Article

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.

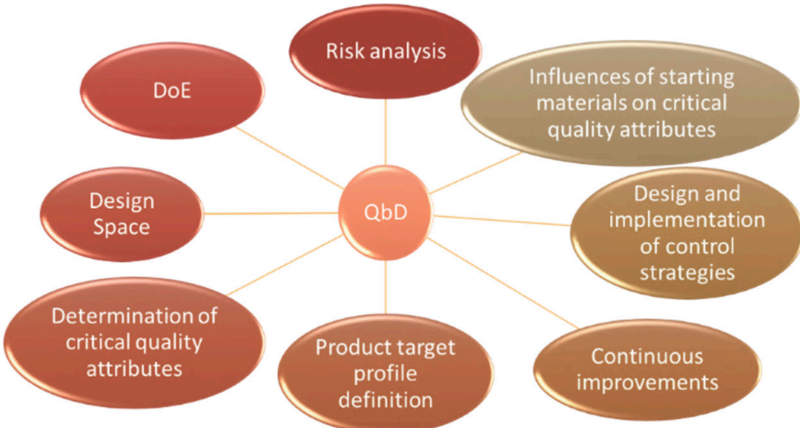

Figure 1. Illustrative summary of key elements of the quality by design study.

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.

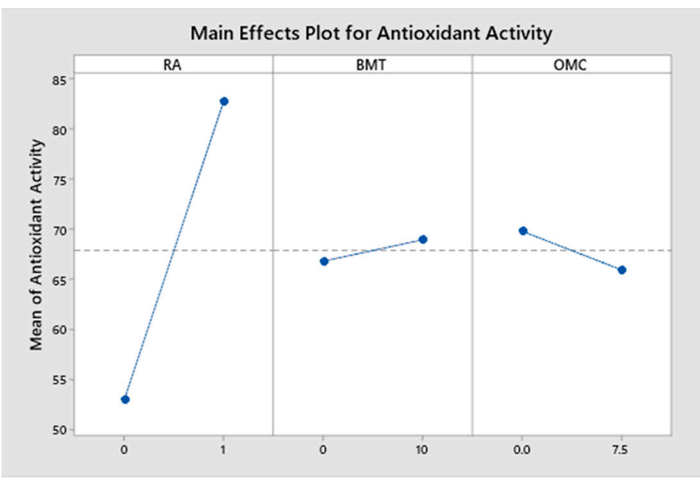

Figure 2. Graph of main effects for the antioxidant activity of the formulations.

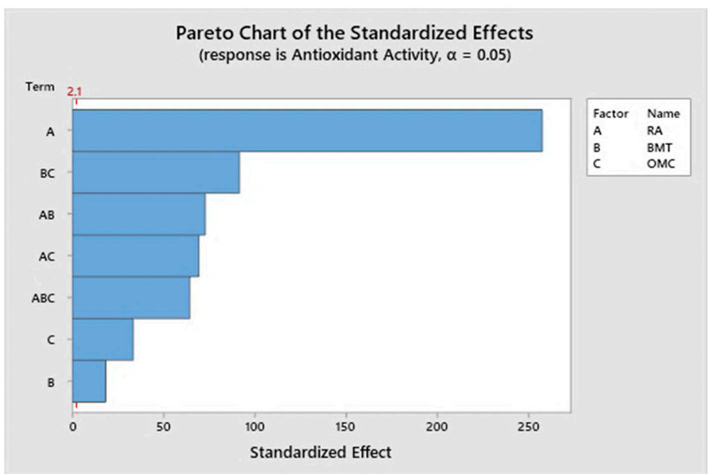

Figure 3. Representation of the effect of isolated and combined variables on the antioxidant activity of formulations (Pareto chart).

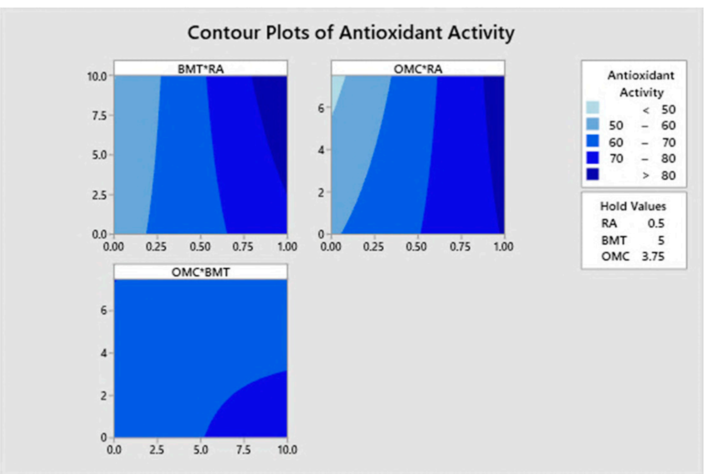

Figure 4. Contour chart for antioxidant activity.

