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Photoprotection and skin irritation effect of hydrogels containing hydroalcoholic extract of red propolis: a natural pathway against skin cancer

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Abstract

The use of natural products in sunscreen formulations as a prophylactic measure against skin cancer is receiving special attention attributed to the photoprotective and antioxidant properties of their chemical components. In this work, we describe the development of topical hydrogel formulations containing hydroalcoholic extract of red propolis (HERP), and the evaluation of the dermal sensitizing effect of the developed products. Sunscreen formulations composed of HERP in different concentrations (1.5, 2.5 or 3.5% w/w) alone or in combination with a chemical (octyl methoxycinnamate) and/or physical (titanium dioxide) filters were developed using poloxamer 407 as gel basis. The preliminary and accelerated stability tests, texture analysis and spreadability tests were performed. All formulations revealed to be stable in preliminary stability assessment. The formulations containing HERP 1.5 and 2.5% alone or associated with the filters showed intense modifications during accelerated stability test, which were confirmed by rheological analyses. The incorporation of HERP and filters in the poloxamer hydrogel decreased the toughness of product ($p<0.05$) and the formulation containing HERP alone presented the lowest adhesivity ($p<0.001$). The incorporation of HERP in the hydrogel decreased the poloxamer transition temperature, showing different rheological behavior with the increase of HERP concentration. The developed formulations were stable, exhibited non-Newtonian and pseudoplastic behavior, showing *in vivo* skin compatibility and no skin irritancy.

Keywords: red propolis; photoprotection; hydrogels; poloxamer; sunscreen

1. Introduction

Excessive skin exposure to solar radiation can trigger cellular events that will result in negative effects, such as sunburn, photoaging and skin cancer. The development of products used to protect the skin against these effects should be directed towards the production of photoprotectors that ensures action in a wide range spectrum of UV radiation [1]. For this reason, these products are developed using two types of active ingredients that provide protection against UV radiation, which can be classified as organic versus inorganic, and have the ability to absorb and/or reflect the radiation[2].

Currently, there is a trend towards the development of these protectors against skin cancer using compounds derived from plants, especially carotenoids and flavonoids. This results from several studies reporting the beneficial effects of these compounds against the harmful effects of radiation, mainly because they have anti-inflammatory and antioxidant activities [3]. In addition, compounds with aromatic rings can absorb UV rays, especially UVA and UVB at a wavelength range of 200-400 nm [4-6].

The antioxidant and anti-inflammatory activities in combination with the absorption capacity of UV radiation, makes these products potential candidates to become multifunctional photoprotectors which, together with organic and inorganic sunscreens, will act synergistically, optimizing the effectiveness of these products [7].

Red propolis, a natural product obtained from buds and plant exudates collected by bees, has in its composition antioxidant compounds such as isoflavones (daidzein, formononetine and biochanin A) [8]. Previous studies have shown that the hydroalcoholic extract of red propolis (HERP), in addition to its antioxidant activity, it shows antimicrobial, anti-inflammatory, immunomodulatory, healing, anticancer and anticariogenic effects [8-11]. The chemoprotective potential of propolis against the damage caused by UV radiation has been demonstrated, however, these studies have focused on green and brown varieties [12,13]. The photoprotective potential of red propolis, from the state of Alagoas (Brazil), was previously demonstrated by us in rat model [14]. In our study, the topical application of the product containing HERP was able to reduce the intensity of the erythema induced by UVB radiation in a similar way to the standard chemical protector oxybenzone [14].

In order to investigate the action of HERP as a photochemoprotective agent for use in humans, the technological development of a product that complies with the stability and safety requirements, according to current legislation, is necessary. Therefore, the selection of the formulation components to develop a product for topical administration on the skin is instrumental to reach safety and effectiveness.

The selection of the formulation components is guided by the stability study, being a fundamental parameter, since it provides evidence of interactions between the active ingredients and formulation excipients and how the quality of a product varies over time under the influence of several environmental factors (e.g., temperature, humidity and light). To assess the safety, skin compatibility studies are carried out. These studies assess the risk of the product to cause irritation under maximized conditions. The aim of this work was to develop semi-solid hydrogels containing HERP, evaluating their immediate and accelerated stability, and confirming their cutaneous compatibility without irritation effects by means of a clinical trial.

