS-EVSC.2023. 49f. Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2023.

Introdução: Considerando a necessidade de melhorar os protocolos de segurança e eficácia para avaliar o estrato córneo humano (SC) e sua interação com formulações tópicas e cosméticas por metodologias minimamente ou não invasivas, o objetivo de nosso trabalho de pesquisa foi melhorar o desempenho da metodologia HPLC-TBARS-EVSC (cromatografia líquida de alta eficiência - substâncias reativas ao ácido tiobarbitúrico - estrato córneo *ex vivo*), explorando os resultados de um grupo de sujeitos.
Métodos: O estudo incluiu 18 indivíduos saudáveis com idades entre 19-57 anos (9 mulheres e 9 homens) com fototipos de II a V. Dois locais no antebraço de cada voluntário foram delimitados aleatoriamente e o SC foi coletado pela técnica de decapagem de fita. Cromatografia líquida de alta eficiência (CLAE) foi utilizada para quantificar o aduto MDA-TBA_2 a partir do SC despojado de fita, irradiado e não por uma câmara simuladora ultravioleta.
Conclusões: Observando os achados de nossa presente investigação e a abordagem estatística aplicada, a utilização da razão entre o local de tratamento e o controle seria uma estratégia adequada para melhor discriminar e avaliar os resultados. Ademais, uma seleção ideal dos voluntários para responder especificamente ao propósito do ensaio *ex vivo* também pode ser considerada vantajosa. Parece que em estudos futuros com foco no impacto da peroxidação lipídica induzida pela irradiação UV, os sujeitos mais adequados são mulheres com idade inferior a 35 anos, com fototipo II.

Palavras-chave: estrato córneo; peroxidação lipídica; CLAE; fototipo; TBARS.

SUMMARY

1. INTRODUCTION

The last years have witnessed incredible progress in the research and development of innovative bioactive and adjuvant ingredients for skin care, both in topical formulations and dermocosmetics. Nevertheless, to support such advances in health products for skin application, ex vivo and in vivo protocols must be developed, aiming at better evaluation of the safety and the efficacy of innovative formulations. Such methodologies must provide reliable and robust results, reinforcing the industry credibility and consumer care, as well as deepening the scientific knowledge about the human skin.

The skin is considered the largest organ of the human body, which has important functions such as physical barrier and, consequently, prevents water loss; and protection against foreign agents such as allergens, viruses and bacteria. It consists of three layers: the epidermis, the dermis and the hypodermis. (BERDYSHEV *et al*., 2018). The epidermis is the most superficial layer of the skin and is divided into sublayers: cornea, granulosa, prickly and basal, the first being the most superficial layer of the epidermis. This is formed by highly protein corneocytes, which are composed of a protein envelope and a lipid. It is a tissue of apolar character, therefore prevents the loss of water through the skin and allows the penetration of organic compounds selectively. It is a layer of low hydration rate in relation to the other layers of the epidermis (BARBOSA *et al*., 2011).

When there is an imbalance between oxidizing organic compounds and the antioxidant defense system, a process called oxidative stress occurs. This imbalance generates excessive production of free radicals, leading to the oxidation of biomolecules of the body, causing the loss of function of these biomolecules and a homeostatic imbalance, which promotes a potential oxidative damage in the cells and tissues of the human body (BARBOSA *et al*., 2010). This process can cause lesions in the lipid bilayer of the skin, causing its lipid peroxidation, which begins with the sequestration of hydrogen ion from the cell membrane, resulting in the formation of a lipid radical. It will react with oxygen and form the peroxila radical, which will then sequestest hydrogen from polyunsaturated fatty acid, resulting, again, in a lipid radical. This cycle ends when the lipid radicals produced multiply until they destroy themselves (ENGERS *et al*., 2011).

For the quantification of oxidative stress, a better method has not yet been defined, but the most used methods are those that use lipid peroxidation products as biomarkers to measure injuries caused by stress (SILVEIRA *et al*., 2015). A widely used method is the TBARS method, which uses the malondialdehyde biomarker (MDA), derived from lipid

peroxidation, which reacts with thiobarbituric acid (TBA), forming a fluorescent-colored compound that can be detected in spectrophotometry or by high-efficiency liquid chromatography (HPLC). It is a simple, easy, low-cost method, but not adequately specific, yet widely used for this purpose (GROTTO *et al*., 2008).

Recently, a protocol was developed to assess the stratum corneum (tape stripped) oxidative stress after exposure to ultraviolet (UV) radiation. Denominated by Sauce and coworkers as HPLC-TBARS-EVSC (high performance liquid chromatography - thiobarbituric acid reactive substances - ex vivo stratum corneum), a liquid chromatography analytical tool was associated to the TBARS assay to measure ex vivo the lipoperoxidation (lipid peroxidation) grade of the subjects' stratum corneum. The assay is based in the quantification (HPLC) of the MDA-TBA_2 (malondialdehyde - thiobarbituric acid) aduct in the tape stripped stratum corneum after UV artificial irradiation (SAUCE *et al*. 2021a).

Tape-stripping is an *ex vivo* technique used to analyze the efficacy and quality of topical formulations and other evaluations of the properties of the stratum corneum. The collection is performed through adhesive tapes directly in contact with the skin, to use it for measurement of lesions caused by oxidative stress (LADEMANN *et al*, 2009; PERES, 2015; OLIVEIRA, 2015; ALONSO *et al.*, 2009).

Also, several topical and cosmetic formulations are designed to provide added protection against oxidative stress damage, especially sunscreens and anti-ageing products. One of the challenges that is often found in the efficacy testing of such products is that the correlation between the results obtained in vitro and the in vivo/ in use scenario is poor. Additionally, the intersubject and intrasubject variability is an inevitability when conducting ex vivo and in vivo studies in human volunteers (OLIVEIRA *et al*., 2016).

2. JUSTIFICATION

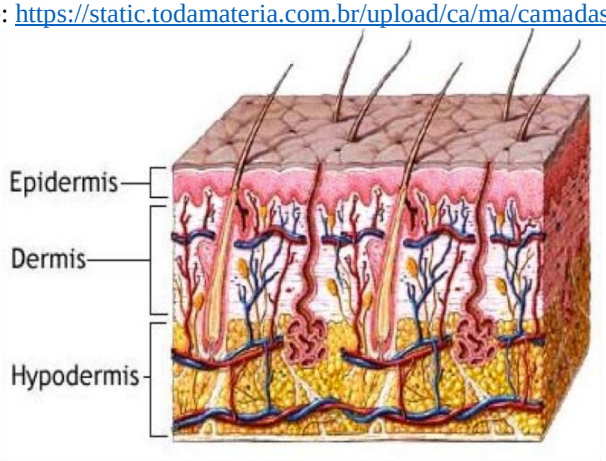
According to data from the Brazilian Institute of Geography and Statistics (IBGE, 2022), the Brazilian population, following a global trend, has been aging at an accelerated pace due to the increase in life expectancy in recent decades resulting from the concomitant decrease in mortality and birth rates. This process has triggered a greater search for quality of life and physical, emotional and social well-being during aging. This search has fostered the rapid advance in research and technological information related to the development of new anti-aging products and methods that prove their effectiveness, in universities and industries of the pharmaceutical and cosmetic sector, aiming to meet the avid market. Although aging is a natural, gradual and universal process, it is assumed that it occurs differently between men and women of different ages, according to their genetic, physiological and lifestyle characteristics. The skin, being an exposed organ subject to environmental damage, is an ideal marker of aging, and the quantification of its oxidative stress is an indirect way to ascertain such a process. Thus, it can be said that investing in the development of dermocosmetics and topical products that can specifically combat oxidative stress and lipid peroxidation of the skin of different niches of individuals, would be a way to delay skin aging significantly. However, studies and development of methods and/or new protocols to establish, in a non-invasive and safe way, the effectiveness of such products in order to meet the growing demand of the current market are still scarce and insufficient. In this sense, the present work contributes to the investigation of how age, gender and phototype of the skin can influence the skin aging process by measuring the level of lipid peroxidation of the stratum corneum removed by tape stripping of the skin of each group of donors, using the TBARS method coupled to HPLC. The results obtained may collaborate with the future selection of volunteers for efficacy tests of new anti-aging products, involving the skin oxidative stress data generated here.

