UNIVERSIDADE DE SÃO PAULO
FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Programa de Pós-Graduação em Fármacos e Medicamentos
Área de Produção e Controle Farmacêuticos

Development, safety and efficacy of sunscreen systems composed of rosmarinic acid, bemotrizinol and octyl *p*-methoxycinnamate

Desenvolvimento, segurança e eficácia de sistemas fotoprotetores compostos por ácido rosmarínico, bemotrizinol e *p*-metoxicinamato de octila

Thalita Marcílio Cândido

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

São Paulo

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ABSTRACT

CÂNDIDO, T.M. **Development, safety and efficacy of sunscreen systems composed of rosmarinic acid, bemotrizinol and octyl *p*-methoxycinnamate.** 2023. 53 f. Thesis (PhD) – Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2023.

Progressively growing diagnoses of skin cancer trigger public and public health concerns about excessive sun exposure, awareness of the deleterious effects of ultraviolet (UV) radiation on the skin, and the proper use of sunscreens. Studies show that bioactive molecules, with special attention to phenolic compounds, such as rosmarinic acid (RA), may act to potentiate the photoprotective and antioxidant activity of cosmetic formulations. Through the review paper in Article 1, it is shown that, due to the range of methodologies available to verify skin conditions, it was possible to prove the effects of supplementation on the skin. A study involving supplementation with rosmarinic acid demonstrated its effects in protecting the skin against UVB rays, increasing skin elasticity and decreasing the depth of wrinkles; results attributed to the antioxidant properties of this molecule. Regarding the topical use of rosmarinic acid, the concepts of quality by design (QbD) were successfully used to design the formulations. From the results of the sun protection factor (SPF) and antioxidant activity, *in vitro*, prediction equations involving rosmarinic acid, bemotrizinol and octyl *p*-methoxycinnamate were obtained. It was verified that the presence of rosmarinic acid increased the antioxidant activity of the formulations, while decreasing the SPF. An increase in the photostability of the formulation containing the two sunscreens was also observed for the rosmarinic acid. Four samples were chosen for the assessment of comet assay and skin biocompatibility. Also, the evaluation of anti-inflammatory activity *in vivo* using laser doppler flowmetry was performed. Through comet assay, all samples protected DNA fragmentation, however, we did not attribute this finding to the presence of rosmarinic acid exclusively. By the *in vivo* tests, none of the formulations caused an alteration of the pH value of the skin surface or an increase in redness. All formulations provided increase in skin hydration after 30 minutes of application. However, only the formulations without rosmarinic acid provided increase in skin hydration after 7 days of application. An increase in transepidermal water loss (TEWL) was observed for F1, F2 and F4. We may conclude that both results (skin hydration and TEWL) were related to the composition of the vehicle containing or not the RA. In respect of the *in vivo* evaluation of anti-inflammatory efficacy, no decreases were observed in the inflammatory reaction caused by ethyl nicotinate, however, the sample containing rosmarinic acid presented an increase in the tangent value. In view of the specialized literature and our results, we may suggest that new *in vivo* tests should be carried out, with modifications in the protocol of application of the samples on the skin and even the investigation of other vehicles for the active ingredients.

Keywords: rosmarinic acid, QbD, antioxidant activity, photoprotection.

RESUMO

CÂNDIDO, T.M. **Desenvolvimento, segurança e eficácia de sistemas fotoprotetores compostos por ácido rosmarínico, bemotrizinol e *p*-metoxicinamato de octila.** 2023. 53 f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2023.

Os diagnósticos de câncer de pele, em crescimento progressivo, desencadeiam a preocupação da população e órgãos públicos de saúde com o excesso da exposição solar, conscientização dos efeitos deletérios da radiação ultravioleta (UV) na pele e com uso devido de protetores solares. Estudos revelam que moléculas bioativas, com especial atenção aos compostos fenólicos, como o ácido rosmarínico, podem atuar potencializando a atividade fotoprotetora e antioxidante de formulações cosméticas. Por meio do artigo de revisão (Artigo 1), mostra-se que, devido à variedade de métodos disponíveis para verificar as condições da pele, foi possível comprovar os efeitos da suplementação na pele. Um estudo envolvendo a suplementação com ácido rosmarínico demonstrou seus efeitos na proteção da pele contra os raios UVB, aumentando a elasticidade da pele e diminuindo a profundidade das rugas; resultados atribuídos às propriedades antioxidantes desta molécula. Em relação ao uso tópico do ácido rosmarínico, os conceitos de qualidade por design (QbD) foram usados com sucesso para projetar as formulações. A partir dos resultados do fator de proteção solar (FPS) e atividade antioxidante, *in vitro*, foram obtidas equações de predição envolvendo ácido rosmarínico, bemotrizinol e *p*-metoxicinamato de octila. Verificou-se que a presença do ácido rosmarínico aumentou a atividade antioxidante das formulações, enquanto diminuiu o FPS. Um aumento na fotoestabilidade da formulação contendo os dois protetores solares também foi observado para o ácido rosmarínico. Quatro amostras foram escolhidas para a avaliação do ensaio cometa e biocompatibilidade da pele. Além disso, foi realizada a avaliação da atividade anti-inflamatória *in vivo* por meio de fluxometria a laser doppler. Através do ensaio cometa, todas as amostras protegeram a fragmentação do DNA, porém, não atribuímos esse achado à presença exclusiva do ácido rosmarínico. Pelos testes *in vivo*, nenhuma das formulações causou alteração do valor do pH da superfície da pele ou aumento da vermelhidão. Todas as formulações proporcionaram aumento na hidratação da pele após 30 minutos de aplicação. Porém, apenas as formulações sem ácido rosmarínico proporcionaram aumento na hidratação da pele após 7 dias de aplicação. Um aumento na perda de água transepidérmica (TEWL) foi observado para F1, F2 e F4. Podemos concluir que ambos os resultados (hidratação da pele e TEWL) estiveram relacionados com a composição do veículo contendo ou não o AR. Com relação à avaliação *in vivo* da eficácia anti-inflamatória, não foram observadas reduções na reação inflamatória causada pelo nicotinato de etila, porém, a amostra contendo ácido rosmarínico apresentou aumento no valor da tangente. Diante da literatura especializada e de nossos resultados, podemos sugerir que novos testes *in vivo* sejam realizados, com modificações no protocolo de aplicação das amostras na pele e até mesmo a investigação de outros veículos para os ingredientes ativos.

Palavras-chave: ácido rosmarínico, QbD, atividade antioxidante, fotoproteção.

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Introduction

1. INTRODUCTION

The contact of the sun radiation with the skin is responsible for important benefits for humans, such as the feeling of well-being and comfort, improved sleep quality – since the perception of sunlight acts on the circadian rhythm; and in the synthesis of vitamin D – which plays an important role in the quality of bone structure. However, the occurrence of skin cancer cases reflects the concern with unprotected and unplanned sun exposure (BURNETT & WANG, 2011; MORABITO et al., 2011).

The sun emits an electromagnetic spectrum in the ultraviolet (UV) range, with wavelength between 100 and 400 nm. According to the wavelength, the spectrum of UV radiation is subdivided into three bands: UVA, UVB and UVC. UVA radiation is the one that has the longest wavelength, from 320 to 400 nm, and is an inducer of oxidative processes. Such radiation maintains its intensity practically constant throughout the day, being subdivided into UVA I (less erythematous and wavelength between 340 and 400 nm) and UVA II (more erythematous and photosensitizing and wavelength between 320 and 340 nm). UVB is partially filtered by the ozone layer and has wavelength between 290 and 320 nm. This radiation is responsible for direct damage to the DNA molecule, erythema, stratum corneum thickening, melanogenesis and photoimmunosuppression. UVC rays have the shortest wavelength (100 to 290 nm), are carcinogenic and contain the peak absorption of pure DNA. This radiation is absorbed by the ozone layer. However, with its reduction, UVC radiation has been getting closer to the atmosphere, in the same way that UVB radiation has been showing a higher incidence. UVA radiation is the only one whose incidence does not depend on the ozone layer and, consequently, causes skin cancer in individuals who are continuously exposed to the sun radiation. In view of the damage caused by the incidence of solar radiation on the skin, the use of photoprotectors has become crucial in preventing and reducing the risk of skin cancer, whether melanoma or non-melanoma (GASPARRO, 2000; FDA, 2007; ARAÚJO & SOUZA, 2008).

Concern about sun exposure has shown exponential growth due to the increasing number of skin cancer cases. In Brazil, skin cancer is the most frequent, according to data from the José Alencar Gomes da Silva National Cancer Institute (INCA) and its occurrence is closely related to unprotected sun exposure, either due

to the absence of adequate clothing or the non-application of photoprotective cosmetics (INCA, 2018).

Considering the damage caused by the incidence of solar radiation on the skin, the use of photoprotectors has become essential in preventing and reducing the risk of skin cancer (INCA, 2022). Photoprotective formulations are developed with the incorporation of a chemical (organic) filter or a physical (inorganic) one, and both may also be incorporated in the formulation. The chemical structure of organic filters makes it possible to absorb UV rays. UV radiation excites the chemical filter molecule that reaches a state of resonance, therefore, there is the conversion of high energy radiation into more innocuous radiation, as in the form of heat. Inorganic filters, such as titanium dioxide and zinc oxide, form a barrier on the skin that can reflect and disperse UV radiation (PALM & O'DONOGHUE, 2007; SEITÉ & FOURTANIER, 2008)

Hypersensitivity reactions can be related to the presence of organic filters in photoprotective formulations. In this way, the search for bioactive molecules that can act by absorbing UV radiation becomes interesting for the dermocosmetic market. The use of these molecules in association with already known and widely used UV filters may reflect the use of lower concentrations of those to obtain sun protection factor (SPF) values as high as when large concentrations of UV filter are used. Furthermore, it is known that bioactive molecules can provide dual activity to formulations, due to the antioxidant potential that some of them may have. In this category, we can mention rosmarinic acid (FERNANDO et al., 2016; SEITÉ & FOURTANIER, 2008; VELASCO et al., 2008).

Rosmarinic acid, (R)-1-carboxy-2-(3,4-dihydroxyphenyl) ethyl ester of 3,4-dihydroxycinnamic acid), is an ester of caffeic and 3,4-dihydroxyphenylacetic acids, which can be found in plant species, mainly in rosemary (*Rosmarinus officinalis*). The molecular structure of the bioactive molecule is represented by **Figure 1** (FERNANDO et al., 2016; LEE, KIM, & PARK, 2007).

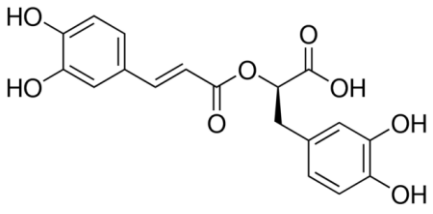

Figure 1 – Molecular structure of rosmarinic acid ($C_{18}H_{16}O_8$).

Rosmarinic acid has several biological activities, acting as an antiviral, antibiotic, anti-inflammatory and antioxidant molecule. Recent studies have shown that rosmarinic acid is able to delay vitamin E depletion, decrease the production of pro-inflammatory lysophosphatidylcholine, and prevent LDL oxidation. Fernando et al., 2016, even demonstrated that this phenolic acid was able to attenuate cellular damage against oxidative stress induced by UVB radiation by increasing the antioxidant effects on keratinocytes (FERNANDO et al., 2016; SÁNCHEZ-CAMPILLO et al., 2009). In view of the molecular structure of rosmarinic acid and its antioxidant properties, this molecule becomes a candidate in the development of multifunctional photoprotective formulations.

Based on the above, the relevance of researching molecules is verified, especially those of plant origin, with properties that provide multifunctionality and that can act in the improvement of photoprotective cosmetic formulations. Furthermore, it is understood the need for the rational development of formulations that ensure representative statistical analises of the effects and interactions of the components of a formulation on the desired critical quality attributes. Thus, the use of rosmarinic acid in the development of photoprotective formulations is justified, as well as the investigation of their safety, photoprotective and antioxidant potential, skin biocompatibility and anti-inflammatory activity.

Objectives

2. OBJECTIVES

This research aimed to evaluate the use of rosmarinic acid in a sunscreen formulation optimized by QbD, in association with octyl *p*-methoxycinnamate and bemotrizinol.

2.1. Specific objectives

(i) Development and optimization of photoprotective formulations, containing two UV filters commonly used in the market and the bioactive compound (Article 2).

(ii) Evaluation of the functional properties of the formulations through the analysis of *in vitro* photoprotective efficacy, photostability and antioxidant activity (Article 2).

(iii) *In vitro* evaluation of DNA fragmentation from human cells exposed to UV radiation by the comet assay method (Article 3).

(iv) Evaluation of the skin biocompatibility and anti-inflammatory activity of the formulations, *in vivo* (Article 3).

Publications

3. PUBLICATIONS

The Thesis was organized in accordance with three published papers, derived from this investigation work:

- Article 1 (“Dietary supplements and the skin: focus on photoprotection and antioxidant activity”): a review of dietary supplements, also involving rosmarinic acid, and their effects on human skin, published at **Nutrients** (ISSN: 2072-6643).
- Article 2 (“Prospecting *in vitro* antioxidant and photoprotective properties of rosmarinic acid in a sunscreen system developed by QbD containing octyl *p*-methoxycinnamate and bemotrizinol”): a full-length article about the influence of rosmarinic acid in the photoprotective and antioxidant activity of sunscreen formulations, *in vitro*, published at **Cosmetics** (ISSN: 2079-9284).
- Article 3 (“Rosmarinic acid multifunctional sunscreen: comet assay and *in vivo* establishment of cutaneous attributes”): a full-length article about the safety and efficacy of multifunction prototype sunscreens containing rosmarinic acid, octyl *p*-methoxycinnamate and bemotrizinol, published at **Cosmetics** (ISSN: 2079-9284).

Article 1

Dietary supplements and the skin: focus on photoprotection and antioxidant activity

Nutrients

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nutrients

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Review

Dietary Supplements and the Skin: Focus on Photoprotection and Antioxidant Activity—A Review

Thalita Marcílio Cândido [1], Maíra Bueno Ariede [1], Fabiana Vieira Lima [1], Luciana de Souza Guedes [1], Maria Valéria Robles Velasco [1], André Rolim Baby [1,*] and Catarina Rosado [2,*]

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Abstract: Skin health is not only significantly affected by ageing, but also by other lifestyle-related factors, such as sun exposure, exercise and eating habits, smoking or alcohol intake. It is known that the cutaneous tissue can exhibit visible signs of senescence, in the form of, for example, dull complexion, loss of firmness, or changes in pigmentation. Consumers attempt to improve skin health and appearance not only by cosmetic products, but also with the consumption of food supplements. Recently, there has been an increase in the amount of food supplements with claims that are related to skin and hair health. Nevertheless, the literature is still scarce in evidence of the efficacy of this type of products. Considering this scenario, we aim in this review to assemble studies and methodologies that are directed at the substantiation of the cutaneous health claims of food supplements. For example, we reviewed those that were indicative of antioxidant properties, improvement in pigmentation disorders, increased hydration or protection against the damages caused by ultraviolet radiation.