2. Materials and Methods

2.1. Materials

Red standard propolis hydroalcoholic extract (LBMat/2016) was previously obtained by us [8]. The chemical composition of HERP, containing 4.68 µg/ml daidzein, 31.81 µg/ml formononetine and 9.58 µg/ml biochanine A, was previously reported by us [14], using the method described by Silva et al.[15]. All other materials, if not otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Methods

2.2.1 Production of hydrogels

Poloxamer 407-based hydrogels containing HERP in different concentrations, associated or not with a chemical (octyl methoxycinnamate) and a physical (titanium dioxide) sunscreen (Table 1), were developed. To prepare the hydrogels, Poloxamer 407 was previously hydrated at a concentration of 20% and kept in the refrigerator for 24 hours. The solid components (HERP, butyl hydroxy toluene and titanium dioxide) were crushed and dispersed in ethoxyldiglycol. To obtain the dispersion, a 20% Poloxamer 407 solution was added, at a temperature of 5ºC, under constant manual stirring, until the complete formation of the gel at room temperature. Then the liquid components were incorporated, until complete homogenization (see Table 1). The formulations were analyzed macroscopically 24 hours after preparation, in order to check for possible changes. The appearance (homogeneity, phase separation), color and odor change were evaluated. The formulations that remained macroscopically stable were submitted to preliminary stability tests.

Table 1. Composition of the developed hydrogel formulations.
Caption: **POL**: Poloxamer 407 gel base; **POLFQ**: Poloxamer gel base / Chemical filter (7.5% octyl methoxycinnamate); **POLFF**:

	FORMULATIONS											
COMPONENTS (%, w/w)	**POL**	**P1.5**	**P2.5**	**P3.5**	**PFF 1.5**	**PFF 2.5**	**PFF 3.5**	**PFQ 1.5**	**PFQ 2.5**	**PFQ 3.5**	**POLFF**	**POLFQ**
Ethoxyldiglycol	8	8	8	8	8	8	8	8	8	8	8	8
Silicone – Decamethyl cyclopenta siloxane	4	4	4	4	4	4	4	4	4	4	4	4
Elastomer of silicone	4	4	4	4	4	4	4	4	4	4	4	4
Butylated hydroxytoluene	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
HERP	-	1.5	2.5	3.5	1.5	2.5	3.5	1.5	2.5	3.5	-	-
Micronized titanium dioxide	-	-	-	-	3	3	3	-	-	-	3	-
Octil methoxycinnamate	-	-	-	-	-	-	-	7.5	7.5	7.5	-	7.5
Fenoxyethanol + Methyl paraben + Propyl paraben	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Poloxamer 20% solution up to	100	100	100	100	100	100	100	100	100	100	100	100

Poloxamer gel base / Physical filter (3% titanium dioxide); **P1.5, P2.5** and **P3.5**: Poloxamer / HERP gel base 1.5%, 2.5% and 3.5%, respectively; **PFF1.5**, **PFF2.5 and PFF3.5**: Poloxamer / TiO2 gel base 3% / HERP 1.5%, 2.5% and 3.5%, respectively; **PFQ1.5, PFQ2.5** and **PFQ3.5**: Poloxamer gel base / octyl methoxycinnamate 7.5% / HERP 1.5%, 2.5% and 3.5%, respectively.

2.2.2 Stability assay

2.2.2.1 Preliminary stability tests

(a) Centrifugation

For the centrifugation test, 5 g aliquots of each sample, in triplicate, were subjected to centrifugation (CGoldenWall 80-2B centrifuge, San Francisco, CA, USA) at a rotation speed of 3000 rpm for 30 minutes at an ambient temperature of 22 ± 2.0ºC.

(b) Thermal Stress

For the thermal stress test, the formulations were submitted to different temperatures in a thermostatic bath (Ika Eco Pro12, Staufen, Germany) with an initial temperature of 40 ºC. The temperature was raised every 10 °C until the final temperature of 80 °C was reached. The samples were kept for 30 min at each temperature. In the ice-thaw cycle, the samples were subjected to thermal stress in order to accelerate the appearance of possible signs of instability. For the test, alternating cooling and heating cycles were used. The values adopted for the cycle were: cycles of 24 hours in the greenhouse (Tecnal - TE 394/3, Piracicaba, São Paulo, Brazil) at 45 ± 2 °C and 24 hours at 5 ± 2 °C, for 12 days (6 cycles).

(c) Macroscopic analysis

For the macroscopic analysis, the organoleptic characteristics of the samples were evaluated in terms of appearance, color and odor, and the homogeneity of the samples, identifying possible macroscopic instabilities [16]. The evaluation of the organoleptic characteristics (appearance, color and odor) was made at the end of the test (80 °C), being evaluated after the sample returned to room temperature. The criteria for the classification of the macroscopic analysis are shown in **Table 2**. Samples that did not show changes in their appearance, or showed slight phase separation, were considered suitable to proceed to accelerated stability tests.