3. LITERATURE REVIEW

3.1 Skin

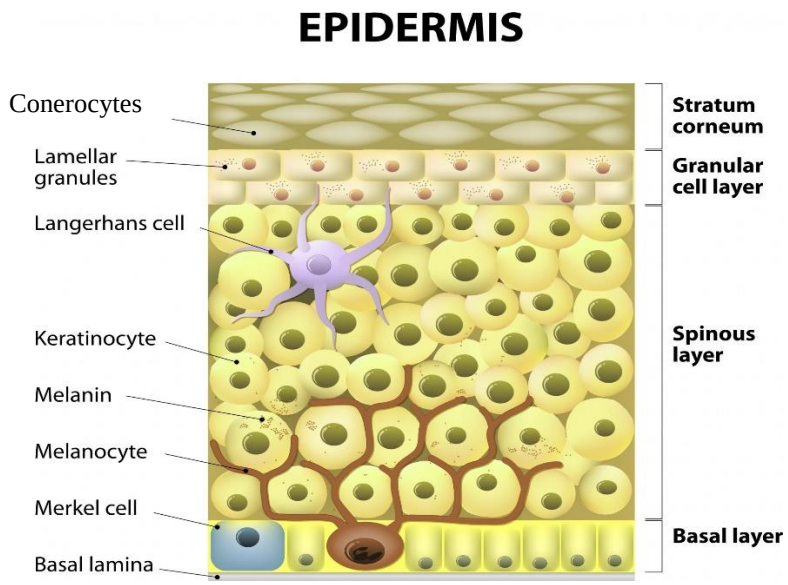
The skin is considered the largest organ of the human body, with coating function and protecting other organs. It is a sensitive, versatile and dynamic fabric, with high capacity of interaction with the external environment (VIRCHOW *et al.*, 2009). The skin consists of three layers: the epidermis, the dermis and the hypodermis. The first is the most superficial layer of the skin, followed by the dermis and after the hypodermis. **Figure 1** illustrates the layers of the skin.

Figure 1. Main structures that make up the skin.
Source: https://static.todamateria.com.br/upload/ca/ma/camadasdapele.jpg



The epidermis has sublayers: cornea layer, granulosa layer, spinous layer and basal layer. They are covered by keratin, being called keratinized stratified epithelial tissue (BARBOSA *et al.*, 2011). The most superficial layer of the epidermis is the cornea or stratum corneum. It is formed by highly protein corneocytes, which are composed of a protein envelope and a lipid. It is a tissue of non-polar character, therefore, prevents the loss of water through the skin and allows, selectively, the penetration of organic compounds, in addition to having the functions of protective barrier against external substances supposedly harmful to the body. It is a layer of low hydration rate compared to the other (BARBOSA *et al.*, 2011; GU *et al.*, 2020). **Figure 2** shows the layers of the epidermis.

Figure 2. Layers of the epidermis.
Source: https://static.todamateria.com.br/upload/ce/lu/celulasdaepiderme.jpg



3.2 Differences between skin properties as a function of gender, age and phototype

3.2.1 Gender

The skin has a high capacity to interact with the external environment and its appearance and properties may depend on this medium, genetics and hormones, the latter being directly related to gender. Male and female skin do not differ essentially until puberty. At this stage, it is possible to notice essential differences in the male and female skin, as hormonal changes begin to occur. The male skin becomes thicker, there is greater dilation of the pores, the sebaceous glands are larger than those of the female skin are and have a greater amount of collagen. Due to the testosterone present in males, there is the growth of greater amount of body hair (PEREIRA *et al*., 2018). Hormones called estrogen and progesterone influence the female skin. The former is able to decrease the loss of collagen from the skin and can increase the water retention capacity of the epidermal barrier. Estrogen is also able to increase skin vascularity by increasing its superficial irrigation, which does not occur on the male skin, which does not have the hormone in question (PEREIRA, 2009).

In a study conducted by Rahrovan *et al.* (2018), it was found that male produce more sebum in several parts of the face, except on the forehead, where the highest level of sebum belongs to the female. In this same study, it was concluded that collagen loss is responsible for increasing skin thickness in both men and women. In the case of males,

the thickness of the skin decreases constantly from the age of 20, while in females, it remains the same until the age of 50, when it begins to decrease.

3.2.2 Age

The main factor of influence of age on the skin is skin aging. Both male and female skin suffer this influence, and skin aging can be intrinsic or extrinsic. Intrinsic aging occurs not only in the skin, but also in all organs of the human body, because it is caused by factors that are independent of the external environment, such as genetics and hormones, in addition to the gradual loss of collagen suffered by men and women over the years (RAHROVAN *et al*., 2018). The common characteristics of intrinsic aging are the appearance of wrinkles, expression lines, dryness, and increased sensitivity to temperature, thinning of the skin and decreased sweating (GU *et al*., 2020). Extrinsic aging, on the other hand, occurs by factors directly linked to the external environment, such as air pollution, smoking, and especially exposure to sunlight, which is known as photo aging (KOOHGOLI *et al*., 2017). The main cause of photo aging is UV radiation (UVA, UVB and UVC), from the sun's rays, which are responsible for sunburn, and for increasing the chance of developing skin cancers, when very exposed to the sun (RITTIE *et al*, 2015). In addition, UV radiation produces ROS's (reactive oxygen species), which lead to skin oxidative stress, accelerating skin aging (GU *et al*., 2020). Both extrinsic and intrinsic aging cause damage to the physiological function of the skin, making it dry and more likely to develop serious skin diseases such as melanoma (GU *et al*., 2020).

One of the main effects of skin aging is the dryness of this organ. Estrogen, for example, causes, in the female skin, increased mucopolysaccharide acid and hyaluronic acid, which are substances with an important role in the hydration of the skin and, as age progresses, women gradually lose the amount of hormone in the body, causing gradual tissue dryness (PEREIRA, 2009). Because it is an exposed organ, the skin is subject to damage caused by the external environment, which are the major responsible for extrinsic aging. On the other hand, intrinsic aging occurs mainly by flattening the dermoepidermal junction, decreasing the superficial contact between the epidermis and the dermis, however, the thickness of the epidermis remains the same over the years, while that of the dermis gradually decreases after reaching eighty years of age. Another contributing factor to skin aging is the gradual loss of dermal collagen. Factors such as fibroblast

reduction, reduction of mast cells and blood vessels, shortened capillary loops and abnormal nerve endings promote decreased collagen synthesis types I and III, which reduces dermal cell adtake. These changes usually begin from the age of seventy (ORTOLAN *et al*., 2013).