Keywords: food supplement; skin health; photoprotection

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1. Introduction

The food supplement market has an extensive range of products with the most diverse health benefit claims. Among these benefits, a few are aimed at maintaining skin health and improving its appearance, but often scarce scientific studies are found to support such claims. However, several methodologies applicable to in vitro and in vivo studies are currently available to demonstrate the beneficial effects of the substances contained in these products on the skin, such as hydration improvement, protection against ultraviolet (UV) radiation or the reduction in wrinkles and spots. As examples of methodologies, we can mention the measurement of gross and net elasticity of the skin using the equipment Cutometer®, the evaluation of erythema and melanin with the use of a Mexameter®, the evaluation of wrinkle depth using a three-dimensional microtopography imaging system (PRIMOS 3D lite) or the Visia® equipment, among others, which will be mentioned in this review [1–9].

Exposure to solar radiation is one of the main factors that contribute to skin aging, since ultraviolet (UV) radiation stimulates the formation of free radicals, which can cause oxidative stress. Oxidative stress in the skin is related to mutations and photoaging. Photoaging is an accumulative process and dependents on the rate and frequency of sun exposure, as well as skin phototype (skin pigmentation). UVA radiation can penetrate through the epidermis to the dermis, causing damage to the connective tissue, or extracellular matrix (ECM), represented here by collagen, elastin and glycosaminoglycanes (GAGs). Damage to these structures generates visible damage in the skin, such as loss of elasticity, hydration and skin firmness, resulting in the appearance of wrinkles and increased fragility,

for example. UVB radiation, on the other hand, is absorbed in the epidermis by DNA, RNA and protein aromatic amino acid molecules, among other molecules, causing direct DNA damage, erythema, stratum corneum thickening, melanogenesis and photoimmunosuppression. The absorption of UVB radiation by DNA and RNA bases can lead to various mutations and affect cellular protein synthesis. The accumulations of unrepaired mutations can cause cell cycle arrest and apoptosis. Furthermore, mutations can nullify the apoptotic capacity of cells and thus increase the formation of malignancies. Such a result will depend on the skin cell type, UV wavelengths and cumulative UV dose [10–13].

In this review, we present studies that established efficacy evidence and support skin claims for some of the most common bioactives used in dietary supplements. Although there are several other food supplements that have a positive influence on skin health conditions, such as Vitamins C and E, Coenzyme Q10 and probiotics [14], the authors chose rosmarinic acid, *Polypodium leucotomus*, Pycnogenol®, astaxanthin, lutein, tranexamic acid, pomegranate (*Punica granatum* L.) extract and orthosilic acid since these supplements are potential trends in the market and studies demonstrate their efficacy in the improvement of the aspect and healthy of the skin. For each compound or substance, a specific introduction is provided, highlighting the mechanism that may be associated with their skin bioactivity, followed by the results obtained in relevant in vitro and in vivo investigations. Another objective of this review is to provide useful material for researchers aiming to further establish, particularly in vivo, the skin beneficial effects of dietary supplements.

2. Rosmarinic Acid

Rosmarinic acid, or (R)-1-carboxy-2-(3,4-dihydroxyphenyl) ethyl ester of 3,4-dihydroxy cinnamic acid, is an ester of caffeic acid and 3,4-dihydroxyphenylacetic acid, being found in plants, such as rosemary (*Rosmarinus officinalis*). The molecular structure of the molecule is represented in Figure 1 [15–17].

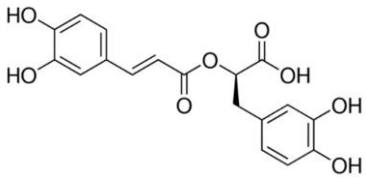

Figure 1. Rosmarinic acid molecular structure ($C_{18}H_{16}O_8$).

Rosmarinic acid molecule has interesting biological activities, such as antiviral, antibiotic, anti-inflammatory, immunomodulatory (tested in the treatment of atopical dermatitis), anticarcinogenic and antioxidant properties [18]. Recent studies showed that rosmarinic acid can reduce proinflammatory lysophosphatidylcholine production, hinder vitamin E depletion and inhibit the oxidation of low-density lipoproteins (LDL). Fernando et al. [16] demonstrated the cytoprotective effect of rosmarinic acid against oxidative stress induced by ultraviolet B (UVB) radiation. HaCaT (Cultured Human Keratinocyte) cells treated with rosmarinic acid prior to UVB irradiation showed an increase in the activity of the antioxidant system and a lesser oxidative damage to the biological macromolecules, observed through intracellular ROS detection, lipid peroxidation and DNA fragmentation assays, for example in [19].

It is known that rosemary has a high content of rosmarinic acid. For this reason, several laboratories have developed rosemary supplements claiming benefits, such as skin quality improvement and skin photoprotection, which are based on the antioxidant property of the rosmarinic acid to effectively prevent damage caused by UV radiation [2,7,16,20–22].

Pérez-Sánchez et al. [21] studied the protective activity of the NutroxSun® product, containing extracts of *Rosmarinus officinalis* and grapefruit (*Citrus paradisii*) on HaCaT keratinocytes. To evaluate the effect of RA on HaCaT cells in ROS generation, for example, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) was used to monitor intracellular

UVB-induced ROS generation. For this purpose, the cells were treated with a thin layer of PBS containing the RA extract (75–100 lg/mL), followed by exposure to UVB light (800 or 1200 J/m^2) and labeled with H_2DCFDA (fluorescent probe that becomes fluorescent when oxidized by free radicals). The treatment with RA extract was able to decrease ROS formation. Additionally, its photoprotective activity in volunteers was assessed by the determination of the minimal erythema dose (MED) after oral ingestion at different time points. The skin protective effect against UV radiation was determined by the calculation of the MED before treatment and after 29, 57 and 85 consecutive days of ingestion of the food supplement (250 mg, one capsule per day). The MED was calculated inducing erythema by an artificial light source with a filtering system (Multiport® Solar Simulator; Solar Light Co., Glenside, PA, USA). An increase of 37% was observed in the MED ($p < 0.05$) after 8 weeks of treatment and an even greater MED was obtained after 12 weeks (56%, $p < 0.01$).

In a similar fashion, Nobile et al. [7] investigated the effect of rosemary and grapefruit extracts on skin photoprotection through a MED assay. For the assessment of the minimal erythemal dose, series of UVB doses were applied on small subsites of the skin of the back of the volunteers. The skin redness caused by UVB radiation was measured by spectrophotometer/colorimeter CM-700D (Konica Minolta, Milano, Italy) in the CIELab color space (a*, red-green parameter). Volunteers were divided in three groups—untreated control, administration of 100 mg of capsules containing the extracts, and administration of 250 mg of the same sample. The results showed in both dose groups, a significant rise of the MED. In the 100 mg dose group, MED increased 15.2%, 20.5%, and 29.8% mJ/cm^2, after 0.5, 1, and 2 months of treatment, respectively. A similar efficacy was noticed in the other dose group. Although the variation of MED was not statistically significant when 100 and 250 mg data were compared, a significance was reached when these were compared with the placebo group. The authors also verified other skin parameters, such as lipid peroxidation, elasticity and wrinkle depth. The latter was measured in the periocular area using a three-dimensional microtopography imaging system (PRIMOS 3D lite, GFMesstechnik GmbH, Teltow, Germany). A noteworthy decrease in wrinkle depth was observed for both dose groups when compared to the control, but, again, the variation of wrinkle depth was not statistically significant when the two-dosage data were compared. For the skin elasticity assessment, the researchers studied the gross and net elasticity, using the Cutometer® MPA 580 (Courage + Khazaka Electronic, Köln, Germany). The skin surface of the face cheeks of the volunteers was analyzed. For this purpose, the skin surface of volunteers' cheeks was drawn into the probe by a negative pressure for 3 s and released for 3 s. The penetration depth of the skin inside the probe, during the suction and the release phase, was measured by an optical system. The same variation of skin elasticity, regarding to gross elasticity, was observed when the two dosages were compared. The results of the lower-dose group indicated an increase in the skin elasticity, 1.8, 3.2, and 4.6%, after 0.5, 1, and 2 months of treatment, respectively, whereas in the 250 mg dose group, it increased by 1.5, 2.9, and 3.7%. Towards skin net elasticity, a significant increase was also observed for both dosage groups. For the assessment of basal and UVA-stimulated (10 J/cm^2) lipid peroxidation (LPO), a tape-stripping technique was employed to determine the concentration of malondialdehyde (MDA) using Corneofix® foils (Courage + Khazaka Electronic, Köln, Germany) to obtain stratum corneum samples from the back of volunteers. The stratum corneum MDA content after 4 h of UVA exposure decreased by 9.7, 16.2 and 20.1%, and a similar decrease was observed after 24 h.

In summary, skin improvements were observed in the volunteers that received the treatment, although no statistic differences were seen between the 100 and 250 mg dose treatment, for the parameters investigated. The presumptive mechanism for the differences between the control and the other two groups has been attributed to rosmarinic acid and its antioxidant properties, causing the inhibition of reactive oxygen species (ROS) induced by UV radiation and concomitant inflammatory markers (LPO and cytokines) combined with a blockage of intracellular signaling pathways, which cause extracellular matrix degradation [2,16,23–26].

3. *Polypodium leucotomus*

Polypodium leucotomus (PL) is a tropical fern species in the Polypodiaceae family [27,28]. Its aqueous extract is obtained from the leaves and known commercially as Fernblock®. It is used as an oral and topical photoprotector agent [29]. Phytochemical studies showed a high concentration of polyphenols, monosaccharides (mainly fructose and glucose), quinic, shikimic, glucuronic, and malic acids [30,31]. The high antioxidant activity and photoprotective properties were attributed to the high content of phenolic components: p-coumaric, caffeic, ferulic, and chlorogenic acids [32–34]. Several studies have been conducted to unveil the mechanisms of PL photoprotective effect, mainly through in vitro or in vivo (animal model) studies [29,31].

Various investigations attributed the PL mechanism of action to an antioxidant action inhibiting ROS formation, especially those induced by UV [34,35], as well as an immunomodulatory pathway and anti-inflammatory effect [27,32,36–38].

Both the caffeic and ferulic acids presented in PL extract are antioxidants, inhibit UV-mediated peroxidation and are effective in protecting the skin from UVB-induced erythema [31,39]. The caffeic acid has anti-inflammatory properties by 5-lipoxygenase inhibition followed by leukotriene biosynthesis [40]. Furthermore, PL inhibited the photoisomerization of trans-urocanic acid, a chromophore found in the skin, which after UV-induced isomerization has immunosuppressive activity [41]. However, the exact mechanism is not clear, and could be based on the antioxidant effect of PL, but the literature does not attribute ownership to a specific group or component of the extract.

A PL beneficial effect was found on the regulation of matrix metalloproteinase that transformed growth factor-B and fibrillar collagen using a culture of dermal fibroblasts, melanoma cells and UV-radiated fibroblasts. The authors attributed the effect to the TGF-β expression on cell-specific regulation and to the antioxidant effect on the fibroblasts and melanoma cells [42].

Regarding toxicological data by Murbach et al. [43], no evidence of toxicity, genoxicity, and mutagenicity was found in a repeated oral dose animal study.

Kohli et al. [44] investigated the molecular and photobiological effects of oral PL administration. Subjects were irradiated with visible light, UVAI, and UVB for 4 days, and on days 3 and 4, the evaluation occurred after ingestion of PL. Clinical and colorimetric trials showed a decrease in erythema after UVB-induced irradiation in 17 out of the 22 subjects after PL oral administration. UV damage biomarkers related to apoptosis and DNA injury were found to be decreased in all subjects through the histological findings. These results seemed to suggest that PL could be used as an adjuvant against UVB photobiological effects.

Oral PL was investigated as a complement in the treatment of vitiligo and presented relevant results in repigmentation improvement. However, no studies assessed its effectiveness as the only form of treatment [35,45]. Some clinical studies investigated PL as a treatment of melasma using the melasma severity index evaluation, showing promising results [45]. However, Ammar investigated oral PL and compared it with a placebo, not obtaining differences between the two groups by narrowband reflectance spectrophotometry, nor by the severity index [46].

To date, no specialized studies have been published to confirm the post-inflammatory hyperpigmentation treatment with PL. However, due to the proven compound anti-inflammatory properties, the treatment of post-inflammatory hyperpigmentation could be possible with this compound [45].

4. Pycnogenol®

Pycnogenol® is a product obtained from *Pinus pinaster* bark extract, containing phenolic acids (p-hydroxy benzoic, protocatechuic, gallic, vanillic, p-coumaric, caffeic and ferulic acids); condensed flavonoids (procyanidins); and monomeric phenolic compounds (catechin, epicatechin and taxifolin). Due to its composition, Pycnogenol® is known for its anti-inflammatory and antioxidant activities [47–51].

Ni et al. [52] studied Pycnogenol® for melasma treatment. Thirty women with melasma took a 25 mg tablet of Pycnogenol® three times a day for 30 days. These volunteers were assessed clinically by melasma area index, pigmentary intensity index and by routine blood and urine tests. Efficacy was assessed at the 30th day of treatment, following the criteria: if a decrease in pigmentary intensity of melasma by two units or of the initial melasma area by more than one third was achieved and no new melasma appeared, the overall result was scored as 2; if the decrease in the pigmentary intensity was one unit and initial size was minor than one third, and no new melasma appeared, the overall result was scored as 1; when no change occurred in the pigmentary intensity, the result was scored as 0. The average pigmentary intensity of the volunteers decreased significantly on day 30, indicating an improvement of 0.47 units, and the average melasma area was also significantly reduced. Moreover, routine blood and urine test results were normal (blood cell count, hemoglobin and white blood cell count, urinalysis, among others).

Devaraj et al. [53] studied the effect of supplementation with 150 mg Pycnogenol® for 6 weeks in the antioxidant capacity of the plasma and in its lipoprotein profile. During the study, the diet and routine activities of the volunteers were unchanged, but consumption of products rich in flavonoids was restricted. After a few weeks of supplementation with Pycnogenol®, blood samples of the volunteers were screened to evaluate the supplementation effects, and the same procedure was repeated when supplementation stopped. The results indicated an increase in plasma polyphenol levels during the supplementation period, demonstrating that the phenols from Pycnogenol® were absorbed. On the other hand, a reverse effect on plasma polyphenol levels was observed when supplementation was interrupted. Other important findings were a significant decrease in LDL-cholesterol levels and an increase in HDL-cholesterol levels during supplementation. In contrast to LDL levels, HDL levels kept increasing after supplementation stopped, and this effect was observed in 66% of the volunteers. Even though the antioxidant effects of Pycnogenol® were demonstrated in the study, the researchers pointed out that there was no evidence that these results could be entirely attributed to the antioxidant activity of Pycnogenol®.

