
Article

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 probe putative synergies 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 vehicle containing or not the RA. 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. Altering the vehicle qualitative and quantitative composition is also a pertinent perspective.

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

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 production of such species, like exposure to ultraviolet (UV) radiation, ozone, X-rays and air pollutants [1–4]. In the skin, free radicals are constantly generated by cutaneous cells, like 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 amount of free radicals produced in the tissues exceeds antioxidant defenses, what is known as oxidative stress occurs. As a result, peroxidation of lipid membranes,

damage to DNA, protein and enzymes appear, and these damages, in turn, worsens 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, damages in the cells may occur by photosensitization processes, where endogenous or exogenous sensitizers absorb UV photons. Depending on the type, distribution and concentration of skin chromophores, epidermal thickness and its functional condition, photobiochemical reactions occur, provoking changes in 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].

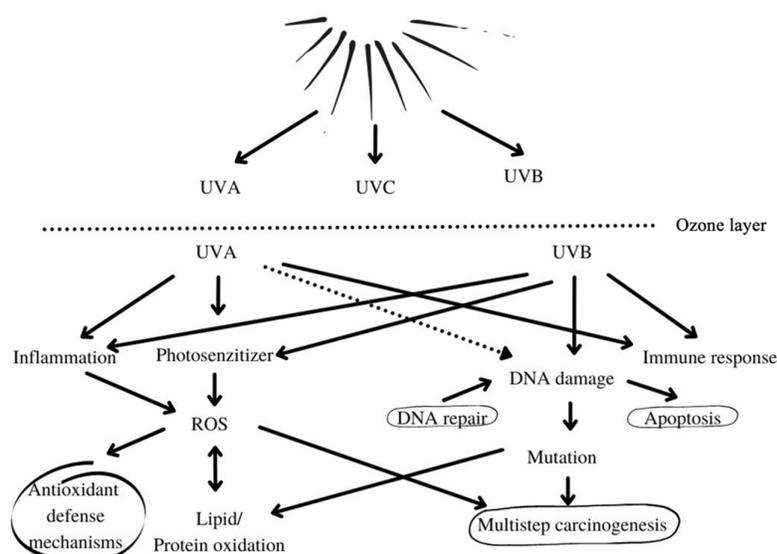


Figure 1. Action of UV radiation on cellular biomolecules (modified from ref. [5])

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 harmful 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 oxidation propagation chain reaction; and prevention of oxidative damage. Hydroxyls attached to aromatic rings, as in polyphenols, can react with free radicals, as shown in Figure 2 [8].

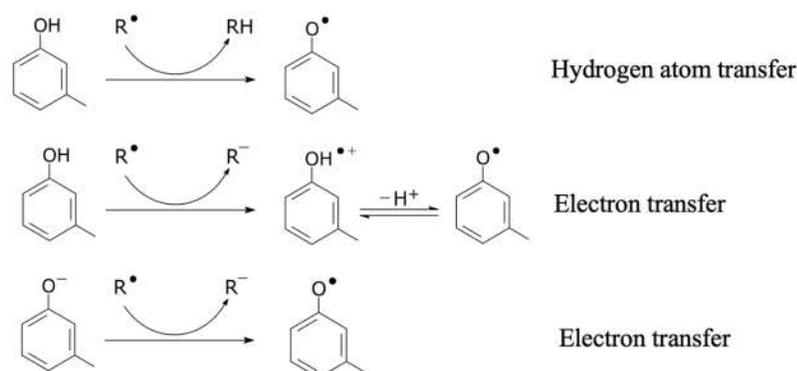


Figure 2. Schematic of reactions between phenolic compounds and free radicals (modified from ref.[8]).

Rosmarinic acid (RA) is a polyphenol which molecular structure was first elucidated in 1958. RA molecule was first isolated from the leaves of the *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 acid and 3,4-dihydroxyphenylacetic acid. 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 molecule [12–16]. The daily topical application of sunscreens can protect the skin from UV rays and, thus, delay or reduce the occurrence of skin damage, 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 on the skin [9,14,15,17,18].

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 probe putative synergies of this polyphenol with two known and widely used UV filters, the bemotrizinol and the 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 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 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 [18]. Formulations' active ingredients are presented Table 1.

Table 1. Active ingredients' composition of the samples *.