3.2.3 Phototype

The main difference of skin phototypes is due to a protein called melanin, which is produced by melanocytes, located in the epidermal region of the skin, more specifically in the basal layer. Melanin is the substance responsible for the coloration and tone of the skin, making it lighter or darker according to its quantity and quality, and plays an important role in the filtration of ultraviolet (UV) rays harmful to the skin. Skin tone may also be related to external factors, such as exposure to sunlight, which can impair skin health, depending on exposure time and wavelength. There are three types of UV radiation: UVA (320 and 400 nm), UVB (290 to 320 nm) and UVC (100 to 290 nm), the first of which can cause changes in the genetics of the body's cells and the second is the cause of sunburn, photoaging and melanomas (MOTA, 2006). Currently, the Fitzpatrick classification, 1976, is used to differentiate skin phototypes, ranging from I (lighter skin) to VI (darker skin). Type I represents individuals who burn easily in the sun and never tan, type II represents individuals who burn easily and tan with difficulty, and type III individuals who burn moderately and also tan moderately with 60 minutes of exposure to the stronger sun (GUPTA *et al*, 2019). **Table 1** observes Fitzpatrick's classification in relation to skin phototypes.

Table 1. Fitzpatrick classification of skin phototypes.

Source: Fonte: (GERALDO *et al.*, 2016)

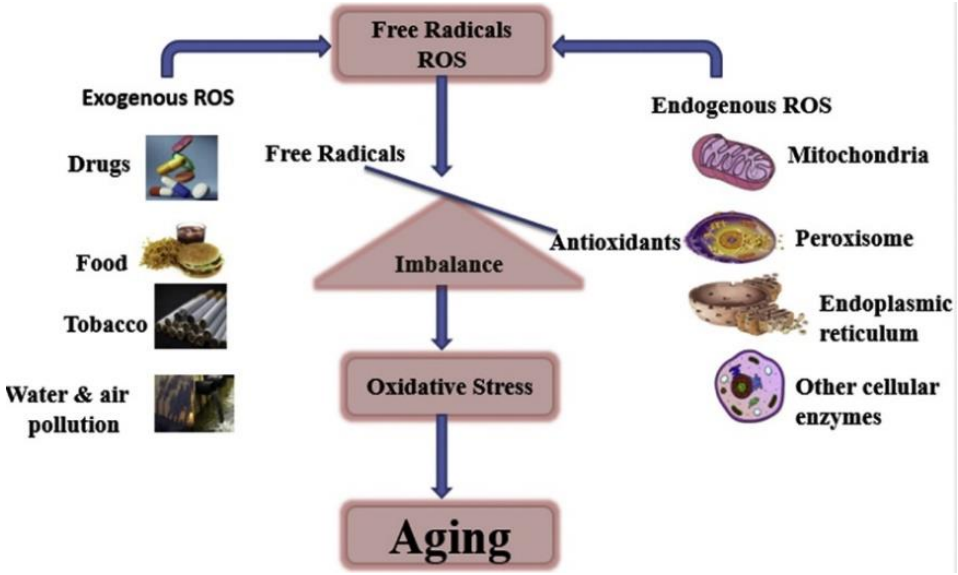
Phototypes - Fitzpatrick Classification				
Phototype	**Skin tone**	**UV sensitivity**	**Erythema**	**Suntan**
I	White	Very sensitive	Always	Never
II	White	Very sensitive	Always	Sometimes
III	White to light dark	Sensitive	Moderate	Moderate
IV	Light dark	Moderately sensitive	Little	Always
V	Dark	Minimally sensitive	Rare	Always
VI	Black	Insensitive or less sensitive	Never	Rarely, due to hyperpigmentation

3.3 Cutaneous oxidative stress and its implications

Oxidative stress is a process resulting from the imbalance between oxidizing organic compounds and the antioxidant defense system. This imbalance generates excessive production of free radicals, causing oxidation of biomolecules of the organism, causing the loss of function of these biomolecules and a homeostatic imbalance, which leads to a potential oxidative damage in the cells and tissues of the human body, as can be seen in **Figure 3**. Such consequences can become chronic and result in some diseases such as atherosclerosis, diabetes, obesity, neurodegenerative disorders and cancers (BARBOSA *et al*., 2010; KATERJI *et al*., 2019).

Figure 3. Oxidative stress process.

Source: (WARRAICH *et al.*,2020)



In order for this imbalance not to occur and oxidative stress to occur, the amount of ROS's must be controlled by enzymatic and non-enzymatic antioxidant defense mechanisms. An antioxidant molecule is able to prevent the oxidation of macromolecules, thus slowing down the process of excess formation of free radical chains (DUARTE; LUNEC, 2005). Some antioxidant agents can be found in foods or plants, such as vitamins C and E, however, the human body contains powerful enzymatic and non-enzymatic antioxidant agents.

The enzymatic antioxidant agents of the body consist of enzymes such as catalases, superoxide dismutases (SOD) and glutathione peroxidases (GPX), which play an important role in maintaining cellular homeostasis, reducing the excessive production of free radicals, responsible for oxidative stress (AZAB et al., 2019). As for non-enzymatic antioxidant agents, one can cite reducing agents such as polyphenols and thiols, which are often used as a defense against oxidative stress in the body (MOSIALOU et al., 1993). In addition, ascorbic acid is able to reduce ROS, vitamin E acts as an antioxidant protector of cells, preventing the excess formation of free radicals, and beta-carotene, which is a protective antioxidant agent against excessive free radicals. These molecules can be found in the diet and in plants (LINSTER et al., 2006; SEN et al., 2006).

Free radicals from oxidative stress come from endogenous and exogenous factors. Major endogenous factors include air pollution, chemicals such as medicines, toxins and cosmetics, gases harmful to human health, solar radiation and infections by pathogens such as viruses and bacteria (EMANUELLI *et al*., 2022). Among the endogenous factors, oxygen is the main generator of free radicals, so much so that these are formed mainly by mitochondria, which produce water with oxygen reduced in stages (WARRAICH *et al*., 2020). The enzymatic activities of the organism can also contribute indirectly to the formation of ROS's, which, in excess, can accelerate aging and cause inflammation in the skin, generating damage to DNA, proteins and lipids, which can lead to serious diseases such as skin cancer (EMANUELLI *et al*., 2022).

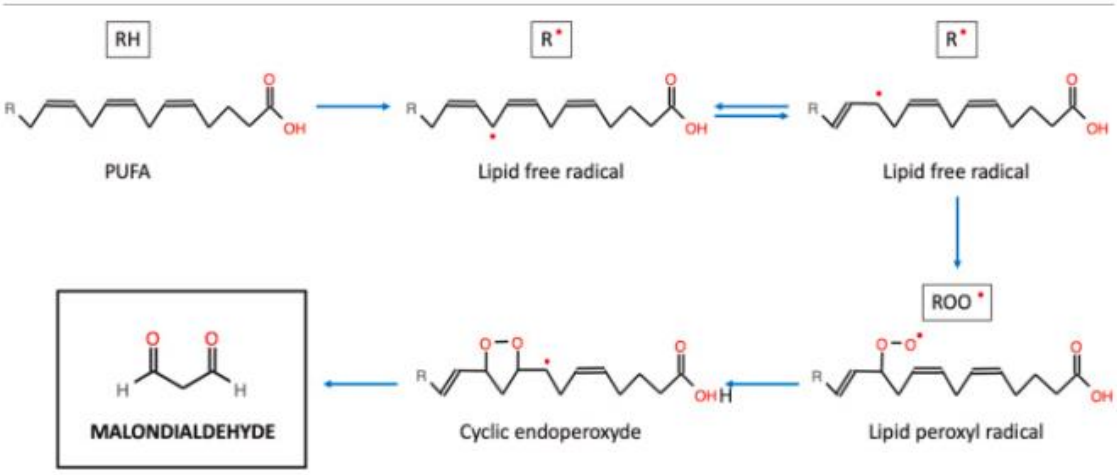
To avoid the action of exogenous factors, it is important that there is a good cellular functioning, and this is directly linked to cell membranes. These are covered by a lipid bilayer, and when free radicals formed by the oxidative stress process act on this bilayer, an oxidative lesion occurs, called lipid peroxidation. This process begins with the sequestration of the hydrogen ion from the cell membrane, resulting in the formation of a lipid radical. It will react with oxygen and form the peroxila radical, which will then sequest the hydrogen from polyunsaturated fatty acid, resulting, again, in a lipid radical. This cycle is concluded when the lipid radicals produced multiply until they destroy themselves (ENGERS *et al*., 2011). These processes, such as excessive formation of free radicals and consequent lipid peroxidation, lead to changes in permeability, fluidity and integrity of cell membranes and even cell death. Free radicals are important for the proper functioning of cell signaling and for the defense against microorganisms, besides also acting as meters for electron transfer in numerous biochemical reactions of the organism. However, excessive production of it can result in oxidative stress and its consequent lesions, such as lipid peroxidation and its consequences for the body, including the skin (FRANÇA *et al*., 2013).