Ryan et al. [54] studied the effect of Pycnogenol® supplementation in an elderly population through cognitive and biochemical measures. The study showed that supplementation had a beneficial effect on the cognitive performance of the volunteers, more specifically an improvement in the working memory factor was observed. The working memory encompasses the capability of temporarily holding items from the spatial and numeric memories. However, no changes were determined on concentration and psychomotor abilities. In relation to the biochemical measures, a decrease in plasma F2-isoprostane concentrations suggested the biological effect of the antioxidant property of Pycnogenol® to inhibit oxidative reactions.

Although these last investigations were not directly related to skin health, we decided to keep them in this review to highlight the Pycnogenol® benefits in volunteers.

5. Carotenoids

Carotenoids are pigments present in plants, fungi, algae and several bacteria. In plants, for example, carotenoids play important roles in antioxidation, light absorption, providing color, and photoprotection [14,55,56].

The human body presents ways to protect itself against ROS using antioxidant system, such as endogenous antioxidants and enzymes. Exogenous antioxidants also play important role in this defense. Since carotenoids are potent antioxidants and humans do not synthesize them, supplementation is necessary when the intake of carotenoids in the diet is poor [55,57].

Several dietary supplements containing carotenoids are available on the market. For this review, we chose the carotenoids lutein and astaxanthin to discuss.

5.1. Astaxanthin

Astaxanthin is a ketocarotenoid, 3,30-dihydroxy-b,b-carotene-4,40-dione, which was first isolated from lobsters by Kuhn and Sorensen in 1938 [58]. This red pigment is synthesized by plant, microorganisms and algae, and has several commercial applications, such as in food, nutraceuticals, pharmaceuticals, cosmetics and aquaculture industries. In aquaculture, for example, astaxanthin is used as a feed supplement to provide color to the salmon flesh [59]. Studies have demonstrated that astaxanthin performs many essential biological functions in marine species, such as providing pigmentation, protection against UV light damage and oxidation of macromolecules, and increase stress tolerance. Another important feature of astaxanthin is its antioxidant property, which is considered higher than that of β-carotene, zeaxanthin and lutein. Astaxanthin's strong antioxidant capacity is attributed to the presence of keto and hydroxyl groups on its terminal ionone rings [60–62], as observed in Figure 2.

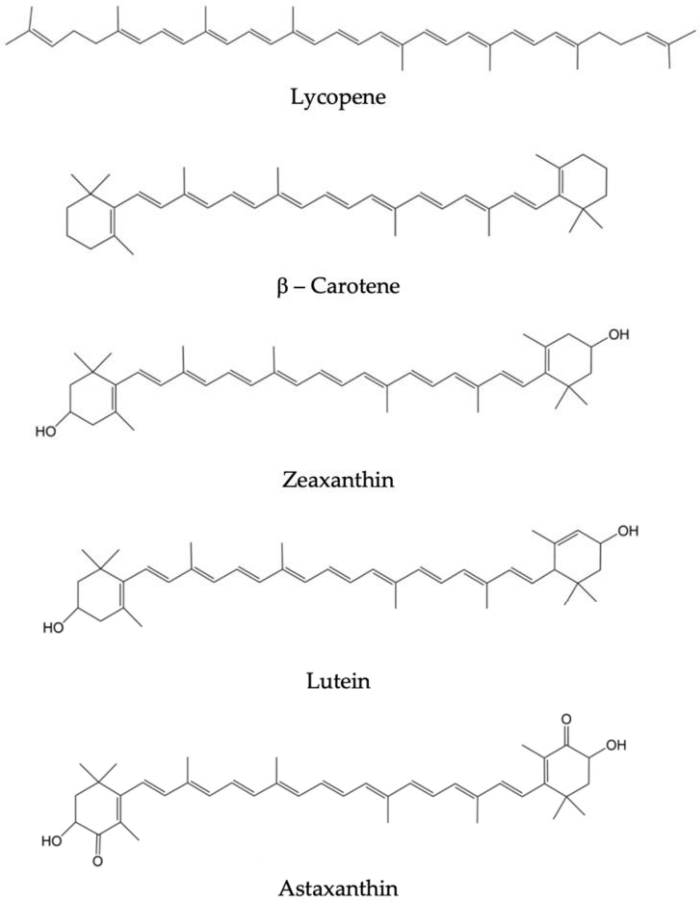

Figure 2. Molecular structures of selected carotenoids—adapted from [62].

McNulty et al. [63] compared the activity of polar and apolar carotenoids (astaxanthin, β-carotene, lutein, lycopene and zeaxanthin) on the lipid peroxidation of membranes containing cholesterol. The results indicated a correlation between changes on the membrane structure caused by the addition of carotenoids and the extent of the lipid peroxidation. All carotenoids disturbed the membrane structure except astaxanthin, and these changes were attributed to the orientation and location of the carotenoids within the membrane.

Furthermore, an increase in lipid peroxidation was noticed with the addition of β-carotene, lutein, lycopene and zeaxanthin, while astaxanthin led to its reduction. The researchers pointed out that the study was carried out with a membrane system less complex than biological membranes, but the findings still provide insights on the role of carotenoids on membrane lipid peroxidation.

Similarly, in a specialized review, Pashkow et al. [64] discussed the antioxidant property of astaxanthin in modulating oxidative stress. It was suggested in the study that a specific orientation of astaxanthin molecule in the cellular membranes allows its interactions with reactive species, which help to prevent membrane lipid peroxidation.

Another important feature of astaxanthin that has been extensively investigated is its cosmetic benefit to the skin. Tominaga et al. [65] evaluated the effect of astaxanthin administration to the skin of female and male volunteers. The female group received oral supplementation and topical treatment, while the male group only oral supplementation. Both groups showed wrinkle reduction and elasticity improvement after treatment. Additionally, the parameters age spot, skin texture and moisture content were assessed in the female group. A reduction in the age spot, an improvement in the skin texture and no significant difference in the moisture content were observed. Similar to the female group, the male group did not show any difference in the moisture content, but showed a significant improvement in the transepidermal water loss after treatment.

The same research group carried out in vitro and in vivo studies to determine the effects of astaxanthin administration on skin deterioration. Regarding the in vitro studies, the suppression of inflammatory cytokine secretion in keratinocytes and matrix metalloproteinase production by fibroblasts in samples treated with astaxanthin prior UVB irradiation was observed. Further studies, with volunteers, indicated a deterioration in the wrinkle's parameters and moisture content in the placebo group; however, no significant changes were observed in the groups treated with a 6 mg and 12 mg dosage of astaxanthin. The level of inflammatory cytokine, IL-1α, increased in the placebo and low dosage groups, but not in the high dosage group. Moreover, an improvement in skin elasticity was observed only in the group treated with 12 mg of astaxanthin [58].

Another study on the effects of astaxanthin supplementation to the skin was developed by Chalyk et al. [3]. In the study, a group of middle-aged volunteers received astaxanthin supplementation, and their skin was examined before and after treatment. Changes on the components of the skin surface were evaluated, more specifically, changes on the morphology of the surface lipids, corneocyte desquamation and microbial presence. Additionally, the plasma level of malondialdehyde, a biomarker of the oxidative stress, was assessed to evaluate the antioxidant effect of astaxanthin. Regarding the surface lipids of the skin, their droplet size and crystal structure were evaluated. A slightly increase in droplet size was observed, whereas no clear changes on the crystal structure could be detected. Changes on the skin surface were indicated by a decrease in corneocyte desquamation and microbial presence. During astaxanthin supplementation, a steadily decrease in malondialdehyde concentration in the plasma was observed.

5.2. Lutein

Lutein and zeaxanthin belong to the group of carotenoids, lipophilic molecules that preferentially accumulate in the luteal macula, while other carotenoids, such as beta-carotene and lycopene, accumulate in the skin [5,57]. The molecular structures of lutein, zeaxanthin and lycopene are shown in Figure 2, and it is possible to observe hydroxyl groups on theirs terminal ionone rings, as a similarity [62].

A study developed by Palombo et al. [5] investigated the beneficial effects of lutein and zeaxanthin supplementation to the skin. Clinical trials employed topical, oral or combined (topical and oral) routes to administer the carotenoids. The study evaluated the supplementation effects on superficial skin lipids, hydration, lipid peroxidation, photoprotective activity and skin elasticity. All routes indicated a positive effect of lutein and zeaxanthin supplementation on the investigated parameters. However, the combined route showed

the highest effect upon all skin physiological parameters except the skin elasticity. Overall, the results indicated that lutein and zeaxanthin supplementation was beneficial to the skin.

Another clinical study investigated the efficacy of lutein and zeaxanthin oral supplementation in healthy adults. The assessment parameters were skin tone and photoprotective activity. Skin lightening and luminance were improved with lutein/zeaxanthin supplementation when compared to the placebo group. Similar results were observed for the mean minimal erythema dose and the individual typological angle, since these parameters increased. Hence, the supplementation showed to improve skin conditions [57].

A comparative study of oral supplementation with lutein and lycopene evaluated human skin protection against UV radiation. This work investigated the influence of UVA/B and UVA 1 radiation in gene expression (HO1, ICAM1, and MMP1). The volunteers were divided in four groups: two groups started the treatment by either taking lutein or lycopene and then switched to placebo, while the other two groups first received placebo followed by carotenoid supplementation. Furthermore, the carotenoids were assessed in the blood samples during the treatment. Before and at the end of the supplementation period, the skin was irradiated and, subsequently, biopsies were performed after 24 h for gene expression analysis. The results suggested that both actives were protective against health damage induced by solar radiation, although lutein proved to be less effective when supplemented in the second period of the treatment. In addition, carotenoid blood levels significantly increased during the supplementation and returned to background levels when placebo was administered [4].

6. Tranexamic Acid

Tranexamic acid (Figure 3) is a synthetic derivative of the amino acid lysine and presents antifibrinolytic action. Its mechanism of action is due to its adhesion to the lysine binding sites, through a reversible blockade, in the plasminogen molecule, preventing it from binding to the activating factor of plasminogen. Once this blockade is created, plasmin (the main agent responsible for fibrinolysis) is no longer formed [66–69].

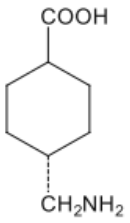

Figure 3. Molecular structure of tranexamic acid.

Another use of the tranexamic acid is on the treatment of melasma where its mechanism of action is associated with the blockage of the melanin synthesis. The effectiveness of the tranexamic acid in hyperpigmentation inhibition is attributed to its capacity to decrease tyrosinase activity and reverse melasma-related dermal changes [69–72].

Maeda and Naganuma [73] studied the effect of tranexamic acid on skin pigmentation induced by UV radiation in guinea pigs. The researchers topically applied solutions of tranexamic acid in different concentrations to the regions of animal skin already exposed to UV radiation. Histologically, by evaluating the amount of melanin in the control skin and treated with tranexamic acid, researchers found a suppression of melanin in the basal layer in the samples treated with the two more concentrated tranexamic acid solutions.

In a study developed by Karn et al. [74], the oral administration of tranexamic acid along with a routine treatment (topical hydroquinone and sunscreen) was evaluated on the treatment of melasma. The results indicated that the volunteers receiving the combined treatment showed a significant decrease in the mean melasma assessment severity index score, whereas those given the routine treatment had a significant decrease in the mean

score only in the beginning, while the score remained stable in the last period of the treatment. Furthermore, the satisfaction assessment showed that 45.4% of the volunteers treated with the combined treatment graded it as excellent compared to only 8.5% of those who received the routine treatment.

Na et al. [75] conducted a clinical trial to investigate the effects of oral and topical administration of tranexamic acid on melasma treatment. Skin pigmentation and erythema were assessed to evaluate the treatment efficacy. Additionally, histological changes on the skin of selected volunteers were measured. The outcomes showed that the mean melanin index scores for the lesioned skin decreased during treatment, while the scores for the perilesional skin increased. The increase in the latter parameter was attributed to the period (spring to summer) that the study was carried out. Similar results were observed for the erythema index scores. Regarding the histological changes, the epidermal pigmentation showed a significant reduction, as well as a reduction in vessel numbers, vascularity and mast cell counts. Moreover, most of the volunteers observed an improvement on the skin appearance.

Another study, from Nagaraju et al. [76], also demonstrated the effects of tranexamic acid in skin with melasma. Thirty patients with refractory melasma received 500 mg of tranexamic acid orally, twice a day, associated with the use of a sunscreen. The assessment was based in the modified melasma area severity index (mMASI) and melasma quality of life index (MELASQOL) that were registered at baseline and after treatment. In addition, a skin biopsy was performed for immunohistochemistry and histopathology evaluation both before and after tranexamic acid supplementation. After a 4 month treatment, the results for mMASI assessment indicated that only one patient showed an excellent response. However, moderate and good improvement responses were perceived in 14 patients. In contrast, a slight improvement in the MELASQOL was observed in 4 patients, while a moderate improvement was seen in 23 patients and good improvement in 3. Concerning the histopathological evaluation, an epidermal pigmentation decrease was observed in 13 cases (representing 92.8% of the volunteers that went through skin biopsy). In addition, the inhibition of melanocyte proliferation was observed in 50% of the cases, and a reduction in melanin incontinence in 35,71% of the cases. Concerning the immunohistochemistry, among other interesting results, the researchers verified that Melan-A staining decreased with a statistical significance in all patients' post-treatment.

7. Pomegranate (*Punica granatum* L.) Extract

Punica granatum L. extract is composed of several constituents, such as anthocyanins, polyphenols and tannins, for which multiple health benefits have been established. This plant is found in tropical and subtropical regions and is widely used for hypertension and atherosclerosis. A few in vitro and in vivo animal studies have already been carried out to evaluate the cutaneous impact of this extract, mainly in inhibiting melanin synthesis, improving hydration and elasticity and preventing skin ageing, in addition to antioxidant and anti-inflammatory activity and protection against damage from the UV exposure [1,77–83].

Kasai et al. [8] evaluated if the use of the pomegranate extract orally could protect the skin from UV radiation. In this study, volunteers aged from 20 to 40 years old were divided in three distinct groups with thirty volunteers each: placebo, those who received a higher dosage (200 mg) and those administered a lower dosage (100 mg) of the pomegranate extract. Before, during and after treatment (carried out for four weeks), the effect of UV irradiation on forearm skin was measured using a dose equivalent to 1.5 MED. Weekly measurements of erythema and melanin were established with the use of a Mexameter®. Even though no significant changes in the erythema and melanin values were observed, the volunteers reported an improvement on the skin appearance.