Table 2. Classification of the macroscopic analysis of the formulations performed after the stability tests.

CLASSIFICATION	DESCRIPTION
Normal	No changes in appearance
Slightly Modified	Slight phase separation presentation
Modified	Presentation of total phase separation
Intensely Modified	Presentation of total phase separation, changes in organoleptic characteristics and consistency

2.2.2.2 Accelerated stability tests

(a) Macroscopic Analysis

The samples considered stable in the preliminary tests were stored under different temperature conditions: 5 °C ± 2 °C; 20 - 25 °C (room temperature); 45 °C ± 2 °C. The macroscopic and spreadability analyzes took place at 24h, and 15, 30, 60 and 90 days after the beginning of the accelerated stability tests. The pH determination was performed 24h after the preparation of the formulations. Analyses were carried out to assess the organoleptic characteristics (appearance, color and odor) considering the criteria previously used (**Table 2**). Mild modifications have been accepted under extreme temperature conditions. Changes in consistency were also allowed, as long as they did not affect the cosmetic application of the samples.

(b) pH Determination

To determine the pH, the samples were diluted in water to a concentration of 10% (w/v) and the potentiometric analysis was performed using the pH meter (MS Tecnopop - mPA-210) in triplicate. Variation of the values obtained greater than 10% was not admitted, compared to the initial value in all storage conditions.

(c) Spreadability Analysis

For the determination of the spreadability, the sample was introduced into a circular mold, (1.2 x 1.0 cm) based on a support plate (20 x 20 cm) on graph paper. After removing the mold, smaller glass plates with known weight were placed on the sample and after one minute, the height / width of spreading of the sample was read to determine the average diameter. Nine plates were added, and the spreadability value (Ei) was calculated using the following equation 1 for each diameter measurement (n = 3) (Eq. 1):

$$Ei = \frac{[(d^2).\pi]}{4} \qquad \text{(Eq. 1)}$$

in which Ei is spreadability (mm^2) and d is the average diameter (mm).

2.2.3. Rheological properties as a function of temperature

The influence of temperature on the rheological behavior of the formulations was studied using a Physica MCR 301 rheometer (Anton-Paar, Graz, Austria). The tests were performed on a parallel plate with P25 geometry (25 mm in diameter), controlled by the Peltier system and 0.5 mm gap. About 1 g of each formulation was used, a temperature ramp between 0ºC to 100ºC and a heating rate of 0.3 °C/s at constant frequency (10 Hz). The storage module (G'), the loss module (G") and the complex viscosity (η) were recorded. The formulations were classified as high, medium and low viscosity, according to criteria established by van Hemelrijck and Müller-Goymann (2011) [16].

2.2.4 Analysis of the texture profile of formulations

The analysis of the texture profile of the formulations was performed using a TA-XT plusC Texture Analyser (Stable Microsystems, Surrey, UK). Approximately 15 mL of each formulation was subjected to compression of a cylindrical probe (25 mm in diameter) at a speed of 1.5 mm/s until reaching a depth of 10 mm at room temperature. The hardness was considered as the maximum force required to achieve a given deformation of 10 mm. The adhesion was obtained by the force as a function of time, which represents the work required to overcome the attraction forces between the sample surface and the probe surface. The analyzes were performed in triplicate [17].

2.2.5. Determination of SPF *in vitro*

The determination of the Sun Protection Factor (SPF) *in vitro* was performed as described by Cefali et al. [4,6]. For the formulations containing HERP and chemical filter, absorbance was determined in a UV/VIS spectrophotometer. For the formulations containing titanium dioxide (physical filter), the absorbance was determined using a diffuse reflectance spectrophotometry. The formulations with HERP and chemical filter were diluted with absolute ethanol to a final concentration of 0.2 mg/mL.

The absorbances of the solutions were determined in the range of 290 to 320 nm, at 5 nm intervals, with three determinations being made for each wavelength. To obtain the absorbances by diffuse reflectance, the formulation containing the physical filter was homogenized on the surface of the sample holder. The readings were taken between 200 and 800 nm. The SPF calculation was done using the following equation. The weighting used in the SPF calculation is described in **Table 3** (Eq. 2).

$$FPS=FC+\sum_{290}^{320} EE(\lambda) \cdot I(\lambda) \cdot Abs(\lambda) \qquad \text{(Eq. 2)}$$

in which FC is the Correction Factor (= 10); EE (λ) is the erythematogenic effect of solar radiation at each wavelength (λ); I (λ) is the intensity of solar radiation at each wavelength (λ); Abs (λ) I the spectrophotometric reading of the absorbance of the sample solution at each wavelength (λ).