3.4. Methods for quantifying oxidative stress

To date, there have been no conclusions about which is the best, most reliable and specific method for detecting different types of oxidative stress, but studies have been conducted on markers and methods that are the most used to quantify it. Typically, products formed by lipid peroxidation are the most used for the measurement of oxidative

lesions caused by oxidative stress (SILVEIRA *et al*., 2015; SAUCE *et al*., 2021a). One of the methods involves the use of the biomarker called malondialdehyde (MDA). This is a secondary product of lipid peroxidation, derived from the beta-rupture of endocyclization of polyunsaturated fatty acids. Currently, MDA is used as a biomarker for oxidative damage in plasma. The method used with this biomarker is called the TBARS method, which consists of the reaction between MDA and thiobarbituric acid (TBA), as shown in **Figure 4**. The result of this reaction is a fluorescent pink chromogen, which can be made in spectrophotometry and whose absorption occurs at wavelength 532 nm and fluorescence at 553 nm. The TBARS method is simple, easy to perform and has low cost in relation to other methods, but is nonspecific for MDA, and can interact with other substances, such as sugars, amino acids, proteins, amines and bilirubin. To reduce the possibility of cross-contamination due to its lack of specificity, the technique can be adapted with the combination of the compound formed by the MDA-TBA reaction by means of high-performance liquid chromatography (HPLC) or HPLC coupled to mass spectrometry (LC-MS/MS) (FRANÇA *et al*., 2013; SAUCE *et al*., 2021a).

Figure 4. Formation of MDA through lipid peroxidation.
Source: (MAS-BARGUES *et al*., 2021).



Another method used to quantify oxidative stress is the detection of isoprostans, products that can also be used as biomarkers. They are secondary derivatives of lipid peroxidation, chemically stable, and can be even dosed in the urine. Among the advantages of this biomarker, its stability and the fact that isoprostans are considered specific products of lipid peroxidation, and can be easily detected in any fluid or body

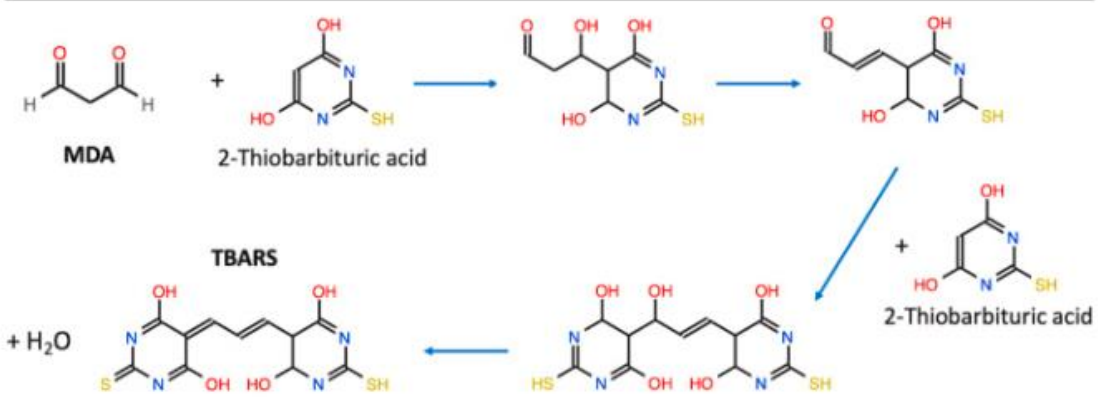
tissue. The methods used for the detection of isoprostans are radioimmunoassays, enzyme immunoassays (both with low specificity, as there may be cross-contamination with other substances), and chromatographic separation associated with mass spectrometry (specific technique, but difficult to perform). The most used methods are radiometric and immunological methods due to greater convenience (JANICKA *et al*., 2010). Another similar method with the detection of isoprostans is the detection of hydrocarbons, such as ethane and pentane, also derived from lipid peroxidation, but these are more volatile. The most used method for the detection of these compounds is chromatography associated with mass spectrometry, using the technique of detecting the concentration of hydrocarbons present in expired air. To this end, there are several techniques that differ according to the form of collection, storage and processing of samples. For this reason, there are variations in the results of the studies of these samples, which may be cross-contaminated with ethane and pentane present in the air (FRANÇA *et al*., 2013; ZHANG *et al*., 2021).

Another test is that of orange in xylene, which consists of measuring the compound hydroperoxide, the primary product of lipid peroxidation. For this measurement, the xylenol-orange compound is used. Hydroperoxides oxidize iron II to iron III and this, reacting with xylenol-orange, will form a chromophore whose maximum absorption occurs at 560 nm wavelength. This method can be used with hydroperoxides in their liquid or lipid forms, it is simple, there is no use of expensive and sophisticated appliances, but it is not specific (ARAB *et al.,* 2004). Finally, another method used to quantify oxidative stress is high-efficiency liquid chromatography (HPLC). It can be with chemiluminescence detection or by association with mass spectrometry. The first is used in the measurement of lipid hydroperoxides, being a specific method, without interference of other substances and of high sensitivity. The second is recent, however, has advanced in recent years. Among its advantages, include the separation, quantification and structural elucidation of the substances in the samples continuously, without the need for purification, making the method faster and more efficient. There is the possibility of analyzing nonvolatile or thermolabile substances, in addition to being a technique of high sensitivity and specificity (KALINOVIC *et al*., 2019).

3.5 TBARS Method

TBARS is a method of quantifying oxidative stress with the use of the malondialdehyde biomarker (MDA), which consists of the reaction of MDA with thiobarbituric acid (TBA) that can be detected in spectrophotometry or HPLC. The acronym TBARS is associated with reactive substances of thiobarbituric acid (GROTTO *et al*., 2008). In conditions of high temperature and acidity for a long period of time, an MDA molecule will react with two TBA molecules forming the compound MDA-TBA2, which is a visible and fluorescent light absorber, and is therefore used for measurement of MDA by spectrophotometry coupled to high-efficiency liquid chromatography, as shown in **Figure 5** (MAS-BARGUES *et al*., 2021).

Figure 5. MDA-TBA$_2$ reaction.
Source: (MAS-BARGUES *et al*., 2021).



4. OBJECTIVES

The objective of this research work was to improve the HPLC-TBARS-EVSC (high performance liquid chromatography – thiobarbituric acid reactive species – *ex vivo* stratum corneum) *ex vivo* protocol for the determination of the lipid peroxidation profile of the stratum corneum of participants with focus on the gender, age and skin phototype. Additionally, as a perspective, this exploration will enable the identification of future participants' characteristics that are better suited to this methodology and also the optimization of the statistic treatment to present the results in a more significative approach.