Henning et al. [77] evaluated in vivo the performance of oral supplementation of juice and capsules containing pomegranate extract against premature aging caused by UV radiation. This trial was carried out for 12 weeks on female volunteers aged between 30 to 40 years old and with skin phototype II to IV, which were separated into three distinct

groups: placebo, those who consumed pomegranate juice and those who received supplementation with capsules containing 1000 mg of pomegranate extract. The study considered the equivalent number of polyphenols consumed by the two different supplements. The MED test and the skin melanin index (MI) were measured to ascertain the efficacy against the effects of skin radiation, both being determined before and after consumption of the active and/or placebo. Non-invasive equipment was used to measure MI, sebum on the skin surface, hydration and skin erythema before and after treatment. The UVB radiation dose and the exposure time were stipulated according to the skin type listed by the National Biological UVB mJ chart. The radiation was applied using a Dermalight® 90 device in doses, which were in the range of 220–550 mJ/cm^2 for a 100 to 290 s in previously defined areas in the arm. Twenty-four hours after irradiation, erythema formation was investigated through MED calculation. At the end of the study, it was found that treatment with pomegranate juice or extract provided a significant increase in the protection against erythema formation when compared to the placebo group, thus increasing MED. No significant changes in hydration and the amount of sebum in the skin were observed, and despite causing a non-significant reduction in melanin formation, the overall results seemed to indicate that the use of pomegranate extract can protect the skin from damage induced by UVB radiation.

8. Orthosilic Acid

Orthosilic acid (monomeric) is used to replace silicon in the human body since it has an efficient bioavailability when used orally. Silicon is present in the human body in small traces that, with ageing, begin to show a decrease in its levels; thus, its supplementation is recommended to provide skin benefits. Its supplementation has been associated with an improvement on the synthesis of collagen and elastin [9,84]. This compound is found in an accumulative form in the horny layer and in the hair fiber cuticle, since it can contribute to the improvement of the hair strands, stimulating growth and making them more resistant. In vivo studies have shown that the compound improves hair thickness and reduces the loss of elasticity of the capillary thread, promotes nail hardness, in addition to assisting in the skin barrier function and increasing the synthesis of collagen and elastin, preventing premature skin aging [9,84–86].

The use of orthosilic acid has long been the subject of in vivo studies to substantiate skin claims. In one of the most recent trials, it was observed that this component was able to promote a significant improvement in wrinkles and skin anisotropy in women aged 40 to 65 years old with signs of facial photoaging. These results were obtained by non-invasive bioengineering devices: viscoelastic properties and anisotropy by the Reviscometer® MPA 5; skin anisotropy by the mechanical anisotropy indicator; and the presence of wrinkles was evaluated by the Visiometer® SV 600. Measurements were conducted before and after oral supplementation for 20 weeks with capsules containing equivalent doses of 10 mg of silicon in the form of ch-OSA pellets (Bio Minerals n.v., Destelbergen, Belgium) twice daily [87].

In the research developed by Ferreira et al. [9], different forms of oral administration of elemental silicon were studied when stabilizing the structure with maltodextrin or monomethylsilanetriol. Volunteers, aged 40 to 60 years old with skin phototype III to V, were given a dose of 5 mg of silicon twice daily for 5 months and were divided in three groups: placebo, those that used a capsule containing maltodextrin and silicon, and those who were given an oral solution containing monomethylsilanetriol and silicon. Before and after the 3 and 5 month treatment, changes in the skin were evaluated using the Visia® analysis system. At the end of the study, it was found that there was a significant improvement in facial wrinkles when comparing the groups that ingested the active with the placebo group. Both forms of administration promoted a statistically significant improvement of UV-induced pigmentation spots. Other parameters also showed significant differences among the groups, but these results were attributed to seasonal effects, since the study was carried out during different seasons.

Petersen et al. [85] aimed to evaluate the potential of stabilized silicon using a hydrolyzed marine collagen molecule (Exsynutriment®). This work was conducted on 22 male and female volunteers, between 40 and 60 years old, and involved 90 days of supplementation with 1 capsule a day. The volunteers were divided in two groups: those who received the active ingredient and those administered the placebo. Before the study, the volunteers were submitted to an evaluation by a dermatologist to determine the skin phototype according to the Fitzpatrick scale and the degree of skin aging according to the Glogau scale. In addition to these, the volunteers underwent a comparison of the characteristics of their face before and after the 90 days of treatment using images obtained by Visia® (Canfield Imaging Systems Inc., Parsippany, NJ, USA), which allowed the evaluation of wrinkles, pores, damage caused by UV radiation and formation/alteration of skin spots. The skin characteristics of the groups (placebo and treated) showed no statistical differences at the start of treatment. However, after the treatment period, there was a significant difference in skin firmness (assessed by changes in the jowl, nasojulgal and eyelid folds and neck profile), texture (assessed by the presence of fine wrinkles and irregularities on the skin surface) and hydration (assessed by xerosis, scales, crust and shine); thus, it was concluded that this supplementation contributed to an improvement in skin conditions. However, orthosilic acid and hydrolyzed marine collagen were administered together; for this reason, it would be interesting to include a third group of volunteers who would receive only orthosilic acid, helping to understand which of the components, or if the combination of these, was responsible for the beneficial effect observed on the skin.

9. Conclusions

It is known that a healthy and balanced diet is of utmost importance for maintaining the health and functions of the body, including here the skin. Unfortunately, it is often not possible to eat so correctly; for this, food supplements become excellent allies. In this review, we discussed and presented studies on bioactive compounds that help to maintain the health and youthfulness of the skin. A wide variety of methodologies are available to ascertain the impact of food supplements in skin condition. For most of the substances or compounds herein, it was demonstrated that these products can act as adjuvants to obtain healthier skin, contributing to substantiate labelling claims, in addition to providing other significant benefits to the consumer. Overall, the following skin benefits from the consumption of these substances were proven: protection against oxidative stress, improvement in skin firmness, reduction in wrinkles and decrease in the pigmentary intensity of melasma, among others. We strongly believe that studies associating the oral use of food supplements with the application of cosmetic products containing the same bioactive compounds would elucidate and bring very interesting and even synergistic results, causing a positive impact on the skin.

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Article 2

Prospecting *in vitro* antioxidant and photoprotective properties of rosmarinic acid in a sunscreen system developed by QbD containing octyl *p*-methoxycinnamate and bemotrizinol

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Prospecting In Vitro Antioxidant and Photoprotective Properties of Rosmarinic Acid in a Sunscreen System Developed by QbD Containing Octyl *p*-Methoxycinnamate and Bemotrizinol

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Abstract: Progressively growing diagnoses of skin cancer trigger public health concerns about excessive sun exposure, awareness of the deleterious effects of ultraviolet (UV) radiation on the skin, and the proper use of sunscreens. Studies show that bioactive molecules, such as rosmarinic acid (RA), may potentiate the photoprotective and antioxidant activity of topical formulations. This research presents the application of the concepts of quality by design (QbD) to evaluate the critical parameters of quality and the development of an optimized cosmetic formulation with RA by means of an understanding of product design space. Samples were developed using design of experiments (DoE) and they were evaluated for in vitro antioxidant activity and photoprotective efficacy, as well as for photostability through artificial irradiation. We were able to achieve the RA performance regarding antioxidant and SPF properties through in vitro experiments. We obtained the equations for predicting the in vitro antioxidant activity and SPF. Considering our sunscreen system, developed with octyl *p*-methoxycinnamate and bemotrizinol, the presence of RA increased its antioxidant capacity; however, the in vitro SPF was reduced when both UV filters were used. The development of multifunctional sunscreens is of utmost importance; moreover, there is a need for the rational development of formulations that ensure representative statistical tests of the effects and interactions among the components of a formulation on the desired critical quality attributes, including efficacy.

Keywords: rosmarinic acid; antioxidant activity; sun protection factor; sunscreen; quality by design

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1. Introduction

Sun rays' contact with the skin is responsible for important benefits for humans, such as the feeling of well-being and comfort, improved sleep quality (since the perception of sunlight acts on the circadian rhythm), and the synthesis of vitamin D, which plays an important role in the quality of bone structure. However, the occurrence of skin cancer reflects the concern about unprotected and unplanned sun exposure [1,2] Given the damage caused by the incidence of solar radiation on the skin, the use of photoprotectors (sunscreens) has become crucial in preventing and reducing the risk of skin cancer, whether melanoma or non-melanoma [3–6].

Photoprotective formulations are developed with chemical (organic) and/or physical (inorganic) filters. The chemical structure of organic filters allows the absorption of ultraviolet (UV) rays. UV radiation excites the molecule that reaches a state of resonance, so there is a conversion of high energy radiation into a more harmless one, such as heat. Inorganic

filters, such as titanium dioxide and zinc oxide, form a barrier over the skin that is capable of reflecting and dispersing UV radiation [7,8].

Hypersensitivity reactions can be related to the presence of organic filters in photoprotective formulations. Thus, the search for bioactive molecules that can act by absorbing UV radiation becomes interesting for the dermocosmetic market. The use of these molecules in combination with well-known and widely used UV filters may reflect the use of lower filter concentrations to obtain sun protection factor (SPF) values as high as when large concentrations of UV filters are used. Moreover, it is known that bioactive molecules can provide dual activity to the formulations, due to the antioxidant potential that some of them may present. In this category, we can mention rosmarinic acid (RA) [8–10].

RA—(R)-1-Carboxy-2-(3,4-dihydroxyphenyl) ethyl ester (3,4-dihydroxy cinnamic acid) ester—is an ester of caffeic acid and 3,4-dihydroxyphenylacetic acid and may be found in plant species, especially rosemary (*Rosmarinus officinalis*) [10,11]. RA presents several biological activities, acting as an antiviral, antibiotic, anti-inflammatory, and antioxidant. Recent studies have shown that RA is able to slow vitamin E depletion, decrease proinflammatory lysophosphatidylcholine production, and prevent LDL oxidation. Studies demonstrated that this phenolic acid was able to attenuate cellular damage against UVB-induced oxidative stress by increasing the antioxidant effects on keratinocytes [10,12].

Since the release of the Pharmaceutical Development Q8 Guide for Industry by the International Conference of Harmonization (ICH), care has been taken by pharmaceutical researchers, whether in academia or industry, to comply with the recommendations set forth in this document. These integrate the productive need to develop better quality formulations, safety and efficacy, and tools that can be used for this purpose. The focus of this guide is the application of quality by design (QbD) concepts. Figure 1 illustrates the key elements that make up QbD studies.

Figure 1. Illustrative summary of key elements of the quality by design study.

Despite the advantages of QbD and process analytical technology (PAT) approaches in the development and optimization of new products, these are not widely used in the cosmetic industries. Thus, in this research work, we applied QbD and PAT to the development of multifunctional sunscreens containing RA.

2. Materials and Methods

2.1. Materials

Rosmarinic acid (96% purity) was purchased from Sigma-Aldrich (São Paulo, Brazil). Bemotrizinol and octyl *p*-methoxycinnamate were purchased from Brasquim (São Paulo, Brazil) and Mapric (São Paulo, Brazil), respectively. Ethyl alcohol and methanol were supplied by Synth (São Paulo, Brazil). Water was obtained in a Milli-Q-plus System (Merck Millipore, Bedford, MA, USA). The antioxidant activity was measured by spectrophotometry (UV-Vis Evolution 300, Thermo Scientific, Madison, WI, USA). The formulations' SPF

was measured by diffuse reflectance spectrophotometry with an integrating sphere using a UV-2000S UV transmittance analyzer (Labsphere, North Sutton, NH, USA). To access the photostabilization potential, the formulations were exposed in a solar simulator (Suntest® CPS+, Atlas, Germany).

2.2. Development of Photoprotective Formulations

To obtain formulations with broad-spectrum protection, octyl *p*-methoxycinnamate (UVB filter) and bemotrizinol (broad spectrum filter) were used, plus 1.0% (*w*/*w*) of the bioactive compound rosmarinic acid [9,13]. The percentage of 1.0% of RA was chosen according to a previous study of the antioxidant activity of the molecule in solutions (data not shown). Emulsified systems of appearance, coloration, odor, and satisfactory pH value were prepared, using a factorial design (DoE-2^3), as described in Table 1.

Table 1. Experimental design of factorial type (DoE 2^3) applied to the development of photoprotective formulations.

Formulations	Proportion (% *w*/*w*)		
	Octyl *p*-Methoxycinnamate	Bemotrizinol	Rosmarinic Acid
F1	-	-	-
F2	-	-	1.0
F3	7.5	-	-
F4	7.5	-	1.0
F5	-	10.0	-
F6	-	10.0	1.0
F7	7.5	10.0	-
F8	7.5	10.0	1.0

Legend: (-) = Compound not added.

2.3. In Vitro Functional Characterization of the Formulations

2.3.1. Antioxidant Activity

The antioxidant activity of the formulations was evaluated through the percentage of inhibition of the free radical 2,2-diphenyl-1-picrylhydrazyl DPPH•. DPPH• presents a purple color in solution and, in the presence of an antioxidant, it is reduced to diphenylpicrylhydrazine, which manifests a yellowish color. This organoleptic alteration is detectable by spectrophotometric analysis [14,15]. Aliquots of the photoprotective samples were solubilized in methanol and, after treatment with DPPH•, centrifuged for sedimentation of solid matters that could interfere with the spectrophotometric analysis. The supernatant was separated and used for triplicate antioxidant activity assay. Samples were evaluated in a spectrophotometer at 515.0 nm, in quartz cuvettes with an optical path of 1.0 cm. Antioxidant activity was expressed as the percentage of DPPH• inhibition (Equation (1)).

$$\% \text{ Antioxidant activity} = \frac{\text{Control Absorbance} - \text{Sample Absorbance}}{\text{Control Absorbance}} \times 100 \quad (1)$$

Equation (1) Antioxidant activity expressed as percentage of DPPH• inhibition.

2.3.2. Photoprotective Efficacy and Photostability of Formulations

The in vitro photoprotective efficacy of the formulations was determined by a Labsphere® UV2000S. Aliquots of the samples were weighed and uniformly applied, respecting the application rate of 1.3 mg/cm^2, on the surface of polymethyl methacrylate (PMMA) plates [16–18]. After drying for 30 min protected from the light, the plates were subjected to the reflectance spectrophotometric reading and at least five transmittance readings per plate were registered. To evaluate the photostability of the formulations, the same samples applied to the PMMA

plates were irradiated in a photostability chamber with an irradiance of 55 W/m^2 and a fixed dose of 396 KJ/m^2, equivalent to an irradiation period of two hours. The photostability evaluation of the formulations was calculated comparatively against the estimated SPF and critical wavelength obtained before and after the sample irradiation step [16].