Table 3. Weighting used in the calculation of the SPF.

Wavelength (nm)	EE x I (normalized)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180

2.2.6. Primary skin irritation test

Skin compatibility was assessed using the primary skin irritability test and can be considered as the first clinical stage of safety assessment. The study was carried out after approval of the research project by the Research Ethics Committee - CEP of Tiradentes University with opinion number: 1.427.187 and was carried out in accordance with the rules and guidelines of CNS Resolution No. 466/12. All volunteers were informed about the research objective and their agreement was obtained and formalized by reading and signing the Informed Consent Form. The clinical evaluation was performed by a dermatologist. Twenty healthy volunteers of both sexes were selected, aged between 18 and 40 years. Exclusion criteria were: skin marks in the experimental area that may interfere with the evaluation of skin reactions, eczematous reaction, allergy or reactivity for the product category studied, dermographism, skin hyper-reactivity, atopy, allergy to adhesive, treatment with topical corticosteroids in the experimental area, pregnancy or lactation and treatment with anti-inflammatory or antiallergic drugs [18]. The contact test consisted of the topical application of the formulations, in an occlusive way, using Patch Test adhesive type (Alergochambers – ProDermatho, Cotia, Sao Paulo,

Brazil). The chambers adhered to the adhesive tape were filled, in a randomized manner, with the test formulations and then glued to the dorsal area of the volunteers (**Figure 1**). The readings were taken 24 hours after the Patch Test was removed and interpreted by dermatologist, considering the scale of the International Contact Dermatitis Research Group - ICDRG (**Table 4**).

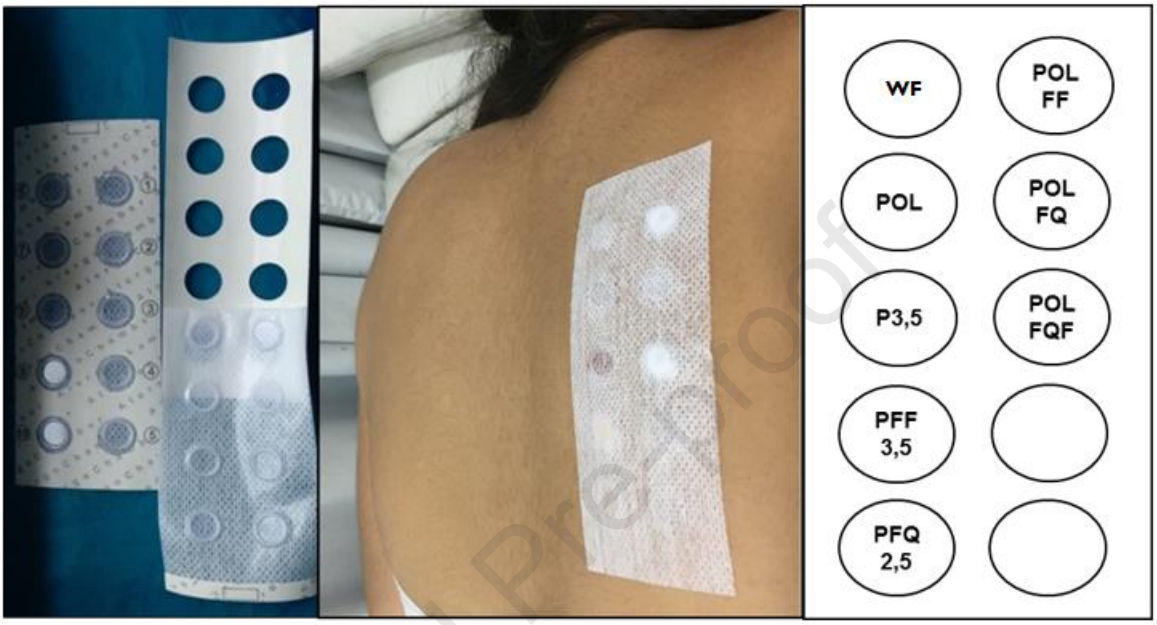

Figure 1. Primary skin irritation test using Patch Test patches (ALERGOCHAMBERS - ProDermatho) (left-hand photo). Formulations tested: WF (without formulation); P3.5 (Poloxamer gel base / 3.5% HERP); PFF3.5 (Poloxamer gel base / 3% TiO2 / 3.5% HERP); PFQ3.5 (Poloxamer gel base / 7.5% octyl methoxycinnamate / 3.5% HERP); POLFF (Poloxamer gel base / 3% titanium dioxide physical filter); POLFQ: Poloxamer gel base / 7.5% octyl methoxycinnamate chemical filter); POLFQF (Poloxamer gel base / 7.5% octyl methoxycinnamate / 3% titanium dioxide physical filter) (right-hand pattern).