5. MATERIAL AND METHODS

5.1. Legal and ethical concerns, and subjects' selection

Our study design included 18 healthy subjects aged between 19-57 years old (mean age = 32.4 ± 14.7 years old), 9 females and 9 males, with phototypes from II to V. The selected volunteers participated of the experiment after receiving all information needed to clarify the trial, being the written consent given by means of its signature. The participant anonymity as well as the departure of the study (at any time) were fully guaranteed. The investigation was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, University of São Paulo, USP, and was in accordance with the principles of the Helsinki Declaration (CAAE: 61716316.4.0000.0067) (ALONSO *et al*., 2009; SAUCE *et al*., 2021a, 2021b). Beforehand to the beginning of the experiment, specifically to ensure the reliability of the tape-stripping procedure, the participants were instructed to not apply any topical product in the volar forearm for 24 hours before the study.

5.2. Tape stripping procedure and sample preparation

Two sites in the volar forearm of each volunteer were randomly delimited as rectangle areas (5.0 x 2.0 cm) compatible with the dimensions of the adhesive tape (Scotch Magic TM Tape, 3M) (SAUCE *et al*., 2021b; ZIOSI *et al*., 2006). The skin was cleansed with dry cotton and the stratum corneum was collected from each site by the tape stripping technique (BENFELDT *et al*., 2007). A sum of six consecutive adhesive tapes per region were used for the stratum corneum removal, being the first tape discarded. The stratum corneum removal process was performed with constant and uniform pressure applied by the researcher.

Samples from one site were used as the control (non-irradiated) and the others were artificially irradiated. Adapted from Alonso and coworkers, the tapes were irradiated in a solar simulator chamber at 5506 $KJ.m^{-2}$ (irradiance of 765 $W.\ m^{-2}$ with temperature control of, approximately, 35 ^{o}C; and 2 hours of UV exposition / 290-400 nm) (ALONSO *et al*., 2009). The UV simulator chamber was an Atlas Suntest CPS+ (Atlas Material Testing Technology, Mount Prospect, IL, USA) equipped with a xenon lamp (1500 W) and a filter that allowed the passage of wavelengths above 290 nm.

The five stratum corneum-containing tapes obtained in each site were transferred to falcon tubes (separately for the control and for the irradiated tapes) after which the sample extraction was performed by adding 10.0 mL of methanol followed by vortex agitation for 1 minute and filtration in 0.22 μm syringe filters. From each falcon tube, a filtrated aliquot of 2.0 mL was added to 288 μL of 0.2% methanolic BHT and of 800 μL H_3PO_4 (0.44 M in ultrapure water) in glass tube that was vortexed for 1 minute. In the sequence, a volume of 1200 μL of TBA (thiobarbituric acid) at 0.6% in 0.44 M H_3PO_4 was added to previous sample, being the agitation once provided by a vortex for 1 minute. From this experimental step, samples were treated in low luminosity due to the TBA sensitivity to the light. After the last agitation process, the tube was heated at 90 ºC in a thermostated bath for 45 minutes. By the end of heating time, samples were left resting to achieve room temperature and 1200 μL of n-butanol was added to it. Vortex agitation was repeated. Later, the sample was centrifuged at 3000 G for 10 minutes. The supernatant was collected and filtered in a syringe filter for further HPLC quantification of the MDA-TBA_2 adduct (ALONSO *et al*., 2009; BASTOS *et al*., 2012; HONG *et al*., 2000; SAUCE *et al*., 2021b).

5.3. HPLC-TBARS-EVSC analytical protocol (chromatographic conditions)

The HPLC analysis was performed in a Shimadzu Prominence (Kyoto, Japan) composed with SPD-M20A diode array detector and CTO-20A column oven (30 ºC). The C18 column (250 x 4.6 mm, 5 mm particle size, Shimadzu, Kyoto, Japan) was preceded by a pre-column (10 x 4.6 mm). We applied an isocratic mode with mobile phase composed of 35.0% methanol and 65.0% phosphate buffer (pH 7.0). The flow rate was established at 1.0 $mL.min^{-1}$ with sample injection of 40 μL. The diode array detector was set at 532 nm (BASTOS *et al*., 2012; SAUCE *et al*., 2021b).

5.4. Mathematical and statistical approach

Participants were categorized according to sex, age and phototype. The area under the curve (AUC) of the MDA-TBA_2 adduct for the non-irradiated and the irradiated stratum corneum was our main variable of interest. The ratio of irradiated/non-irradiated AUC was calculated, giving a measure of the impact of UV exposure per individual participant.

To assess differences of the AUC of the MDA-TBA_2 adduct for the non-irradiated and the irradiated stratum corneum between categories, the t-test for independent samples or the Mann-Whitney test were used, following the use of the Shapiro-Wilk test for the normal distribution. Repeated-Measures ANOVA was used to assess the differences of non-irradiated and irradiated AUC across multiple categorical independent variables. Linear regression was used to assess the association of independent variables with the ratio of AUC. Descriptive and inferential statistics was performed in jamovi 2.3.21 (jamoviproject, Sydney, AU). A significance level of 5% (p<0.05) was used for all the tests (MARQUES *et al.,* 2023).

6. RESULTS

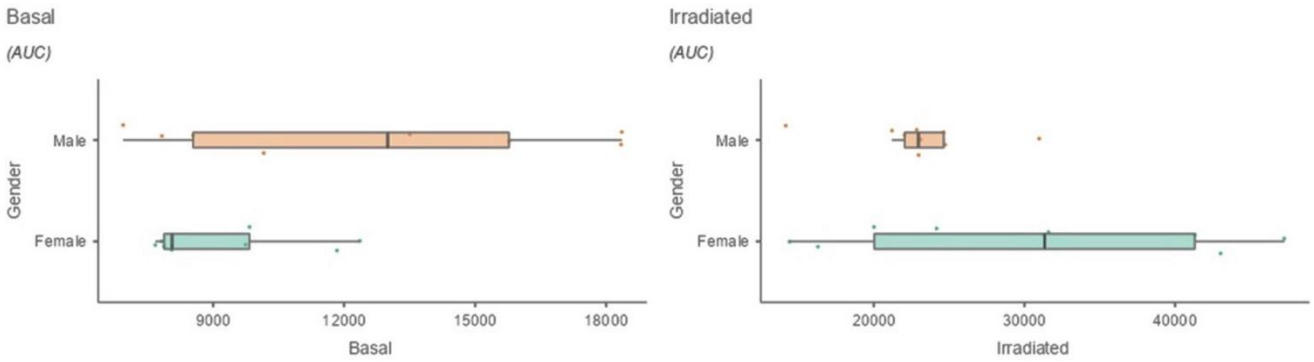
Table 2 describes age (years old, and categorical variable), phototype and area under the curve (AUC) of the MDA-TBA_2 adduct quantified by HPLC for the non-irradiated and irradiated stratum corneum samples from the 18 subjects by sex. No differences were found at baseline in the variables of interest. However, the Ratio of AUC was significantly different between the two sexes (p=0.028). In male volunteers, the AUC of the irradiated stratum corneum samples was increased 2-fold in comparison to the non-irradiated; while in female volunteers the increase was more than 3-fold. Additionally, observing the distribution of non-irradiated and irradiated AUC data by sex (**Figure 6**), another difference is noticeable: at baseline, males had a bigger dispersion of AUC data, while after irradiation data are less dispersed. The inverse appears to happen with females

Table 2. Sex, age (years), phototype and area under the curve of the MDA-TBA_2 adduct for the non-irradiated and the irradiated stratum corneum samples obtained from the subjects.