2.4. Analysis of the Factorial Design

To investigate the impact of the RA and its interaction with bemotrizinol and octyl *p*-methoxycinnamate, the in vitro antioxidant activity and the estimated SPF parameters of the formulations were evaluated. The influence of the factors and their interactions over the results for each of the evaluated parameters was measured by the main effect graphs for each single variable; cause and effect graphs (Pareto) for isolated variables and their combinations; and outline charts. After analyzing the results, regression equations were determined for each parameter evaluated [19].

2.5. Statistical Analysis of Results

The DoE 2^3 analysis was conducted using the Minitab® program, version 18. Experiments were conducted randomly, in triplicate, and with a significance level of 5% ($p \leq 0.05$) for the determination of significant results.

3. Results

3.1. In Vitro Functional Characterization of Formulations

3.1.1. Antioxidant Activity

The antioxidant activity presented by the formulations is shown in Table 2. All samples were dissimilar when compared with each other and, in general, samples containing RA (F2, F4, F6, and F8) resulted in superior antioxidant activity. The formulation that developed the highest antioxidant activity was F8, which presented both UV filters and RA. The synergistic effects of the association between UV filters and RA, both on antioxidant activity and photoprotective efficacy, will be discussed further.

Table 2. Antioxidant activity of formulations F1 to F8.

Formulation	F1	F2	F3	F4	F5	F6	F7	F8
Antioxidant activity (%)	53.00 ± 0.05 ^{G}	73.82 ± 0.05 ^{D}	59.21 ± 0.05 ^{F}	81.14 ± 0.0 ^{C}	64.80 ± 0.05 ^{E}	87.54 ± 0.05 ^{B}	34.86 ± 0.79 ^{H}	88.56 ± 0.08 ^{A}

Legend: Antioxidant activity of the epidermis expressed as mean ± standard deviation. Different letters represent statistically significant differences between the groups. Each letter represents an analysis group statistically different from the other. Groups that share same letter(s) are not different from each other. The results were evaluated according to the one-way ANOVA statistical test, followed by the Tukey test for comparison between groups (significance level = 0.05).

The formulation variables (RA, bemotrizinol, and octyl *p*-methoxycinnamate) were evaluated for their isolated effects on the antioxidant activity results. The graph of main effects (Figure 2) shows that RA was the variable with the greatest impact on the antioxidant activity. By the Pareto graph (Figure 3), the interaction among the variables was clearly observed. In decreasing order of antioxidant activity, there was isolated RA, followed by the association of UV filters, the association between RA and UV filters, and, finally, isolated UV filters. The contour graph (Figure 4) illustrates the regions where there was the best antioxidant activity as a function of the variables employed.

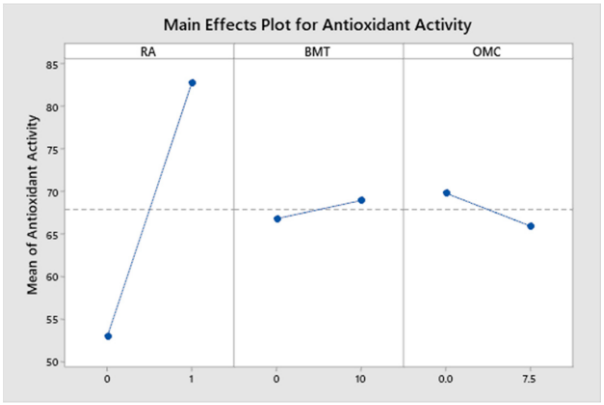

Figure 2. Graph of main effects for the antioxidant activity of the formulations.

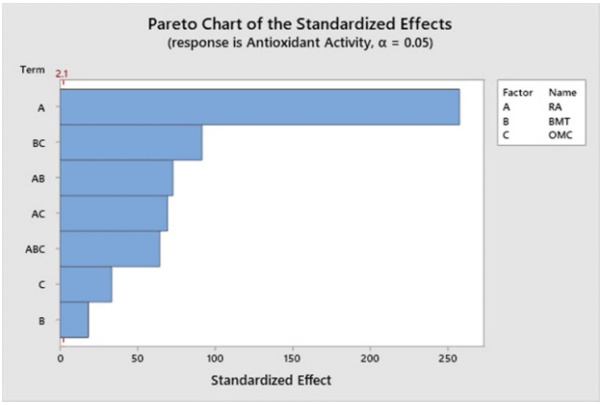

Figure 3. Representation of the effect of isolated and combined variables on the antioxidant activity of formulations (Pareto chart).

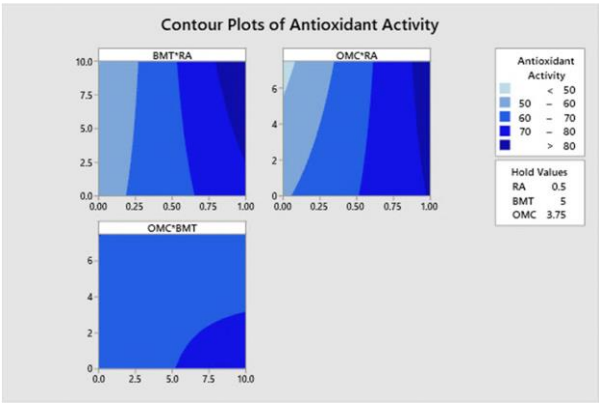

Figure 4. Contour chart for antioxidant activity.

The results of antioxidant activity against the proposed experimental design were analyzed using the Minitab® program, version 18. It was possible to elaborate an equation for predicting antioxidant activity from the verification of the adequate correlation coefficient

value (R^2 = 0.9998), confirmation of normality, homoscedasticity, and independence of the residues, allowing the suitability of the proposed statistical model to be validated.

$$\% \text{ Antioxidant activity} = 53.061 + 20.757\ AR + 1.1742\ BMT + 0.8199\ OMC + 0.1976\ AR \times BMT + 0.1558\ AR \times OMC - 0.48117\ BMT \times OMC + 0.39733\ AR \times BMT \times OMC \quad (2)$$

Legend: RA—rosmarinic acid; BMT—bemotrizinol; OMC—octyl *p*-methoxycinnamate.

3.1.2. Photoprotective Efficacy and Photostability of Formulations

Table 3 presents the results from the in vitro photoprotective assay (SPF and critical wavelength) of the samples. RA, although not having optimized the SPF values, presented a photostabilizing potential for the sample containing both UV filters. The SPF decay was 37.5% for the formulation containing AR (F8) compared to 52.4% SPF loss for the one without the addition of the bioactive compound (F7). Furthermore, the presence of RA in the formulation F4 caused a statistically significant increase in the critical wavelength value when compared with F3 (formulation with only octyl *p*-methoxycinnamate). The negative impact on the estimated SPF value of RA and the positive impact of the UV molecules on this same parameter can be seen in Figures 5 and 6.

Table 3. Sun protection factor (in vitro SPF), critical wavelength (nm), and percentage of SPF decay of formulations F1 to F8.

Formulations	Pre-Irradiation		Post-Irradiation		SPF Decay (%)
	SPF	Critical Wavelength (nm)	SPF	Critical Wavelength (nm)	
F1	1.0 ± 0.0^{F}	320.0 ± 0.0^{G}	1.0 ± 0.0^{F}	320.0 ± 0.0^{G}	-
F2 *(RA)*	1.0 ± 0.0^{F}	320.0 ± 0.0^{G}	1.0 ± 0.0^{F}	320.0 ± 0.0^{G}	-
F3 *(OMC)*	3.7 ± 0.6^{DE}	333.0 ± 0.0^{F}	4.3 ± 0.6^{CDE}	338.7 ± 2.3^{E}	-
F4 *(RA + OMC)*	3.3 ± 0.6^{EF}	344.7 ± 3.1^{D}	4.3 ± 0.6^{CDE}	343.7 ± 2.3^{D}	-
F5 *(BMT)*	5.3 ± 0.6^{CDE}	379.0 ± 0.0^{BC}	5.3 ± 0.6^{CDE}	381.0 ± 0.0^{AB}	0.0%
F6 *(RA + BMT)*	6.0 ± 1.7^{CD}	376.0 ± 0.0^{C}	3.3 ± 0.6^{EF}	382.7 ± 0.6^{A}	44.0%
F7 *(OMC + BMT)*	14.0 ± 2.0^{A}	376.0 ± 0.0^{C}	6.7 ± 0.6^{C}	377.7 ± 0.6^{BC}	52.4%
F8 *(AR + OMC + BMT)*	10.7 ± 1.5^{B}	376.7 ± 0.6^{C}	6.7 ± 0.6^{C}	378.7 ± 0.6^{BC}	37.5%

Legend: Different letters represent statistically significant differences between the groups. Each letter represents an analysis group statistically different from the other. Groups that share the same letter(s) are not statistically different from each other The results were evaluated according to the one-way ANOVA statistical test, followed by the Tukey test for comparison between groups (significance level = 0.05). (-) = Not determined.

Through the slope of the lines in the graph of main effects (Figure 5) and analysis of the Pareto graph (Figure 6), we found that bemotrizinol was the active ingredient that most positively affected the SPF value due to its high efficacy. Bemotrizinol is a molecule with broad spectrum absorption and high molar absorptivity at 310 and 343 nm, including the UVA I wavelength. Its structure contains two hydroxyl groups and one hydroxyphenyl triazine group that allow the phenomenon of tautomerism, responsible for the rapid energy release by the molecule after the absorption of UV radiation [13,20]. Followed by this, there were octyl *p*-methoxycinnamate and the combination of the two filters. The contour graph (Figure 7) corroborates this observation.

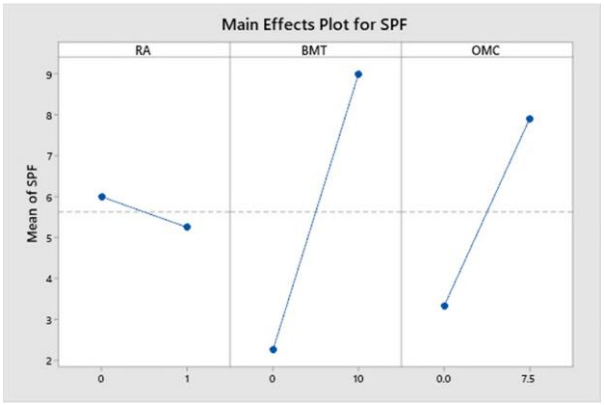

Figure 5. Graph of main effects for in vitro SPF of the formulations.

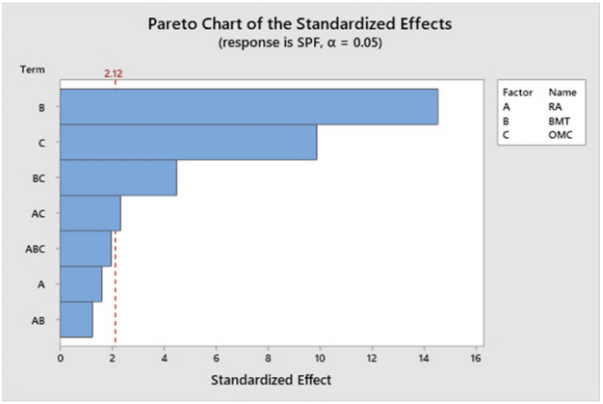

Figure 6. Pareto chart representation of the effect of isolated and combined variables on the in vitro SPF of the formulations.

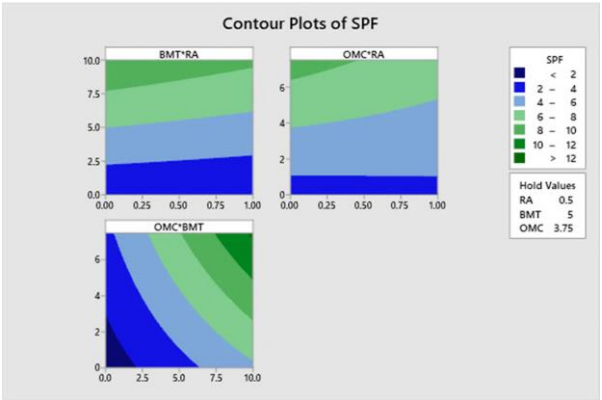

Figure 7. Contour chart for in vitro SPF.

The results of SPF against the proposed experimental design were analyzed using the Minitab® program, version 18. It was possible to elaborate an equation for predicting SPF from the verification of the adequate correlation coefficient value (R^2 = 0.9554), confirmation

of normality, homoscedasticity, and independence of the residues, allowing the suitability of the proposed statistical model to be validated.

$$\begin{gathered}\text{SPF} = 1.000 - 0.000\ \text{RA} + 0.4333\ \text{BMT} + 0.356\ \text{OMC} + 0.067\ \text{RA} \times \text{BMT} - 0.044 \\ \text{RA} \times \text{OMC} + 0.0800\ \text{BMT} \times \text{OMC} - 0.0489\ \text{RA} \times \text{BMT} \times \text{OMC}\end{gathered} \quad (3)$$

Legend: RA—rosmarinic acid; BMT—bemotrizinol; OMC—octyl *p*-methoxycinnamate.

4. Discussion

Rosmarinic acid, according to its properties, can be considered a promising candidate for use in multifunctional sunscreens, acting against free radicals and as an effective antagonist in lipid peroxidation [21–25]. The DoE 2^3 was used to develop the O/W emulsions (F1 to F8). Formulations were macroscopically stable with organoleptic characteristics (odor, color, and appearance) adjusted for their purposes (data not shown). The DoE 2^3 assisted in assessing combinations of compounds that could be applied in the formulations and, in sequence, in evaluating interactions between the UV filters and RA aiming at the maintenance of photoprotection and antioxidant efficacy. Additionally, it was also possible to determine, in relation to the results of the in vitro efficacies, the influence of each single variable (octyl *p*-methoxycinnamate, bemotrizinol, and RA).

Sánchez-Campillo and colleagues studied the antioxidant activity of RA in aqueous systems through thiobarbituric acid reactive substances (TBARS) and Trolox equivalent antioxidant capacity (TEAC) assays. They found that RA showed a 3.24 times higher antioxidant activity than the ascorbic acid in the TBARS test and a 1.60 times higher activity in the TEAC test. Considering that ascorbic acid is a molecule recognized for its high antioxidant activity, these results highlighted the relevant antioxidant activity of the RA [12]. In addition, Lima and colleagues studied the effect of phenolic compounds, such as RA, quercetin, and luteolin, on protection against oxidative damage in HepG2 liver cells. They found increased glutathione levels and decreased lipid peroxidation in HepG2 hepatoma due to the use of these compounds, reinforcing the relevance of RA antioxidant activity [26]. Thus, it can be inferred from the interpretation of the generated predictive equation and the contour plot that the antioxidant activity of the formulations presented in this study increased as a function of the concentration of RA.