Table 4. Criteria for the evaluation of the contact test proposed by the ICDRG used 24 hours after the removal of the Patch Test.

REACTION	RESULT	GRADE
0 - Absent	Negative (-)	0
1 - Mild erythema	Doubtful (?)	1
2 - Clear erythema	Positive (+)	2
3 - Erythema + edema + papules	Positive (++)	3
4 - Erythema + edema + papules + vesicles	Positive (+++)	4

2.2.7 Statistical analysis

Statistical analysis was performed using ANOVA followed by Tukey's post hoc test ($p<0.05$) for independent variables, using the GraphPad Prism 5.0 (Software, Inc., LaJolla, CA, USA).

3. Results and discussion

Water-based formulations, such as gels, are generally not suitable for the incorporation of HERP, as described by Bittencout et al. (2014) [19]. The attempt to incorporate only 0.5% HERP in formulations based on hydroxyethyl cellulose gel and copolymer of sulfonic acid acryloyl dimethyltaurate/vinylpyrrolidone (Aristoflex®) was reported by the authors to show immediate phase separation with precipitation of HERP. In our work, the incorporation of the HERP/ethoxyldiglycol dispersion in the Poloxamer 407 gel was carried out without any technical difficulties. Formulations with homogeneous appearance and gel-like consistency were obtained, as described previously by van Hemelrijck and Müller-Goymann [16].

Poloxamer 407 is a synthetic hydrogel-forming copolymer with surfactant characteristics, non-irritating and stable over a wide pH range. The polymer is composed of tri-blocks of poly (propylene oxide) (PPO)-poly (ethylene oxide) (PEO). The PPO segment is relatively hydrophobic compared to the surrounding PEO segments. This characteristic makes this copolymer to jellify when in contact with water [20]. Therefore, using poloxamer 407 it is possible to incorporate hydrophobic molecules in a gel base.

The Poloxamer 407-based formulations prepared with HERP concentrations of 1.5%, 2.5% 3 3.5% associated or not with the physical filter (titanium dioxide at 3%) and chemical (octyl methoxycinnamate at 7.5%) showed variable colorations, from light pink, dark red to light brown, due to different concentrations of the extract (which has a dark red color), and its association with the filters.

It is known that the micelle formation process of poloxamers is affected by several factors, such as temperature [21,22], the use of co-solvents [23], or other additives [24,25].

Sharma *et al*. (2008) [26] studied formulations based on poloxamer 407 containing different hydrophobic drugs (dibucaine, tetracaine and lidocaine). The authors stated that the drugs would be preferentially placed inside the micellar propylene oxide nucleus, forming an organized structure. Since hydrophobic molecules would have unfavorable interactions with water molecules inside the PPO nuclei, the organization in form of micelles results in the dehydration of the micellar nuclei. The hydrophobic character of these organized structures suggests that HERP will interact with the most hydrophobic part of the molecule (PPO), remaining stable in the gel.

In order to accelerate the potential reactions between the components and demonstrate the most common instability processes of the formulation under study, the tests use extreme conditions of temperature and mechanical stress. The conditions of these studies are not aimed to estimate the useful

life of the product, but to assist and guide the choice of components of the formulation under development.

The formulations were subjected to the tests that make up the preliminary study: centrifugation, thermal stress and ice-thaw cycle. Those that were considered to be slightly modified (moderate phase separation) were kept for additional characterization.

When subjected to centrifugation and thermal stress tests, hydrogels did not change in appearance, being classified as Normal. The temperatures adopted in the thermal stress are in the temperature range in which the poloxamer 407 presents in the gel state (40º to 80ºC), being far from the transition range to sol state, which may have influenced the stability of formulations [17]. However, in the freeze-thaw cycle test, at low temperatures, the formulations P1.5; P2.5; PFF1.5 and PFF2.5 showed intense modification with loss of consistency. This was attributed to the temperatures below the transition temperature (T_t) of the poloxamer at which the formulations have been stored, which, for a concentration of 20% of poloxamer is, on average, 19ºC [26]. Table 5 summarizes all the tests performed with the developed formulations.