Variables of interest	**Male (n)**	**Female (n)**	***p*-value**
Age (Median, Q1-Q3)	26 (19-45)	25 (23-36)	0.755
18-35 y.o	45.5% (5)	54.5% (6)	0.629
>35 y.o	57.1% (4)	42.9% (3)	
Phototype			
II	26.6% (2)	71.4% (5)	0.410
III	66.7% (4)	33.3% (2)	
IV	50.0% (2)	50.0% (2)	
V	100% (1)	0	
Non-irradiated stratum corneum (AUC)* (Mean ± sd)	12493.7 (4392.2)	9251.7 (1809.6)	0.113
Irradiated stratum corneum (AUC)* (Mean ± sd)	22959.2 (4361.0)	29939.61 (12067.3)	0.258
Ratio AUC (Mean ± sd)	2.06 (0.87)	3.22 (1.14)	0.028

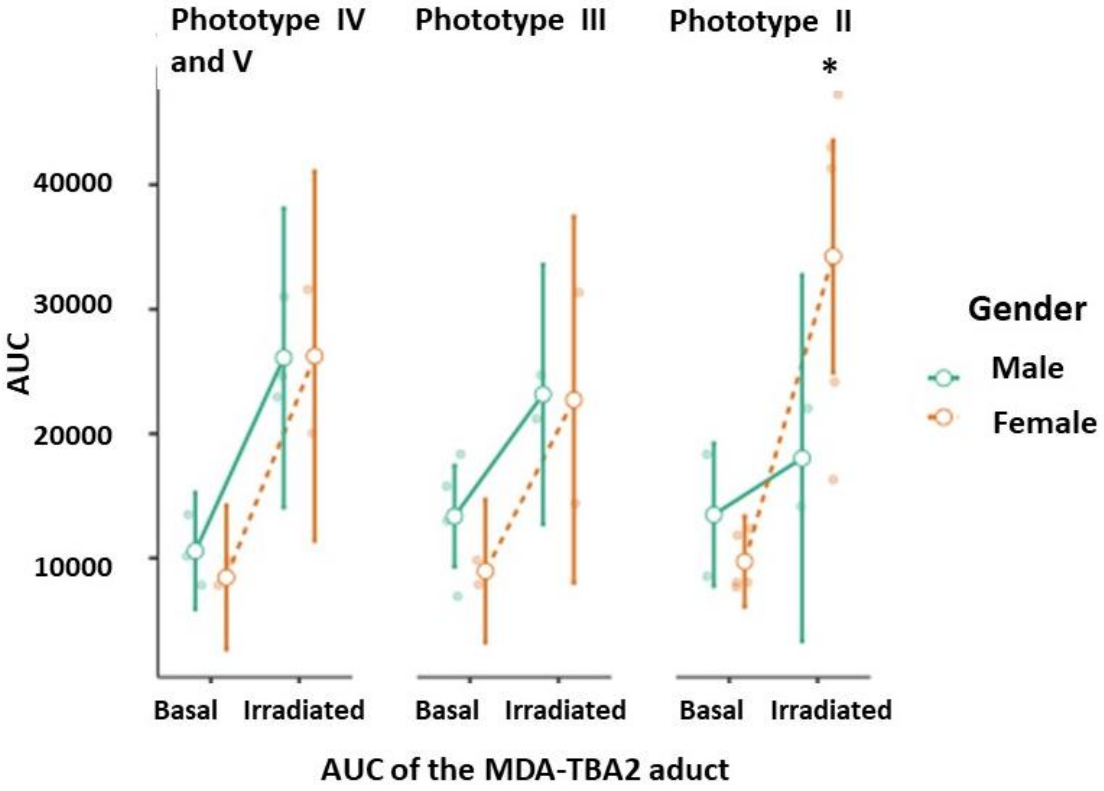
* The adduct was quantified by HPLC and expressed as the area under the curve (AUC). Sd is standard deviation.

Figure 6. Non-irradiated (Basal) and Irradiated AUC data distribution across sexes.



Considering the influence of phototype (**Figure 7**), only females with phototype II showed significant differences between non-irradiated and irradiated AUC (p=0.026). To assess how the independent variables were associated with the Ratio AUC, a linear regression model was performed. This multivariable model would assess the simultaneous contribution of the independent variables sex, age category and phototype to the outcome Ratio AUC. No significant association was found, but females, aged less than 35 years old, and with phototype II had a slightly higher likelihood of presenting increased Ratio AUC (β = 1.07 [-0.036; 2.18], p=0.057).

Figure 7. Non-irradiated (Basal) and Irradiated AUC differences by sex and phototype (* p=0.026, Repeated Measures ANOVA with the Scheffe correction).



7. DISCUSSION

The development and/or improvement of ex vivo and in vivo protocols specially targeted for the stratum corneum and aiming to non-invasively assess the safety and efficacy of new active ingredients and formulations is of utmost importance. These methodologies can ensure better products to the consumers and can also contribute with advances in non-explored skin properties.

The literature has reported the use of the TBARS method to evaluate the oxidative stress in the outermost layers of the human stratum corneum obtained ex vivo by tape stripping (ALONSO *et al*., 2009; SAUCE *et al*., 2021b). Alonso and coworkers evaluated the efficacy of 2 antioxidant emulsions in tape-stripped stratum corneum and quantified the lipoperoxidation extent after UV exposure by TBARS assay through spectrophotometry at 534 nm. The ex vivo experimental protocol was performed in 10 healthy female volunteers, being the overall phototype and age ranges of II to IV and 28-54 years old, respectively. Volunteers pre-treated the specific forearm area with the respective sample during 7 days, twice a day. At the 7^{th} day, the tape stripping technique was used to obtain the stratum corneum and part of the stripped biological sample was irradiated in a solar simulator (ALONSO *et al*., 2009).

Sauce and coworkers developed a protocol to be applied in the skin research, using tape stripped stratum corneum, associating the HPLC analytical method with the TBARS test (HPLC-TBARS-EVSC). By this protocol, the authors investigated the protective capacity against stratum corneum lipoperoxidation of a sunscreen formulation composed of 5.0% of ethylhexyl triazone and 10.0% bemotrizinol vehiculated in an oil-in-water emulsion. UV artificial irradiation was generated by a solar simulator (Suntest CPS+). Prior to quantification of the lipoperoxidation in the samples, the linearity and selectivity of the analytical method was established. The HPLC was in the isocratic mode, being the mobile phase composed of methanol and phosphate buffer. A diode detector at 532 nm was used to quantify the MDA-TBA_2 adduct. This study was also conducted on 10 healthy volunteers, however, the age ranged from 18 to 70 years old and the interval of the phototypes was II to V (SAUCE *et al*., 2021b). Subjects' forearms were pre-treated with the formulation for 2 hours and, after this period of time, the stratum corneum was tape-stripped and irradiated.

The analysis of the lipoperoxidation of biological samples (gingival crevicular fluid, kidney, stratum corneum, liver, plasma, lung etc.) using the TBARS test throughout

the quantification of the MDA-TBA_2 adduct is predominantly performed by spectrophotometry, since it is a simple low-cost analytical method. Nevertheless, TBA is able to react with distinct molecules, for instance, aldehydes (saturated and unsaturated) and several other pigments (BASTOS *et al*., 2012; MATEOS *et al*., 2005). Thus, developing and/or ameliorating a selective analytical tool with the elimination of interferents, such as molecular entities in biological samples or even more complex compounds, like foods, is suitably justified (BASTOS *et al*., 2012; SAUCE *et al*., 2021b). The HPLC method, as corroborated by Bastos and coworkers, has improved the selectivity of the quantification of the MDA-TBA_2 adduct, a chromogen, since the analytical tool is efficient to separate it from the other chromogens (BASTOS *et al*., 2012).