Contrarily, when evaluating the action of RA with the UV filters, several factors influenced the results. We observed that the presence of RA negatively affected the in vitro SPF values of the formulations. For the sample with octyl *p*-methoxycinnamate, the RA addition caused an increase in the critical wavelength, an event not observed for the other formulations. Although RA has a maximum absorption peak between 290 and 330 nm, which could lead to an improvement in the absorption in the UVB region (mainly, at least) presented by the formulations, such evidence was not found in this investigation with the sunscreen system used [27,28]. However, it was observed that the addition of RA to the formulation containing the two UV filters provided the smallest decrease in the estimated SPF value after irradiation, suggesting a photostabilizing activity of RA in this particular system.

Pattananandecha and colleagues investigated the inhibiting effect of a standardized RA extract on reactive oxygen species (ROS), reactive nitrogen species (RNS), and matrix metalloproteinase-1 (MMP-1) production in a cell-based study. The extract presented effects both in the inhibition of the lipid peroxidation, intracellular ROS, and MMP-1 production in human skin fibroblasts induced by H_2O_2 and UVA, and in the elimination of nitric oxide in mouse macrophage cells. The researchers also investigated the cytotoxicity of the RA extract, demonstrating that tested concentrations ranging from 10 to 100 ppm did not exert cytotoxicity to human skin fibroblasts nor mouse macrophage cells. RA extract was also able to reduce collagen degradation in fibroblasts when compared to the control group, demonstrating the RA potent antioxidant activity [29]. Corroborating this finding, the study by Matwiejczuk and colleagues showed that RA was able to prevent or decrease the changes in the metabolism of collagen [30].

Sánchez-Campillo and colleagues studied the photoprotective action of RA against UV and other ionizing radiations. They found that RA increased tyrosinase (an important enzyme in melanin biosynthesis) activity and its expression level in melanoma cells in rats when compared to the control group. Radioprotective and antimutagenic effects were determined by the micronucleus test, where lymphocytes were irradiated with gamma radiation. The lymphocytes treated with RA decreased the micronucleus values, indicating RA's effectiveness against oxidative stress induced by radiations [12].

Fernando and colleagues studied RA against cell damage caused in human HaCaT cells exposed to UVB radiation. They verified the attenuation of keratinocyte damage via improved antioxidant activity and the attenuation of UVB-induced oxidative macromolecular damage, including carbonyl protein content, DNA strand breaks, and 8-isoprostane (oxidative stress marker) level. In addition, RA increased the expression and activity of superoxide dismutase, catalase, heme oxygenase 1, and its transcription factor Nrf2, which were decreased by UVB radiation. The enzymes superoxide dismutase, catalase, and heme oxygenase 1 are relevant for antioxidant defense in most cells exposed to oxygen [10,31]. Most recent studies also demonstrated the RA potential against radiation [29,32–36]. Bispo, for example, investigated the association of RA with octyl *p*-methoxycinnamate and avobenzone, showing an improvement in the in vivo SPF, although this bioactive compound was not able to enhance the photostability of that system [36].

To evaluate the photoprotective activity in vitro, the diffuse reflectance spectrophotometry with an integrating sphere is highly recommended and used, allowing the calculation of several parameters of photoprotection. However, in vivo FPS analysis is based on determining the UVB energy needed to produce an erythema on the protected skin of individuals, divided by the UVB energy required to produce an erythema on unprotected skin [37]. It is known that ingredients that prevent skin erythema, such as antioxidants, could significantly increase sunscreen protection according to specialized literature [38–42]. The difference between the methodology of evaluating in vitro and in vivo SPF of photoprotective formulations could generate divergent results, depending on the nature of the samples. For instance, Tomazelli and colleagues demonstrated an increase in the in vivo SPF of a formulation containing rutin, a known antioxidant ingredient, while in vitro studies did not perform equally [43]. Also, the excipients used in these samples may have been responsible for the absence of improvement in the in vitro SPF. Finally, we suggest the development of new formulations and in vivo assays to confirm the hypothesis, evidencing the potential of RA in multifunctional formulations [40,41].

5. Conclusions

We were able to achieve the RA performance regarding antioxidant and SPF properties through in vitro experiments. Through QbD and PAT, we obtained the equations for predicting the in vitro antioxidant activity and SPF. Considering our sunscreen system, developed with octyl *p*-methoxycinnamate and bemotrizinol, the presence of RA increased its antioxidant capacity; however, the in vitro SPF was reduced when both UV filters were used. The development of multifunctional sunscreens is of utmost importance; moreover, there is a need for the rational development of formulations that ensure representative statistical tests of the effects and interactions among the components of a formulation on the desired critical quality attributes, including efficacy.

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Conflicts of Interest: The authors declare no conflict of interest.

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Article 3

Rosmarinic acid multifunctional sunscreen: comet assay and *in vivo* establishment of cutaneous attributes

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Rosmarinic Acid Multifunctional Sunscreen: Comet Assay and In Vivo Establishment of Cutaneous Attributes

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Abstract: The skin acts as a protective barrier, guarding the body against microorganisms, chemicals, and several environmental factors. Accordingly, this all-important organ must be kept healthy to maintain its optimal functionality. One approach to maintain skin health is the application of multifunction bioactive sunscreens containing antioxidant molecule(s). Rosmarinic acid (RA), a phenolic compound, is known for its antioxidant activity. Herein, the safety and efficacy of a multifunction prototype sunscreen were investigated, aiming to evaluate the performance of this polyphenol with two known and widely used UV filters (bemotrizinol and octyl *p*-methoxycinnamate). Samples protected the DNA fragmentation compared to UV control, by the comet assay, and showed good skin compatibility in subjects. Formulations F1 and F3 were able to increase skin hydration, and, possibly, the RA interfered with this attribute. An increase in transepidermal water loss was observed for formulations F1, F2, and F4, which may be related to the vehicle, containing the RA or not. No decreases were observed in the inflammatory reaction caused by the ethyl nicotinate with any of the samples. As a perspective, we suggest trials with a greater number of subjects or protocol modifications. Altering the vehicle qualitative and quantitative composition is also a pertinent perspective.

Keywords: rosmarinic acid; anti-inflammatory activity; comet assay; sunscreen; skin biocompatibility

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1. Introduction

It is known that reactive species are naturally originated in biochemical processes such as energy production, inflammation, phagocytosis, and cell growth regulation, among others. External factors are also able to provide the production of species such as exposure to ultraviolet (UV) radiation, ozone, X-rays, and air pollutants [1–4]. In the skin, free radicals are constantly generated by cutaneous cells such as fibroblasts and keratinocytes. Nevertheless, healthy skin can eradicate these reactive species with nonenzymatic (glutathione, ubiquinol, vitamins C and E) and enzymatic (glutathione peroxidase, glutathione reductase catalase, superoxide dismutase, and thioredoxin reductase) antioxidants [5–7].

When the number of free radicals produced in the tissues exceeds the antioxidant defenses, what is known as oxidative stress occurs. As a result, the peroxidation of lipid membranes, damage to DNA, protein, and enzymes appear, and these harms, in turn, worsen in cognitive dysfunction, degenerative cardiovascular diseases, and cancer, among other illnesses [8].

Energetic photons of UV light can be transmitted through the layers of the skin and after that, can be absorbed by cellular chromophores. DNA bases are cellular chromophores; thus, they can directly absorb UV photons, initiating photo-induced reactions. Furthermore, damage in the cells may occur by photosensitization processes, where endogenous or exogenous sensitizers absorb UV photons. Depending on the type, the distribution and concentration of skin chromophores, epidermal thickness, and its functional condition, photobiochemical reactions occur, provoking changes in the cell and tissue biology due to the formation of reactive oxygen species (ROS). The interactions between ROS and cellular biomolecules provoke a final biological response, as illustrated in Figure 1 [5,6,9,10].

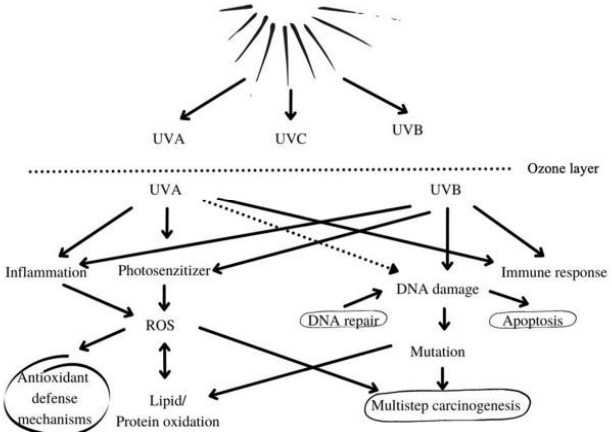

Figure 1. Action of UV radiation on the cellular biomolecules (modified from [5]).

Although the formation of these reactive species is inevitable, it is possible, with the administration of endogenous and exogenous antioxidants, to minimize their formation, thus avoiding injurious effects to the organisms. Molecules must perform one or more of the following actions to act as antioxidants: oxygen depletion; single oxygen extinction; chelation of metal ions that would otherwise catalyze reactions to form reactive oxygen species; elimination of reactive oxygen species or termination of the oxidation propagation chain reaction; and the prevention of oxidative damage. Hydroxyls attached to aromatic rings such as in polyphenols can react with free radicals, as shown in Figure 2 [8].

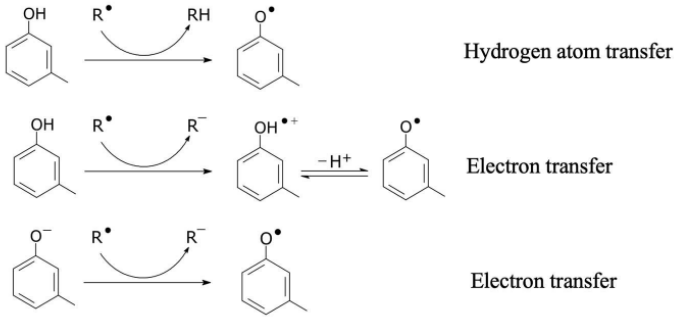

Figure 2. Scheme of reactions between the phenolic compounds and free radicals (modified from [8]).

Rosmarinic acid (RA) is a polyphenol whose molecular structure was first elucidated in 1958. Its molecule was first isolated from the leaves of *Rosmarinus officinalis* L. and later from plants belonging to the families Lamiaceae (e.g., lavender, sage and mint) and Boraginaceae (e.g., cordia and echium). The name "rosmarinic acid" was given precisely since it was first extracted from rosemary (*R. officinalis* L.). It is an ester derived from caffeic and 3,4-dihydroxyphenylacetic acids. The RA molecule has a carboxylic group, two

aromatic rings A and A′ with *ortho*-catechol structures, the unsaturated C=C bond and the ester portion [11]. Different studies have shown that RA can act biologically in several ways, being an antiviral, antibiotic, anti-inflammatory, and antioxidant [12–15].

The daily topical application of sunscreens can protect the skin from UV rays and thus delay or reduce the occurrence of skin harm such as the appearance of melanomas, spots, and wrinkles. Considering that antioxidants are capable of helping to minimize the occurrence of ROS that are related to the damages previously mentioned, the association of such molecules to the photoprotective formulations optimizes the beneficial effects of these products over the skin [9,14–17].

Considering the photoprotective and antioxidant potential of RA, in this research work, the safety and efficacy of a multifunction prototype sunscreen were investigated, aiming to evaluate the performance of this polyphenol with two known and widely used UV filters, bemotrizinol and octyl *p*-methoxycinnamate.

2. Materials and Methods

2.1. Materials

Rosmarinic acid (96%), agarose ethylenediaminetetraacetic acid disodium salt dihydrate, sodium chloride, Trizma, Triton X-100, dimethyl sulfoxide, phosphate buffer, methyl nicotinic, and sodium hydroxide were purchased from Sigma-Aldrich (St Louis, MO, USA). Octyl *p*-methoxycinnamate and bemotrizinol were purchased from Mapric (São Paulo, Brazil) and Brasquim (São Paulo, Brazil), respectively. The water was purified in a Milli-Q-plus System (Merck Milipore, USA). Long-lived, spontaneously immortalized human keratinocyte cells (HaCaT) were obtained from the Cell Bank of Rio de Janeiro. DMEM supplemented with 10% fetal bovine serum, trypsin, and SYBR Gold (1:10,000, Invitrogen—Cat S11494) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Methods

2.2.1. Composition of the Formulations

Four formulations containing commercially available octyl *p*-methoxycinnamate (UVB filter), bemotrizinol (broad spectrum filter), plus 1.0% (*w*/*w*) of the bioactive compound rosmarinic acid were used in the study [17]. The active ingredients of the formulations are presented in Table 1.

Table 1. Composition of the active ingredients of the samples.

	Compounds (% *w*/*w*)		
Formulation	Rosmarinic Acid	Octyl *p*-methoxycinnamate	Bemotrizinol
F1	-	-	-
F2	1.0	-	-
F3	-	7.5	10.0
F4	1.0	7.5	10.0

Complete composition of the samples can be found in Table S1 (Supplementary Materials).

2.2.2. In Vitro Assay

In Vitro Evaluation of DNA Fragmentation of Human Cells Exposed to UV Radiation by the Comet Assay Method

Human Keratinocytes Cell Culture

Human keratinocyte (HaCaT) cells were seeded in 75 cm^2 bottles, cultivated, and expanded in a humid atmosphere, in the presence of 5% CO_2 at 37 °C using DMEM supplemented with 10% fetal bovine serum. Upon reaching 70% confluence, cells were trypsinized and seeded in 6-well plates for later treatments and the further evaluation of DNA fragmentation.

Incubation with the Formulations and Cells Irradiation

Cells were incubated with the samples at two non-cytotoxic concentrations, 0.0049 and 0.00245% (*w*/*v*), for 24 h for subsequent exposure to UV radiation. After the incubation time, cells were submitted to 3 J/cm^2 of UVA/UVB in a sun simulation chamber (Suntest CPS+, Atlas, Linsengericht, Germany). Then, cells were kept in suitable culture conditions for an additional 24 h. Cell lysates were collected and proceeded with the comet assay.

Comet Assay

Cell lysates were centrifuged for 5 min at 1500 rpm. Afterward, the cell pellet was mixed with 1000 µL of 0.75% low melting point agarose. Immediately, 100 µL of this suspension was spread onto slides previously coated with 1% agarose. After solidification, the slides were immersed in a freshly prepared lysing solution [2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA Na_2S), and 10 mM Trizma Base, pH 10], with the addition of 1% Triton X-100 and 10% dimethyl sulfoxide, for at least 1 h. After lysis, slides were incubated for 5 min in cold PBS (free Ca^{2+} and Mg^{2+}) and for 40 min in alkaline solution (pH >13). Cells on the slides were submitted to electrophoresis (300 mA, 25 V) for 30 min. Slides were neutralized by three washes with neutralization buffer (pH 7.5) and the DNA was precipitated. DNA was stained with SYBR Gold (1:10,000, Invitrogen—Cat S11494). A number of 150 cells was randomly selected on each slide and analyzed using an optical fluorescence microscope (Leica, DM 6000 B, Wetzlar, Germany) coupled with a camera of 2.8 MP (Leica, DFC7000 T, Wetzlar, Germany).