Table 5. Tests performed with the samples in the stages of stability, characterization and cutaneous compatibility. Captions: performed (black square); performed over stability time (gray squares); not performed (white squares).

	P1.5	P2.5	P3.5	PFF1.5	PFF2.5	PFF3.5	PFQ1.5	PFQ2.5	PFQ3.5
Preliminary stability	■	■	■	■	■	■	■	■	■	■	■
Accelerated stability				■	▒	▒	■	▒	▒	▒
Rheological characterization	■	■	■	■	■	■	■	■	■	■	■
Texture analysis				■			■			■
FPS *in vitro*				■			■			■
Cutaneous compatibility				■			■			■

Tundisi et al. (2021) have shown that casein concentration affected adhesive strength, viscosity, mechanical properties and drug release, of poloxamer 407 hydrogels, with the increasing concentration of casein increased the adhesive strength, without inflammation or injury by *in vivo* exposure [27]. Li et al. (2019) produced hyaluronic acid- poloxamer hydrogels that changed from sol to gel at a temperature close to body (30°C), and with stable moisturizing properties [28]. These findings could explain the stable behavior of formulations containing a higher concentration of HERP

at low temperatures. The increase in the HERP concentration may have decreased the transition temperature, keeping the formulation more structured, in a gel form at low temperatures, which did not occur, for example, with formulations without HERP (POL, POLFF and POLFQ), that showed an intense change in viscosity.

Therefore, based on the results recorded in the preliminary stability tests, only the formulations P3.5 (Poloxamer / HERP 3.5% gel base), PFF3.5 (Poloxamer / HERP 3.5% gel base) TiO2 3%), PFQ1.5, PFQ2.5 and PFQ3.5 (Poloxamer gel base / HERP 3.5% / octyl methoxycinnamate 7.5%) remained stable according to the established criteria and proceed to the accelerated stability tests.

These tests use more extreme conditions compared to the preliminary tests. Periodic assessment of organoleptic characteristics provides information regarding changes in color, odor and appearance. These analyzes indicate the similarities of the formulations submitted to different storage conditions in relation to their initial characteristics (24h after preparation) [29,30].

In addition to the macroscopic evaluation, the monitoring of pH values provides information regarding the chemical stability of the formulation, since the variations may be related to degradation reactions of the components of the base and/or the active [31].

Macroscopic evaluation, determination of pH and spreadability of the formulations were carried out in 24 h, and after 15, 30, 60 and 90 days. After 24 h, all formulations showed slightly acidic pH values (between 4.0 and 5.0), attributed to the presence of acidic substances (phenolic substances) present in the HERP.

Table 6 presents the macroscopic characteristics of the formulations in the three storage conditions. The control formulations used in the preliminary stability test were also evaluated under different storage conditions. The formulations that did not contain HERP (POL, POLFF and POLFQ) remained stable at room temperatures (20-25ºC) and in the greenhouse (45º ± 2ºC). However, when subjected to a temperature of 5º ± 2ºC, all of theese showed marked loss of viscosity after 24h of storage.

On the 15th day, formulations containing an association of HERP in different concentrations with the chemical filter (PFQ1.5; PFQ2.5 and PFQ3.5) showed intense modification, with phase separation at a temperature of (45º ± 2ºC). In addition to macroscopic instability, these formulations had lower pH values (between 3.0 and 4.0), thus suggesting a thermo-instability when the extract is associated with the chemical filter.

Storage at elevated temperatures for a longer period of time may have compromised the micellar structure of the hydrogel, causing the substances contained in the nucleus of the micelles to be released, following the separation of the aqueous phase. In addition, the increase in temperature for long periods can cause degradation of the components of the formulation, favoring the instability of the product.

Ribeiro *et al.* (2015) [32] found similar results, where the nanoemulsion containing *Opuntia ficus-indica* extract showed a significant decrease in the pH value when kept at the same temperature. The authors suggest that this result is related to the degradation of oily components of the formulation, producing free fatty acids. Thus, variations in pH values may have caused physical-chemical changes in formulations PFQ1,5; PFQ2,5 and PFQ 3,5, compromising its stability, causing loss of viscosity and phase separation [33].

Figure 2 shows the variation of the pH values of the formulations that remained stable from 0 to 90 days (P3.5 and PFF3.5). When correlated with the evaluation time, the formulations showed a significant variation ($p < 0.05$) of the pH values. The two formulations showed a decrease in the pH values in the tested storage. However, the PFF3.5 stored at room temperature and 45 ± 2ºC the variation remained within the range recommended by ANVISA (10%) [34].