Formulation	Compounds (% w/w)		
	<i>Rosmarinic Acid</i>	<i>Octyl p-methoxycinnamate</i>	<i>Bemotrizinol</i>
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

2.2.2.1. In vitro evaluation of DNA fragmentation of human cells exposed to UV radiation by comet assay method

2.2.2.1.1. Human keratinocytes cell culture

Human keratinocytes (HaCaT) cells were seeded in 75 cm² bottles, cultivated, and expanded in a humid atmosphere, in the presence of 5% CO₂, 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 further evaluation of DNA fragmentation.

2.2.2.1.2. Incubation with the formulations and cells irradiation

Cell were incubated with the samples at two non-cytotoxic concentrations, 0.0049 and 0.00245% (w/v), for 24 hours for subsequent exposure to UV radiation. After the incubation time, cells were submitted to 3 J/cm² of UVA/UVB in a sun simulation chamber (Suntest® CPS+, Atlas, Linsengericht, Germany). Then, cells were kept in suitable culture conditions for additional 24 hours. Cell lysates were collected and proceeded with the comet assay.

2.2.2.1.3. Comet assay

Cell lysates were centrifuged for 5 minutes at 1500 rpm. After, 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 Na₂S) and 10 mM Trizma Base, pH10], with the addition of 1% Triton X-100 and 10% dimethyl sulfoxide, for at least 1 hour. After lysis, slides were incubated for 5 minutes in cold PBS (free Ca⁺⁺ and Mg⁺⁺) and for 40 min in alkaline solution (pH > 13). Cells on the slides were submitted to electrophoresis (300 mA, 25 V) for 30 minutes. 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). 150 cells were randomly selected on each slide and analysed 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 [19].

2.2.3. In vivo assays

2.2.3.1. Subjects

The protocol was conducted 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 and the Helsinki Declaration. 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).

2.2.3.2. Skin biocompatibility

Aliquots of the formulations were applied to predefined sites in both forearms (two formulations per forearm of each subject, in a 9.0 cm² area). The application site of the samples was randomized to minimize the occurrence of possible biases. Measurements were taken before and after 30 minutes of application with a Corneometer, a Tewameter TM300, a pHmeter (C&K Electronics, Germany) and a Chromameter (Minolta, Japan). After that, the subjects were instructed to apply the formulations twice a day for 7 days. At the 8th day, new measurements were performed [20–22].

2.2.3.3. Anti-inflammatory activity

Aliquots of the samples were applied to the forearm of each volunteer in a 9.0 cm² 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 8th day, the second phase began, where erythema formation was induced in each pre-treated area by applying a filter paper (2.25 cm²) saturated with an aqueous solution of methyl nicotinate (0.5% w/w), for 60 seconds. Measurements of the skin microcirculation of the individual test sites were recorded continuously for 15 minutes 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 formulations 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.

2.2.3. Statistical analysis

Experiments were conducted randomly, in triplicate and with a significance level of 5% ($p \leq 0.05$), conducted by Minitab program, version 18.