The comet tail length was classified into four classes (scores): 1 = low damage; 2 = medium damage; 3 = high damage; and 4 = almost all DNA in the tail. The final score of each experimental group was performed by multiplying the number that represented the class of the comet and the total number of comets found in this class. The sum of the results from the multiplications was carried out, corresponding to the final score [18].

2.2.3. In Vivo Assays

Subjects

The protocol was concluded with 12 healthy subjects with phototypes II to VI, after oral information and written consent. The procedures were in accordance with the ethical standards on human experimentation (Ethics Committee of Universidade Lusófona's Research Center for Biosciences & Health Technologies) and the Declaration of Helsinki. Exclusion criteria were the presence of dermatitis or other skin or allergic disease and smoking. Subjects were instructed not to apply any topical products to the test sites during the study but were allowed to wash normally. The mean age was 32 ± 12 years old (20 to 44 years).

Skin Biocompatibility

Aliquots of the formulations were applied to predefined sites on both forearms (two formulations per forearm of each subject, in a 9.0 cm^2 area). The application site of the samples was randomized to minimize the occurrence of possible biases. Measurements were taken before and after 30 min of application with a corneometer (stratum corneum hydration), a Tewameter TM300 (skin barrier quantified through the transepidermal water loss, TEWL), a pH meter (pH value of the skin surface) (C&K Electronics GmbH, Germany), and a chromameter (red color, a *, according to CIE Lab system) (Minolta Camera Co., Osaka, Japan). Afterward, the subjects were instructed to apply the formulations twice a day for 7 days. On the eighth day, new measurements were performed. The non-invasive measurements were conducted according to the specialized literature and guidelines (n = 3) [19–22]. To reduce the interindividual variability, the results were treated as the ratio between the response and the basal/control value.

Anti-Inflammatory Activity

Aliquots of the samples were applied to the forearm of each volunteer in a 9.0 cm^2 area. The study was conducted in two phases: a pre-treatment, which consisted in the application of the samples twice a day for 7 days. On the eighth day, the second phase

began, where erythema formation was induced in each pre-treated area by applying filter paper (2.25 cm^2) saturated with an aqueous solution of methyl nicotinate (0.5% *w*/*w*) for 60 s. Measurements of the skin microcirculation of individual test sites were recorded continuously for 15 min using laser doppler flowmetry equipment (PeriFlux System 5000, Perimed, Stockholm, Sweden). Therefore, the in vivo evaluation of the anti-inflammatory effect was based on the ability of the samples to decrease the extent of an erythema-induced response. The area under the curve of the perfusion profile obtained at each site was measured for all subjects, together with the slope of the tangent line in the hyperemia stage [23]. Results were analyzed as the ratio between the values obtained at each sample site and the control values for the volunteers, in the selected parameters.

2.3. Statistical Analysis

Experiments were conducted randomly, in triplicate, and with a significance level of 5% ($p \leq 0.05$), conducted by Minitab software, version 18 (Minitab, Inc., State College, PA, USA).

3. Results

3.1. In Vitro Evaluation of DNA Fragmentation of Human Cells Exposed to Ultraviolet Radiation by the Comet Assay Method

The efficacy of the samples was evaluated in vitro by the comet assay, observing the fragmentation of the treated and untreated human keratinocyte (HaCaT) cells after exposure to UV radiation. Results are shown in Figures 3 and 4.

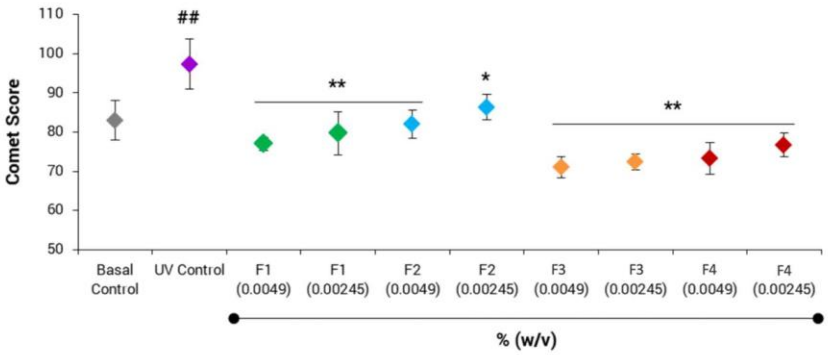

Figure 3. Evaluation of DNA protection potential of samples F1, F2, F3 and F4 in human keratinocytes after exposure to UV radiation. Data represent the mean ± standard deviation of three independent experiments. * $p < 0.01$ compared to the UV control group; ## $p < 0.01$ compared to the basal control group; ** $p < 0.01$ compared to the UV control group (ANOVA, Dunnet, Scotland).

It was possible to observe the protective behavior of F1, F2, F3, and F4. The UV control induced 17% of DNA fragmentation in comparison to the basal one. All tested samples under exposure to UV radiation in both concentrations promoted a significant decrease in the DNA fragmentation of 21 and 18%, 16 and 11%, 27 and 26%, 25 and 21%, respectively, in comparison to the UV control.

3.2. In Vivo Skin Biocompatibility

To evaluate the skin biocompatibility of the formulations, measurements were performed with a corneometer, Tewameter TM 300, pH meter, and chromameter equipment. The results, as the ratio between the response and basal/control, are shown in Table 2.

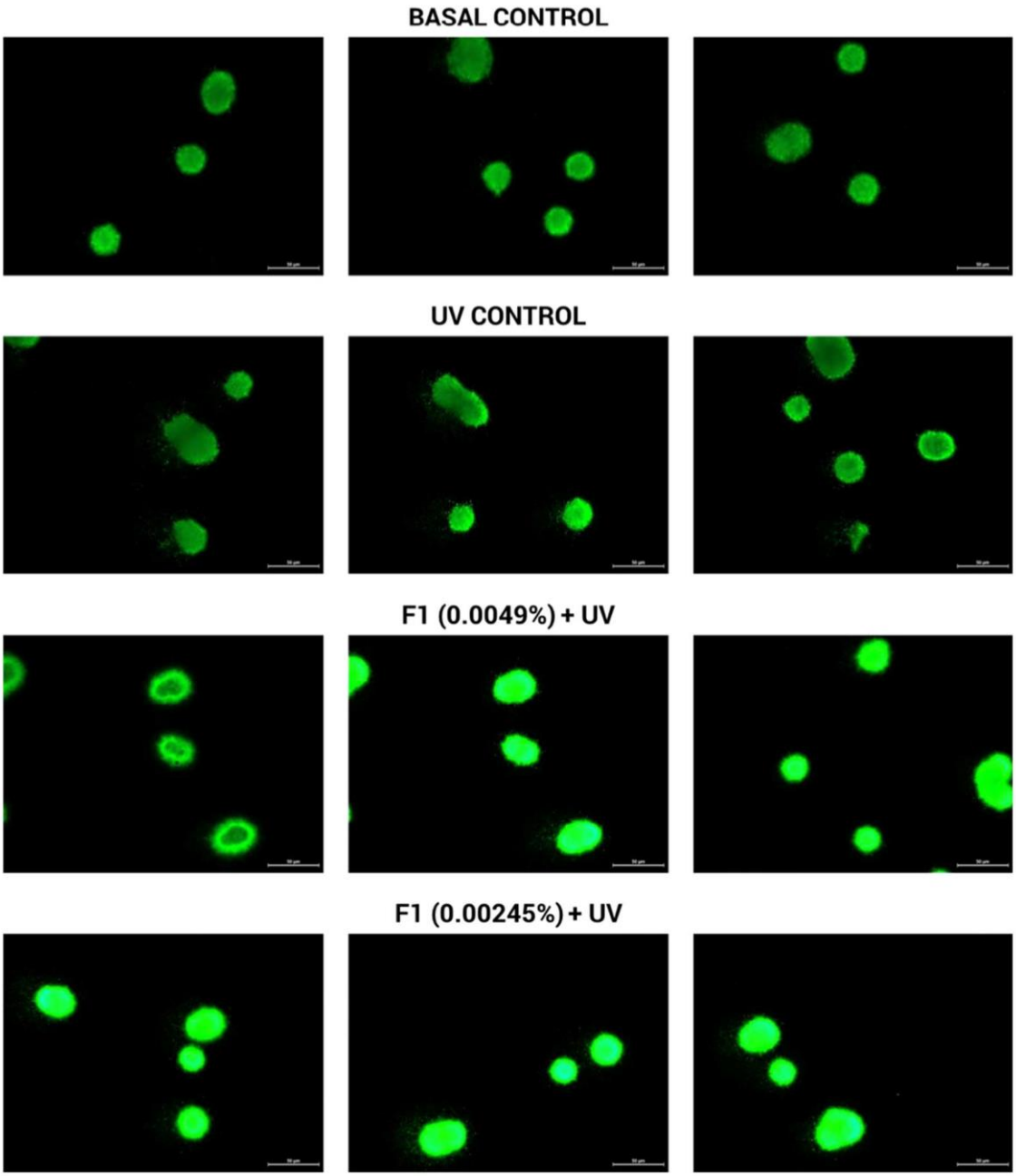

Figure 4. *Cont.*

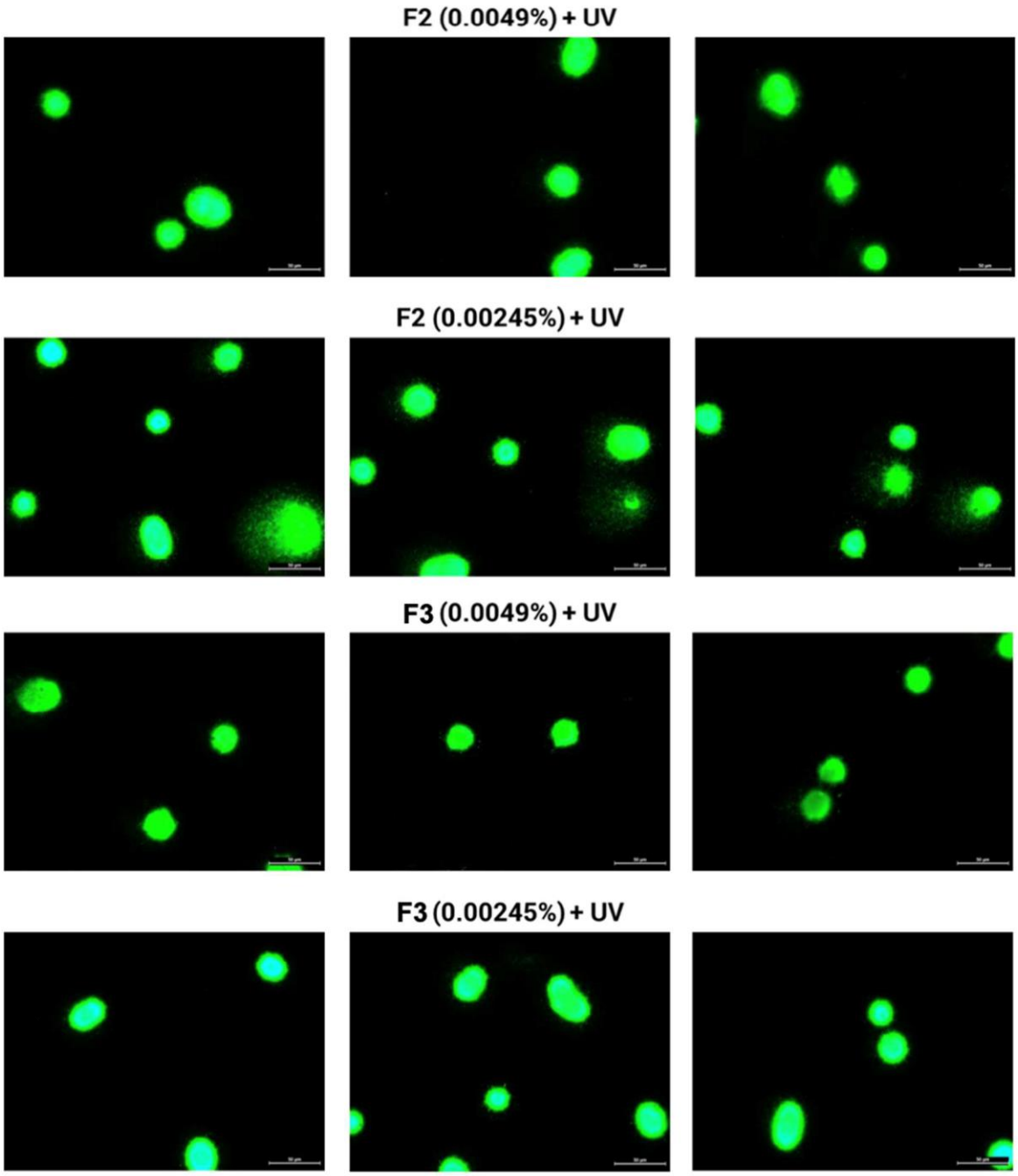

Figure 4. *Cont.*

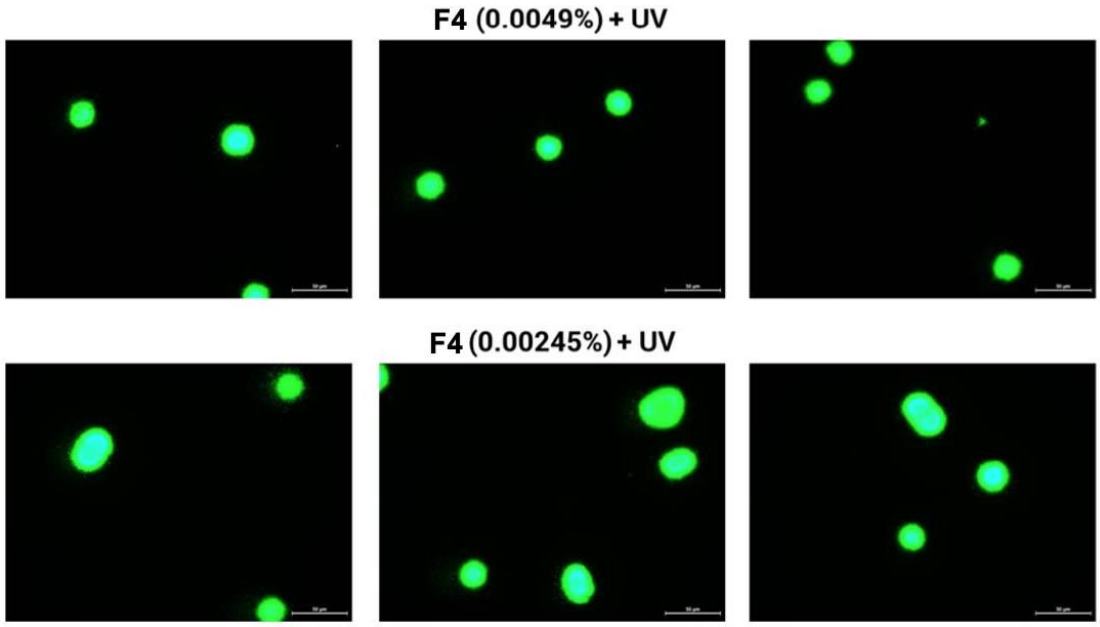

Figure 4. The comet assay method, microscope images at 1000× magnification. Cell DNA damage in human keratinocytes, being basal control (cell culture in regular condition); UV control (cell culture submitted to UV radiation); and the test products F1, F2, F3, and F4 in cell cultures exposed to UV radiation.