Table 6. Classification of macroscopic characteristics of formulations containing HERP associated or not with physical and chemical filters stored at different temperatures.

Formulations	5±2°C					20 a 25°C					45±2°C				
	24h	15 days	30 days	60 days	90 days	24h	15 days	30 days	60 days	90 days	24h	15 days	30 days	60 days	90 days
POL	**M**	**M**	**M**	**M**	**M**	N	N	N	N	N	N	N	N	N	N
POLFQ	**IM**	**IM**	**IM**	**IM**	**IM**	N	N	N	N	N	N	N	N	N	N
POLFF	**IM**	**IM**	**IM**	**IM**	**IM**	N	N	N	N	N	N	N	N	N	N
P3.5	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
PFF3.5	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
PFQ1.5	N	N	N	N	N	N	N	N	N	N	N	**IM**	-	-	-
PFQ2.5	N	N	N	N	N	N	N	N	N	N	N	**IM**	-	-	-
PFQ3.5	N	N	N	N	N	N	N	N	N	N	N	**IM**	-	-	-

POL: Poloxamer 407 gel base; **POLFQ:** Poloxamer gel base / Chemical filter (7.5% octyl methoxycinnamate); **POLFF:** Poloxamer gel base / Physical filter (3% titanium dioxide); **P3.5**: Poloxamer / HERP 3.5% gel base; **PFF3.5**: Poloxamer gel base / 3% TiO2 / 3.5% HERP; **PFQ1.5, PFQ2.5** and **PFQ3.5**: Poloxamer gel base / octyl methoxycinnamate 7.5% / HERP 1.5%, 2.5% and 3.5%, respectively.

Mahmood *et al*. (2013) [35] reported the results of the stability studies of emulsions containing *Camellia sinensis* extract where, at a higher temperature (40ºC), the emulsions showed a significant decrease in pH values over a period of 6 months. They claim that such variations may have occurred due to the hydrolysis or oxidation reactions of the formulation components that generally occur during accelerated stability tests. Li *et al.* (2019) reported similar results when described the highest affinity and adsorption capacity of quercetin, with improved antioxidant and antibacterial activities formulated in a polyamide fiber for healthcare applications [36].

In view of the results of significant variations in pH values, it became evident the need to incorporate buffering agents in the formulation, as well as antioxidant agents that prevent the degradation of the oil components of the HERP present in the formulations.

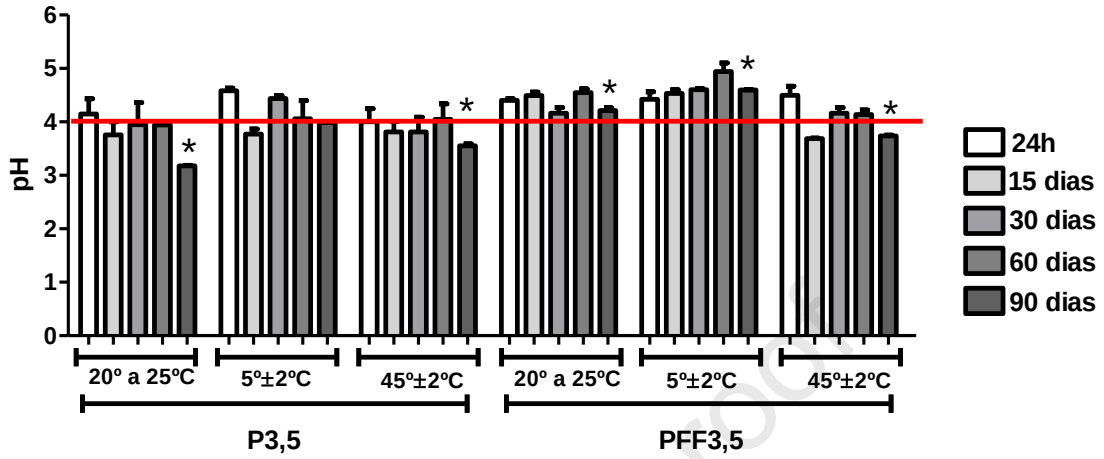

Figure 2. Variation of the pH values of the formulations P3.5 and PFF3.5 in different storage conditions in the time 24h to 90 days. P3.5: Poloxamer / HERP 3.5% gel base; PFF3.5: Poloxamer gel base / 3.5% HERP / 3% TiO2. Data expressed as mean ± standard error of the mean. Significant differences in comparison to the storage time expressed as: * $p < 0.05$ in relation to the 24 h time (ANOVA followed by Tukey's post hoc).