3. Results

3.1. In vitro evaluation of DNA fragmentation of human cells exposed to ultraviolet radiation by comet assay method

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

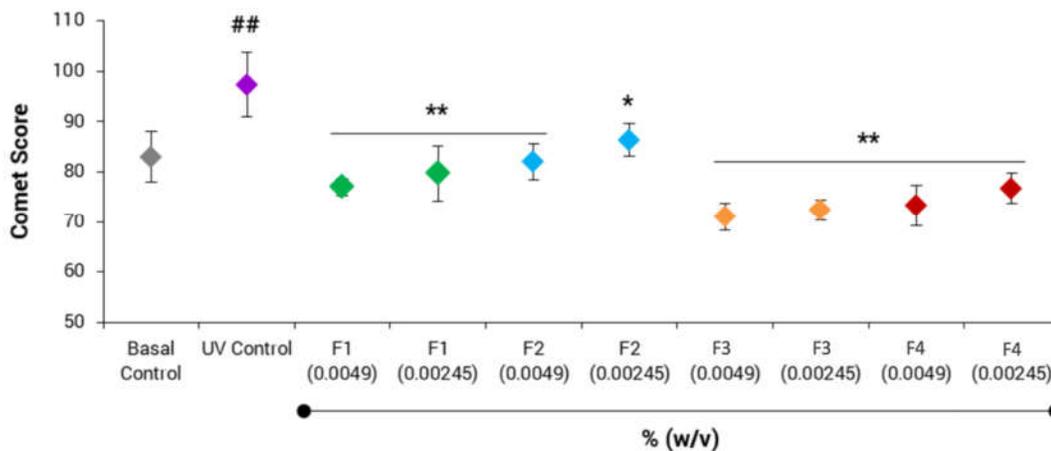
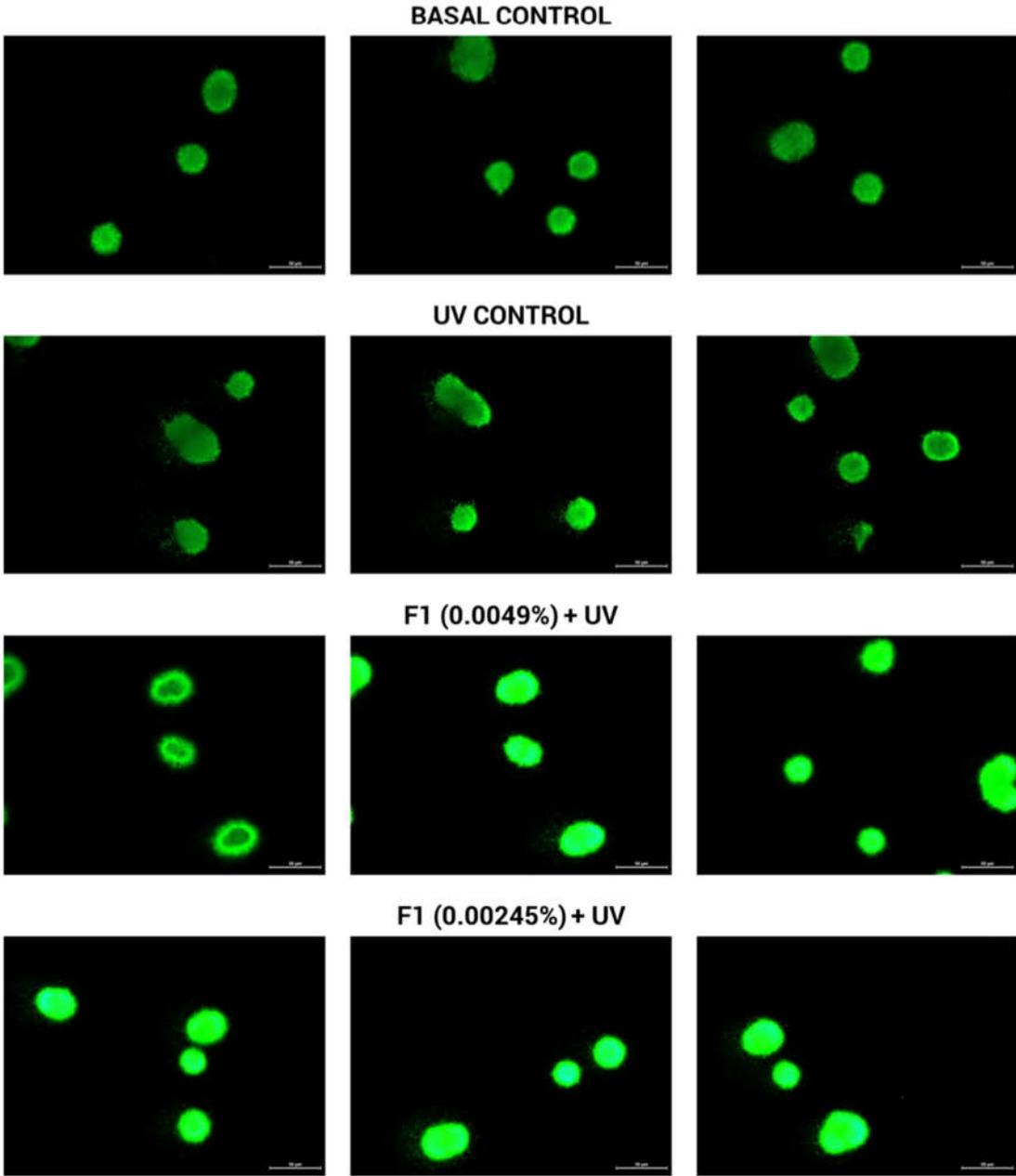
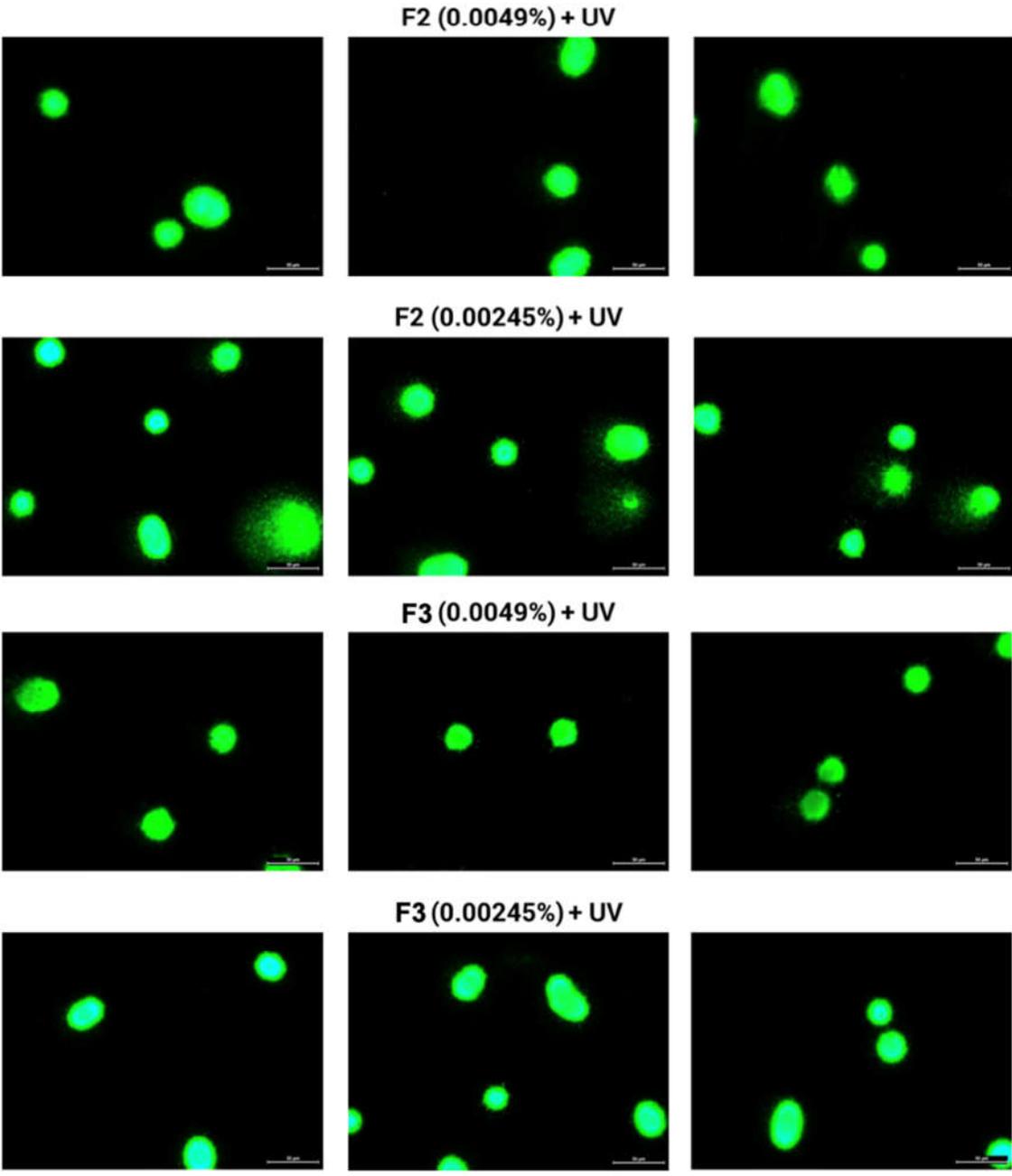