Table 2. In vivo skin biocompatibility by the corneometer, Tewameter TM 300, pH meter, and chromameter.

Assay	Sample	Time after First Application	Mean ± Standard Deviation (Ratio)	*p*-Value
Superficial hydration (Corneometer)	F1	30 min	1.405 ± 0.070	**0.000**
		7 days	1.356 ± 0.266	**0.022**
	F2	30 min	1.285 ± 0.242	**0.034**
		7 days	1.363 ± 0.401	0.077
	F3	30 min	1.557 ± 0.306	**0.007**
		7 days	1.435 ± 0.332	**0.024**
	F4	30 min	1.312 ± 0.141	**0.003**
		7 days	1.328 ± 0.381	0.089
TEWL/Transepidermal water loss (Tewameter)	F1	30 min	1.554 ± 0.337	**0.010**
		7 days	1.702 ± 0.727	0.065
	F2	30 min	1.692 ± 0.635	**0.044**
		7 days	1.168 ± 0.516	0.462
	F3	30 min	1.591 ± 0.598	0.060
		7 days	1.493 ± 0.494	0.058
	F4	30 min	2.213 ± 1.137	**0.048**
		7 days	1.891 ± 0.727	**0.030**

Table 2. *Cont.*

Assay	Sample	Time after First Application	Mean ± Standard Deviation (Ratio)	*p*-Value
a* (redness) (Chromameter)	F1	30 min	1.007 ± 0.143	0.904
		7 days	1.045 ± 0.153	0.502
	F2	30 min	1.001 ± 0.079	0.967
		7 days	1.044 ± 0.063	0.150
	F3	30 min	0.986 ± 0.037	0.386
		7 days	1.056 ± 0.079	0.140
	F4	30 min	0.993 ± 0.046	0.709
		7 days	1.054 ± 0.078	0.153
Skin pH value (pH meter)	F1	30 min	0.994 ± 0.063	0.819
		7 days	0.977 ± 0.089	0.560
	F2	30 min	0.993 ± 0.043	0.703
		7 days	0.983 ± 0.079	0.617
	F3	30 min	1.028 ± 0.082	0.446
		7 days	1.010 ± 0.102	0.813
	F4	30 min	1.040 ± 0.062	0.179
		7 days	1.028 ± 0.077	0.414

Comparisons were made before and after the application.

3.3. In Vivo Anti-Inflammatory Activity

The anti-inflammatory potential of the formulations consisted in their ability to decrease the extent of an induced erythema response in subjects, being the results shown in Table 3.

Table 3. The ratio between the values obtained at each sample site and the control values for the parameters of the area under curve and slope of the tangent line in the hyperemia.

Parameter	Sample	Mean ± Standard Deviation (Ratio)	*p*-Value
Area under the curve	F2	1.572 ± 1.280	0.169
	F3	1.020 ± 0.575	0.910
	F4	1.235 ± 0.993	0.451
Tangent	F2	1.918 ± 1.273	**0.038**
	F3	1.280 ± 0.862	0.307
	F4	1.741 ± 1.600	0.155

Comparisons were between the control and each applied sample.

4. Discussion

Formulations, at both concentrations, into the cell cultures, under the UV stress condition, protected the DNA fragmentation compared to UV control. Although we noticed the decrease in DNA fragmentation, we could not exclusively attribute this finding to the presence of RA in the samples, since F1 and F3 were absent in this phenolic compound. Possibly, the RA concentration at 1.0% was not completely enough to develop a distinctive performance among the samples, since all of them presented some level of DNA protection ranging from 11 to 27% including the blank sample (F1) [14,24,25]. Additionally, the test could have a limitation when complex samples are performed. Furthermore, our protocol similarly indicated that the blank sample and the sunscreen one (without RA)

protected the cells from DNA fragmentation under UV stress, and specialized literature has reported that RA was able to reduce the extant of DNA injury by other mechanisms through doxorubicin or sepsis, for instance [26,27]. Although investigations point to RA as a molecule capable of protecting DNA fragmentation, studies that have investigated its influence in a multifunctional photoprotective formulation are restricted. For this reason, we used a complex multifunctional sample, compatible with the available commercial products, to study DNA fragmentation by the comet assay method. The effect of RA, as an isolated compound, in the protection of DNA damage against UV radiation is reported in the specialized literature. Pérez-Sánchez and coworkers evaluated the RA protective effects in human keratinocytes and reported an increased survival of the cells upon UVB radiation [28]. In similar research, Psotova and coworkers demonstrated that RA exhibited the ability to reduce the decrease in the cell viability of keratinocytes exposed to UVA radiation [29]. Although these results are promising, complex dermocosmetic formulations must be tested in scenarios more adjusted to consumer use.

F1 and F3 provided an increase in skin hydration 30 min after their application and after daily application (p-value < 0.05), twice a day. F2 and F4 had the same effect, however, only after 30 min of application (p-value < 0.05). The increase in skin hydration could be related to the composition of the dermocosmetic sample composed of emollients and humectants such as glycerin, isopropyl myristate, and silicones (cyclopentasiloxane, dimeticone, and trimethylsiloxysilicate). These ingredients could have contributed to the skin moisture by humectancy and occlusion [30,31]. We noticed that samples containing RA, independently of the presence of UV filters, were not able to maintain the cutaneous superficial hydration effect on the seventh day, however, all samples positively interacted with this skin attribute after 30 min. Tomazelli and coworkers and Ruscinc and coworkers observed compatible results from their multifunction bioactive sunscreens containing 0.1% rutin and 5.0% *Vaccinium myrtillus* L. extract, respectively, when in vivo skin hydration was not improved after the application of those products [32,33].

Treatments with F1 and F2 resulted in an increase in the TEWL after 30 min of application (p-value < 0.05). Treatment with the F4 resulted in an increase in TEWL at both times of analysis (30 min and 7 days) and F3 did not interfere in this cutaneous tissue attribute. Considering the scenario of our results, we may infer that our vehicle, containing RA or not, provided some level of disturbance over the barrier attribute of the skin by elevating the TEWL; nonetheless, the presence of UV filters in F3 (absence of RA) may have inhibited such an effect. These findings may also be related to the presence of diethylene glycol monoethyl ether in all samples, which can increase the permeation of substances when applied over the skin as well as cause an increase in cutaneous water loss [32,33]. Additionally, in the pH and a * parameters, no significant differences were found (p-value > 0.05) compared to the control, which indicated that, despite the increase in TEWL, the samples developed a safe profile for topical application as they did not cause redness nor changes on the skin pH value [23].

It has been extensively reported that topical application of nicotinic acid and its esters causes local erythema [34,35] by vasodilation of peripheral blood capillaries in the dermal papillae of dermis through prostaglandin D_2 and E_2 releases. The prostaglandin D_2 liberation also occurs by the incidence of UV radiation on the skin by the generation of ROS [36]. Considering this phenomenon, the association of antioxidants in photoprotective formulations can optimize skin protection. RA is known for its antioxidant potential by free radical scavenging action proven by several experimental protocols such as DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging and TBARS (malonyl dialdehyde formation) [14,17,37]. Its molecule presents two catechol structures conjugated with a carboxylic acid group as an important structural element in the antioxidant activity of this compound [38]. Its activity as a photoprotective adjuvant ingredient was evaluated with successful results in the elevation of the sun protection factor (SPF) in vivo of a sunscreen system and RA also increased the tyrosinase activity and its expression level in B16 melanoma cells [15,39,40].

Regarding the area under the curve, no significant differences were reached when comparing the sites pretreated with the samples and the control (p-value > 0.05). The slope of the tangent line in the hyperemia results followed a similar trend, and F2 slightly accelerated the formation of the erythema caused by ethyl nicotinate when compared with the control sample. Thus, RA formulations, divergent to that expected, were not able to decrease the onset and erythema intensity according to our protocol. Matwiejczuk and coworkers researched a RA protective effect against the influence of methylparaben and propylparaben on collagen in fibroblasts. Parabens can inhibit the biosynthesis of collagen and reduce the proliferation and viability of human skin fibroblasts [41]. In this investigation, RA provided protection against these changes, being the findings related to the RA antioxidant capacity [42]. Fernando and coworkers demonstrated the antioxidant capacity of RA by a reduction in UVB-induced intracellular ROS and weakening oxidative damage to protein and DNA [14]. Pattananandecha and coworkers studied the effects of an extract containing RA on UVA-irradiated human skin fibroblasts. The extract was able to inhibit ROS and matrix metalloproteinase-1 [43]. Several studies involving RA in modified systems has also been reported [44–48]. For instance, Perra and colleagues developed hyalurosomes loaded with an extract rich in RA and proved, in vitro, its protection against oxidative stress in skin fibroblasts [49].

5. Conclusions

The samples in vitro protected the DNA fragmentation compared to the UV control and showed in vivo good skin compatibility. Formulations F1 and F3 were able to increase skin hydration, and, possibly, the RA interfered with this attribute. An increase in TEWL was observed for formulations F1, F2 and F4, which may be related to vehicle composition, containing or not the RA. Regarding the in vivo anti-inflammatory efficacy, no decreases were observed in the inflammatory reaction caused by the ethyl nicotinate with any of the evaluated formulations. As a perspective, we suggest trials with a greater number of subjects or protocol modifications such as the application of a monitored sample, a greater number of applications per day, or even the occlusion of the application site. Altering the qualitative and quantitative composition of the vehicle is also a pertinent perspective.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cosmetics9060141/s1, Table S1: Qualitative and quantitative composition of the formulations.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Universidade Lusófona's Research Center for Biosciences & Health Technologies (protocol code CE.ECTS/P11.21 approved on 16 November 2021) for studies involving humans.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Supplementary Material

Table S1 - Qualitative and quantitative composition of formulations.

	Compounds	Proportion (%w/w)			
		F1	F2	F3	F4
Oil Phase	Bemotrizinol	-	-	10.0	10.0
	Octyl *p*-methoxycinnamate	-	-	7.5	7.5
	Simulgel® INS 100 (hydroxyethyl acrylate/sodium acryleoyldimethyl taurate copolymer; Isohexadecane; polysorbate 60)	5.0	5.0	5.0	5.0
	Cocoate® BG (butylene glycol cocoate)	6.0	6.0	6.0	6.0
	Hostacerin® SAF (mineral oil, isopropyl palmitate, trilauret-4 phosphate, rapeseed oil sorbitol esters and ammonium acrylyldimethyltaurate copolymer)	3.0	3.0	3.0	3.0
Aqueous Phase	Rosmarinic acid	-	1.0	-	1.0
	Transcutol® (diethylene glycol monoethyl ether)	2.5	2.5	2.5	2.5
	Glycerin	2.5	2.5	2.5	2.5
	Parabens + phenoxyethanol	0.5	0.5	0.5	0.5
	Disodium edetate	0.1	0.1	0.1	0.1
	Pemulen® TR-2 (copolymer of acrylates/C10-30 alkyl acrylate)	0.2	0.2	0.2	0.2
	Purified water	80.2	79.2	62.7	61.7

Legend (-) = Compound not added

Conclusions

4. CONCLUSIONS

Through the review paper in Article 1, it is shown that, due to the range of methods available to investigate the skin conditions, it was possible to prove the effects of supplementation on the cutaneous tissue. A study involving supplementation with rosmarinic acid demonstrated its effects in protecting the skin against UVB rays, increasing skin elasticity and decreasing the depth of wrinkles; results attributed to the antioxidant properties of this molecule.

Regarding the topical use of rosmarinic acid, the concepts of QbD were successfully used to design the formulations and the process. From the results of the sun protection factor (SPF) and antioxidant activity, *in vitro*, prediction equations involving the variables rosmarinic acid, bemotrizinol and octyl *p*-methoxycinnamate were obtained. Through the equations and the interference study performed, it was verified that rosmarinic acid increased the antioxidant activity of the formulations, while decreasing the SPF. An increase in the photostability of the formulation containing the two UV filters was also observed for the rosmarinic acid. As a perspective, *in vivo* SPF evaluation is suggested to confirm the action of rosmarinic acid in the formulation.

Four samples were chosen for the assessment of comet assay and skin biocompatibility (stratum corneum hydration, transepidermal water loss, skin surface pH value and redness). Also, the evaluation of anti-inflamatory activity *in vivo*, using laser doppler flowmetry equipment, was performed. Through comet assay, all formulations protected DNA fragmentation, however, we did not attribute this finding to the presence of rosmarinic acid exclusively in the samples, since two of the formulations were absent from this phenolic compound. By the *in vivo* tests, none of the formulations caused an alteration of the pH value of the skin surface or an increase in redness, which were signs of skin compatibility. All formulations provided increase in skin hydration after 30 minutes of application. However, only the formulations without rosmarinic acid (F1 and F3) provided increase in skin hydration after 7 days of application. An increase in TEWL was observed for F1, F2 and F4. We may conclude that both results (skin hydration and TEWL) were related to the composition of the vehicle containing or not the RA. In respect of the *in vivo* evaluation of anti-

inflammatory efficacy, no decreases were observed in the inflammatory reaction caused by ethyl nicotinate with any of the evaluated samples. However, the formulation containing rosmarinic acid (F2) presented an increase in the tangent value, meaning that the response to the inflammatory reaction was faster than observed for the other formulations.

In view of the specialized literature and our results, we may suggest that new *in vivo* tests should be carried out, with modifications in the protocol of application of the samples on the skin and even the investigation of other vehicles for the active ingredients.

Scientific Production

SCIENTIFIC PRODUCTION

I. **CÂNDIDO, T.M.**; ARIEDE, M.B.; PINTO, C.A.S.O.; LOURENCO, F.R.; ROSADO, C.; VELASCO, M.V. R.; BABY, A.R. Prospecting *in vitro* Antioxidant and Photoprotective Properties of Rosmarinic Acid in a Sunscreen System Developed by QbD Containing Octyl *p*-Methoxycinnamate and Bemotrizinol. *Cosmetics*, v. 9, p. 29, 2022.

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