As mentioned in the introduction, one of the biggest challenges associated to ex vivo and in vivo studies is the high inter and intra-variability. This hindrance can be minimized if the data is analyzed using each subject as its own control, i.e., by examining putative differences in the ratios of the results obtained in the treated versus the control site. Several works conducted by our group have employed this strategy with satisfactory results, particularly taking into account that the panels of volunteers were relatively small (between 12-15 subjects) (OLIVEIRA *et al*., 2016; CÂNDIDO *et al*., 2022; MOROCHO-JÁCOME *et al*., 2022; SARRUF *et al*., 2020; OLIVEIRA *et al*., 2015; PERES *et al*., 2016). This study can also be considered as a corroboration of the advantages of using this approach, since the subtle differences between the two sexes were observed in the AUC ratios and not in the mean AUC. Moreover, using the AUC ratios as a dependent variable, it allows to use linear regression models to assess the simultaneous association of multiple independent variables to the outcome of interest.

In another study conducted by our group, where the data, treated as percentage, was used to describe the findings of the adduct formation in the non-irradiated and irradiated samples of tape stripped stratum corneum, the HPLC-TBARS-EVSC protocol did not adequately respond to its main objective, i.e., to find a sensitive manner to present robust and reliable results (GONÇALVES, 2019). This work challenged the capacity of the UVB filters ethylhexyl methoxycinnamate, ethylhexyl salicylate and octocryene, all isolated and incorporated into an oil-in-water emulsion, in preventing the lipid peroxidation of UV irradiated tape-stripped stratum corneum. Results were expressed as the mean $\pm$ standard deviation of the adduct concentration (μM) and, among all samples,

no differences of the lipoperoxidation grade of the stratum corneum were found, even from the blank formulation, free of UVB filters.

The current investigation aimed to fine-tune the HPLC-TBARS-EVSC protocol and provide further insights into how sex, age and phototype of the volunteers could impact the results. It included 9 males and 9 females, as volunteers and no age limit or specific phototype was considered as exclusion criteria, hence, the age range and phototypes presented in our sample. Regarding sex differences, a more significant increase in the AUC of the stratum corneum samples after irradiation was found in female volunteers, where the average increase was more than 3-fold. Interestingly, males had a bigger dispersion of AUC data at baseline, while in female volunteers this dispersion increased after irradiation. A noteworthy impact of female hormones on the blend of stratum corneum ceramides has been reported in the literature (DENDA *et al.*, 1993), which could explain these results. Considering the influence of phototype, significant differences between non-irradiated and irradiated AUC were only observed in females with phototype II. Additionally, when a multivariable model was used to assess the simultaneous contribution of the independent variables sex, age category and phototype to the Ratio AUC, no significant association was found, but females in the younger group with phototype II had a slightly higher likelihood of presenting increased Ratio AUC. These results could be attributable to the synergic effect of the female ceramide profile and the lower melanin content, since in a study conducted with epidermal reconstructs, following UV, in low phototype melanocytes an increased lipid oxidative damage was found (CARIO-ANDRE *et al.*, 2005).

8. CONCLUSIONS

Observing the findings of our present investigation, and the statistical approach applied, the use of the ratio between the treatment site and control would be an adequate strategy to better discriminate and evaluate the results. Additionally, an optimal selection of the volunteers to respond specifically to the purpose of the ex vivo assay also can be considered advantageous. Among our universe of subjects, it seems that in future studies focusing on the impact of lipid peroxidation induced by UV irradiation the most suitable subjects are females aged less than 35 years old, with phototype II.

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ATTACHMENTS

- **SCIENTIFIC ARTICLE:** SU, Chin Yi; MOROCHO-JACOME, Ana Lucia; LIMA, Fabiana Vieira; MARQUES, Gabriela Argollo; RODRIGUEZ, Wendi Lucia Avila; JULCA, Claudia Alejandra Cruz; ROBLES, Esther del Rocio Abanto; ROSADO, Catarina; VELASCO, Maria Valeria Robles; BABY, André Rolim. *In vitro* water resistance evaluation of a bioactive sunscreen containing distinct film/ barrier-forming agents. **Biomedical And Biopharmaceutical Research Journal**, v. 17, n. 2, p. 1-14, out. 2020. http://dx.doi.org/10.19277/bbr.17.2.241.

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In vitro water resistance evaluation of a bioactive sunscreen containing distinct film/ barrier-forming agents

Avaliação in vitro da resistência à água de um fotoprotetor bioativo contendo diferentes filmes / agentes formadores de barreira

Chin Yi Su[1], Ana Lucía Morocho-Jácome[1], Fabiana Vieira Lima[1,2], Gabriela Argollo Marques[1], Wendi Lucia Avila Rodriguez[1], Claudia Alejandra Cruz Julca[1], Esther del Rocio Abanto Robles[1], Catarina Rosado[3], Maria Valéria Robles Velasco[1], André Rolim Baby[1]

[1]Department of Pharmacy, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil
[2]Health Sciences Department, Faculty of Pharmacy, Federal University of Espírito Santo, Brazil
[3]CBIOS – Universidade Lusófona's Research Center for Biosciences and Health Technologies, Lisbon, Portugal

*corresponding author: andrerb@usp.br

Abstract

The use of functional bioactive ingredients and the property of water resistance are differentials in the choice of more effective and safer sunscreens. Water-resistance tests are expensive, time-consuming, and usually performed on subjects that expose them to irradiation and long immersion times. Thus, the study of *in vitro* water resistance using different film/ barrier-forming agents is relevant for obtaining sunscreen resistant to rinsing. We aimed to evaluate the water resistance of a rutin-based bioactive sunscreen containing distinct film/barrier-forming agents by an *in vitro* method. The *in vitro* water resistance assessment (% WRR) was carried out in a water bath. *In vitro* sun protection factor (SPF), critical λ (nm), UVA/UVB ratio, and UVA protection factor (UVA-PF(0)) were evaluated before and after rinsing using a diffuse reflectance spectrophotometer with integration sphere. The formulation with dimethicone showed higher values of SPF after rinsing and achieved the requirement of % WRR greater than 50%. All formulations showed similar variations for all other parameters. The results highlighted the property of water resistance provided by the dimethicone, indicating that this emollient is an interesting ingredient choice for sunscreens.

Keywords: Bioactive photoprotection, water resistance, film/barrier formation

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CHAPTER

10

Cosmetics applications

Andressa Costa de Oliveira[a], *Ana Lucía Morocho-Jácome*[a], *Cibele Ribeiro de Castro Lima*[b], *Gabriela Argollo Marques*[a], *Maira de Oliveira Bispo*[a], *Amanda Beatriz de Barros*[a], *João Guilherme Costa*[c], *Tânia Santos de Almeida*[c], *Catarina Rosado*[c], *João Carlos Monteiro de Carvalho*[d], *Maria Valéria Robles Velasco*[a], *André Rolim Baby*[a]

[a]Department of Pharmacy, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil, [b]Physics Institute, University of São Paulo, São Paulo, Brazil, [c]CBIOS—Universidade Lusófona's Research Center for Biosciences and Health Technologies, Lisbon, Portugal, [d]Department of Biochemical and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil

1 Introduction

Skin is considered the major organ in the human body and its principal function is protecting body against the external environment (e.g., microorganisms and other harmful invasive agents). The more superficial skin layer is stratified to act as a protective barrier to retain water and prevent dehydration and dryness. When the *stratum corneum* is compromised, it results in transepidermal water loss (TEWL) and gradual skin dehydration (Berardesca, Loden, Serup, Masson, & Rodrigues, 2018).