Figure 3 Evaluation of DNA protection potential of the samples F1, F2, F3 and F4, in human keratinocytes after exposure to UV radiation. Data represent the mean \pm standard deviation of 3 independent experiments. ## $p < 0.01$, compared to the basal control group; ** $p < 0.01$, compared to the UV control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





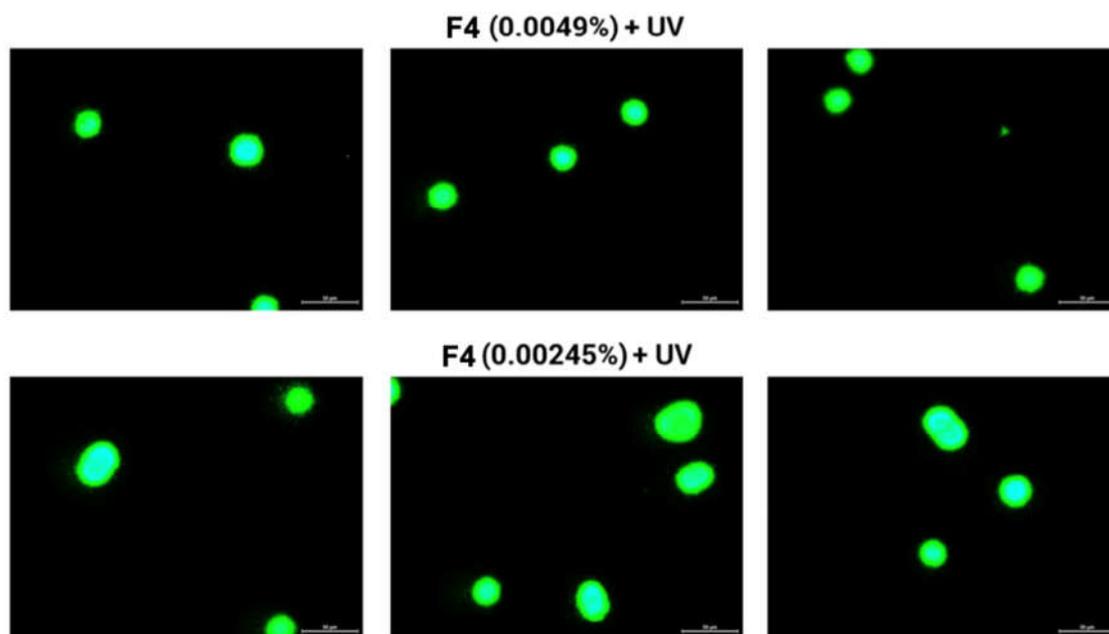


Figure 4. Comet assay method, microscope images at 1000x magnification. Cell DNA damage in human keratinocytes, being basal control (cell culture in regular condition); UV control (cell culture submitted to UV radiation); and the test products F1, F2, F3 and F4 in cell cultures exposed to UV radiation.

It was possible to observe the protective behaviour 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 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 skin biocompatibility of the formulations, measurements were performed with Corneometer, Tewameter TM 300, pHmeter and Chromameter equipment. Results are shown in Table 2.

Table 2. In vivo skin biocompatibility by Corneometer, Tewameter TM 300, pHmeter and Chromameter.

Assay	Sample	Time after first application	Mean \pm standard deviation	p-value
Superficial hydration (Corneometer)	F1	30 minutes	1.405 \pm 0.070	0.000
		7 days	1.356 \pm 0.266	0.022
	F2	30 minutes	1.285 \pm 0.242	0.034

		7 days	1.363 ± 0.401	0.077	
	F3	30 minutes	1.557 ± 0.306	0.007	
		7 days	1.435 ± 0.332	0.024	
	F4	30 minutes	1.312 ± 0.141	0.003	
		7 days	1.328 ± 0.381	0.089	
TEWL / Transepidermal water loss (Tewameter)	F1	30 minutes	1.554 ± 0.337	0.010	
		7 days	1.702 ± 0.727	0.065	
	F2	30 minutes	1.692 ± 0.635	0.044	
		7 days	1.168 ± 0.516	0.462	
	F3	30 minutes	1.591 ± 0.598	0.060	
		7 days	1.493 ± 0.494	0.058	
	F4	30 minutes	2.213 ± 1.137	0.048	
		7 days	1.891 ± 0.727	0.030	
	a* / Redness (Chromameter)	F1	30 minutes	1.007 ± 0.143	0.904
			7 days	1.045 ± 0.153	0.502
F2		30 minutes	1.001 ± 0.079	0.967	
		7 days	1.044 ± 0.063	0.150	
F3		30 minutes	0.986 ± 0.037	0.386	
		7 days	1.056 ± 0.079	0.140	
F4		30 minutes	0.993 ± 0.046	0.709	
		7 days	1.054 ± 0.078	0.153	
Skin pH value		F1	30 minutes	0.994 ± 0.063	0.819