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.

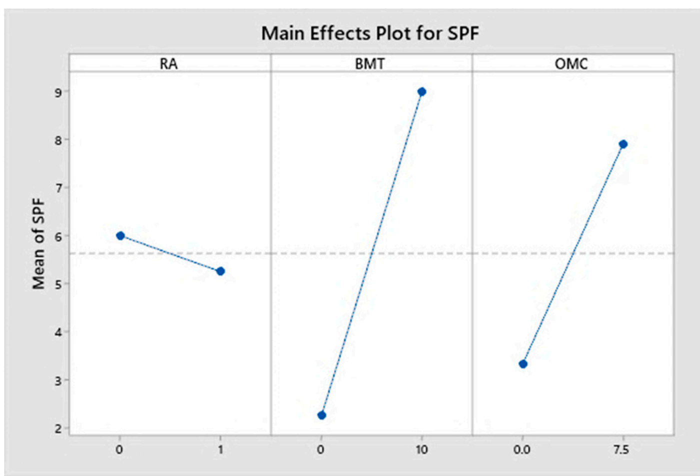

Figure 5. Graph of main effects for in vitro SPF of the formulations.

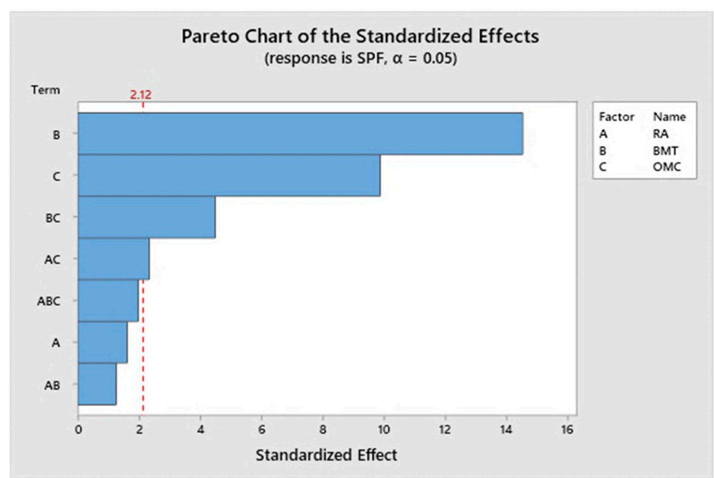

Figure 6. Pareto chart representation of the effect of isolated and combined variables on the in vitro SPF of the formulations.

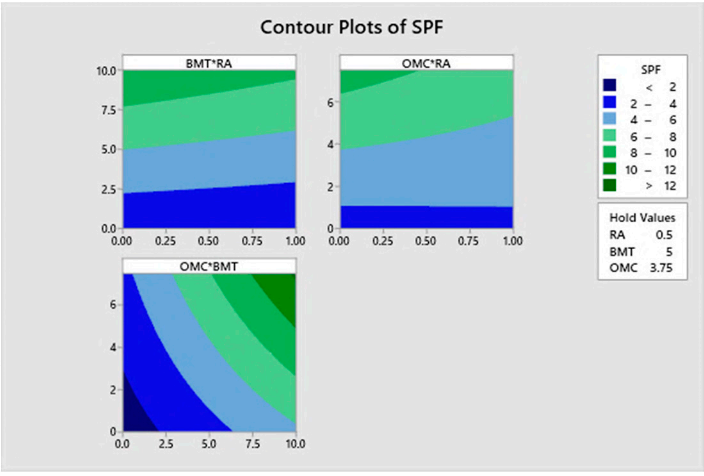

Figure 7. Contour chart for in vitro SPF.

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.

$$SPF = 1.000 - 0.000\ RA + 0.4333\ BMT + 0.356\ OMC + 0.067\ RA \times BMT - 0.044\ RA \times OMC + 0.0800\ BMT \times OMC - 0.0489\ RA \times BMT \times OMC \quad (3)$$

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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