The appeal for products from natural origins has been growing dramatically in the last decade, leading the cosmetic industry to look for more natural and sustainable ways to develop cosmetic products. Thus algae extracts are promising and valuable in the cosmetic market (Khanra et al., 2018). Moreover, microalgae have compounds that can be used in the manufacture and development of cosmetics with particular beneficial properties for the prevention or treatment of skin aging (Ariede et al., 2017).

Only about 10% of microalgae species are identified and described, but a few species are produced at an industrial level (i.e., *Dunaliella salina*, *Haematococcus*, *Arthrospira*, *Chlorella*, *Aphanizomenon*) (Couteau & Coiffard, 2018). Moreover, only a very few macroalgae species have been cultivated for industrial purposes

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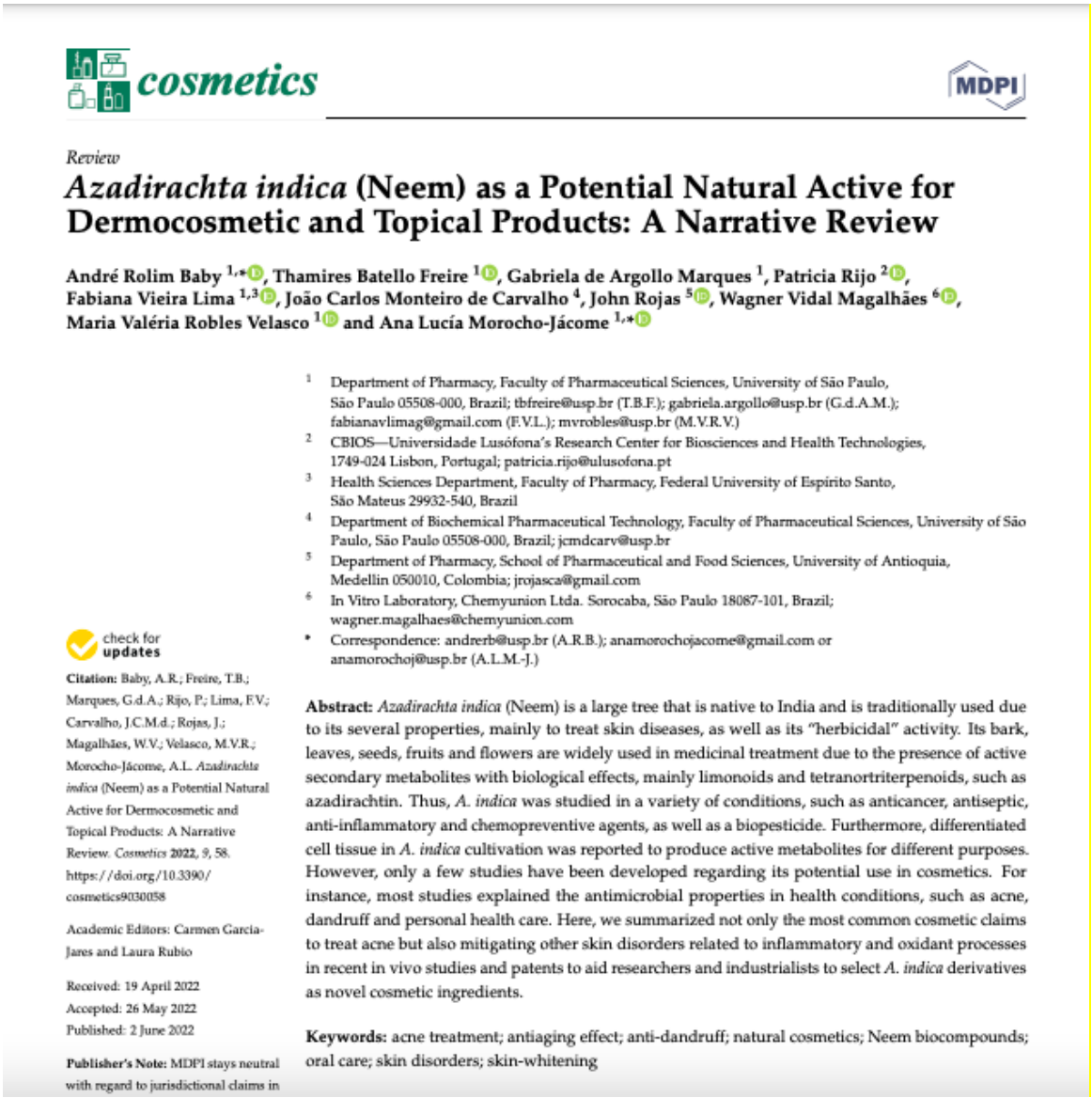

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Review

Azadirachta indica (Neem) as a Potential Natural Active for Dermocosmetic and Topical Products: A Narrative Review

André Rolim Baby [1,*], Thamires Batello Freire [1], Gabriela de Argollo Marques [1], Patricia Rijo [2], Fabiana Vieira Lima [1,3], João Carlos Monteiro de Carvalho [4], John Rojas [5], Wagner Vidal Magalhães [6], Maria Valéria Robles Velasco [1] and Ana Lucía Morocho-Jácome [1,*]

[1] Department of Pharmacy, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo 05508-000, Brazil; tbfreire@usp.br (T.B.F.); gabriela.argollo@usp.br (G.d.A.M.); fabianavlimag@gmail.com (F.V.L.); mvrobles@usp.br (M.V.R.V.)
[2] CBIOS—Universidade Lusófona's Research Center for Biosciences and Health Technologies, 1749-024 Lisbon, Portugal; patricia.rijo@ulusofona.pt
[3] Health Sciences Department, Faculty of Pharmacy, Federal University of Espírito Santo, São Mateus 29932-540, Brazil
[4] Department of Biochemical Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo 05508-000, Brazil; jcmdcarv@usp.br
[5] Department of Pharmacy, School of Pharmaceutical and Food Sciences, University of Antioquia, Medellin 050010, Colombia; jrojasca@gmail.com
[6] In Vitro Laboratory, Chemyunion Ltda. Sorocaba, São Paulo 18087-101, Brazil; wagner.magalhaes@chemyunion.com
* Correspondence: andrerb@usp.br (A.R.B.); anamorochojacome@gmail.com or anamorochoj@usp.br (A.L.M.-J.)

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Abstract: *Azadirachta indica* (Neem) is a large tree that is native to India and is traditionally used due to its several properties, mainly to treat skin diseases, as well as its "herbicidal" activity. Its bark, leaves, seeds, fruits and flowers are widely used in medicinal treatment due to the presence of active secondary metabolites with biological effects, mainly limonoids and tetranortriterpenoids, such as azadirachtin. Thus, *A. indica* was studied in a variety of conditions, such as anticancer, antiseptic, anti-inflammatory and chemopreventive agents, as well as a biopesticide. Furthermore, differentiated cell tissue in *A. indica* cultivation was reported to produce active metabolites for different purposes. However, only a few studies have been developed regarding its potential use in cosmetics. For instance, most studies explained the antimicrobial properties in health conditions, such as acne, dandruff and personal health care. Here, we summarized not only the most common cosmetic claims to treat acne but also mitigating other skin disorders related to inflammatory and oxidant processes in recent in vivo studies and patents to aid researchers and industrialists to select *A. indica* derivatives as novel cosmetic ingredients.

Keywords: acne treatment; antiaging effect; anti-dandruff; natural cosmetics; Neem biocompounds; oral care; skin disorders; skin-whitening