(pHmeter)		7 days	0.977 ± 0.089	0.560
		F2	30 minutes	0.993 ± 0.043
7 days	0.983 ± 0.079		0.617	
F3	30 minutes	1.028 ± 0.082	0.446	
	7 days	1.010 ± 0.102	0.813	
F4	30 minutes	1.040 ± 0.062	0.179	
	7 days	1.028 ± 0.077	0.414	

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 in Table 3.

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

Parameter	Sample	Mean \pm standard deviation	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

4. Discussion

All 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 the F1 and F3 were absent of this phenolic compound. Possibly, the RA concentration at 1.0% was not completely enough

to develop a distinction performance among the samples, since all of them presented some level of DNA protection ranging from 11-27%, including de blank sample (F1) [14,24,25]. Besides our protocol also indicated that blank sample and the sunscreen one (without RA) protected the cells from DNA fragmentation under UV stress, specialized literature reported that RA was able to reduce the extent of DNA injury by other mechanisms through doxorubicin or sepsis, for instance [26,27].

F1 and F3 provided an increase in skin hydration 30 minutes 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 minutes 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). Those ingredients could have contributed with the skin moisture by humectancy and occlusion [28,29]. 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 7th day, however, all samples positively interacted with this skin attribute after 30 minutes. 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 application of those products [30,31].

Treatments with F1 and F2 resulted in an increase in the TEWL after 30 minutes of application (p-value < 0.05). Treatment with the F4 resulted in an increase in TEWL at both times of analysis (30 minutes 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 or not RA provided some level of disturbance over the barrier attribute of the skin by elevating the TEWL, nonetheless, the presence of the UV filters in F3 (absence of RA) may have inhibited such effect. These findings may be also related to the presence of diethylene glycol monoethyl ether in all samples, which is capable of increasing the permeation of substances when applied over the skin, as well as causing 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 skin pH value [23].

It was 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 D₂ and E₂ releases. The prostaglandin D₂ liberation also occurs by incidence of UV radiation on the skin by the generation of reactive oxygen species (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, like, DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging and TBARS (malonyl dialdehyde formation) [14,18,37,38]. Its molecule presents two catechol structures conjugated with a carboxylic acid group as an important structural element in the antioxidant activity of this compound [38,39]. Its activity as a photoprotective adjuvant ingredient was evaluated with successful results in the elevation the sun protection factor (SPF) in vivo of a sunscreen system and, also, RA increased the tyrosinase activity and its expression level in B16 melanoma cells [15,16,40].

Regarding the area under the curve, no significant differences were reached, 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 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 reduction of 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]. Also, several studies involving RA in modified systems has 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 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 application of a monitored sample, greater number of applications per day or even occlusion of the application site. Altering the vehicle qualitative and quantitative composition is also a pertinent perspective.

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

Author Contributions: Conceptualization, T.M.C., A.R.B. and C.R.; methodology, T.M.C.; investigation, T.M.C.; N.M.E.P.; G.P.; B.S.S.; resources, M.V.R.V. and A.R.B.; writing—original draft preparation, T.M.C.; M.B.A.; N.M.E.P.; G.P.; B.S.S.; W.V.M.; C.R. and A.R.B.; writing—review and editing, T.M.C., M.B.A. and A.R.B.; supervision, A.R.B. and C.R.; project administration, T.M.C., C.A.S.O.P.; W.V.M.; A.R.B. and C.R.; funding acquisition, A.R.B.; M.V.R.V. and C.R. All authors have read and agreed to the published version of the manuscript.

Funding: FAPESP (Brazil), CNPq (Brazil), FCT (Portugal) and CAPES (Brazil).

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 November 16, 2021) for studies involving humans.

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

Data Availability Statement: Not applicable.

Acknowledgments: This research was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Process 2019/16169-0), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Process 305250/2019-1), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES, Finance Code 001). Portuguese funds were from the FCT-Foundation for Science and Technology to CBIOS, under the projects UIDB/04567/2020 and UIDP/04567/2020. Authors also thank CAPES PrInt for financial support (Process number: 88887.371188/2019-00).

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

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