After the 24h period of the beginning of the accelerated stability tests, the spreadability test of the formulations P3.5, PFF3.5, PFQ1.5, PFQ2.5 and PFQ3.5 was carried out at room temperature. It was observed that there was an increase in spreadability due to the added weights (**Figure 3**). It was found that there was no significant difference between the spreadability of the formulations ($p > 0.05$). The spreadability test makes it possible to determine the ability of a formulation to spread when subjected to a certain force, trying to reproduce the spreading characteristics on the skin. This characteristic, besides being important in the sensory point of view, for photoprotective formulations, directly influences the effectiveness of the product, since the uniformity of the film formed on the skin can compromise the Sun Protection Factor of the same [32,37].

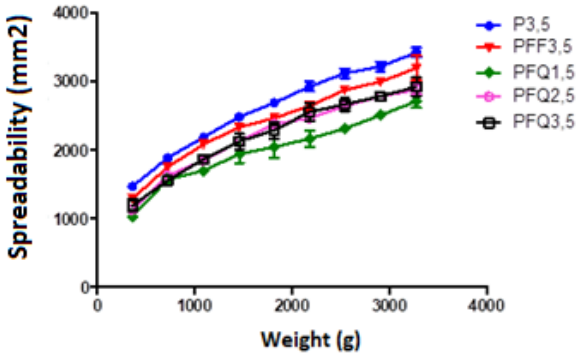

Figure 3. Spreadability (mm^2) of formulations P3.5, PFF3.5, PFQ1.5, PFQ2.5 and PFQ3.5 as a function of weight (g), at room temperature over 24 hours.

For the remaining time-points (15th, 30 th, 60 th and 90 th days), the spreadability tests were repeated with the formulations P3.5 and PFF3.5, since they were the only ones that remained stable during this period. The results are shown in **Figure 4**. Poloxamer 407 has thermo-reversible viscosity characteristics in aqueous media. As the temperature increases, the polymers are added in micelles to minimize the free energy of the solution. After heating, there is a balance between the monomers and the micelles, resulting in elastic micellar packaging [38]. P3.5 showed a decrease in spreadability, with significant variation ($p < 0.05$), after 90 days. The formulation PFF3.5, on the other hand, it did not show significant variation between spreadability as a function of time. All formulations were subjected to the characterization of the apparent viscosities as a function of temperature (**Figure 5**). It can be seen that at storage temperatures (20 to 40ºC), the formulations have high to medium viscosity [16]. The results showed that the association of HERP with the chemical filter promoted an increase in viscosity values (**Figure** 5C).

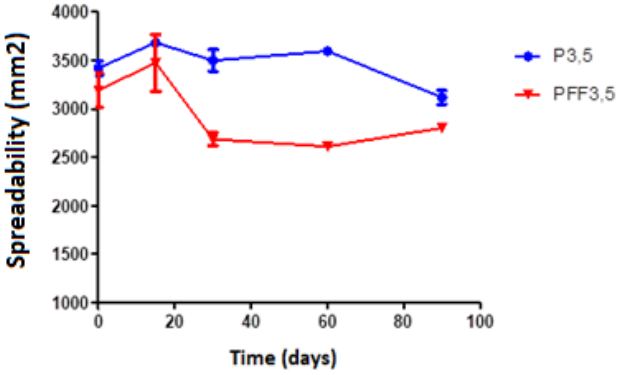

Figure 4. Spreadability (mm^2) of formulations P3.5 and PFF3.5 as a function of time (days) using a heavier plate.

The octyl methoxycinnamate (chemical filter) has lipophilic character, and as previously mentioned, hydrophobic substances can interact with the nucleus of the hydrogel micelles, promoting an increase in the degree of dehydration of the nuclei, favoring the organization of the micellar system, which would promote an increase in the consistency of the same [16,26].

In order to identify the influence of adding HERP and the association with different sunscreens on the phase transition temperature of the poloxamer, the rheological properties of the formulations were characterized in the temperature range from 0ºC to 100º C. From the rheological point of view, a "a" value of G' greater than G" is one of the inherent characteristics of the solid elastic behavior and the process of changing the phase from liquid to semi-solid can be described through the increment in G', surpassing G" [39]. Thus, the gelation temperature is identified as the temperature at which the curves of G' and G'' cross [17,22].

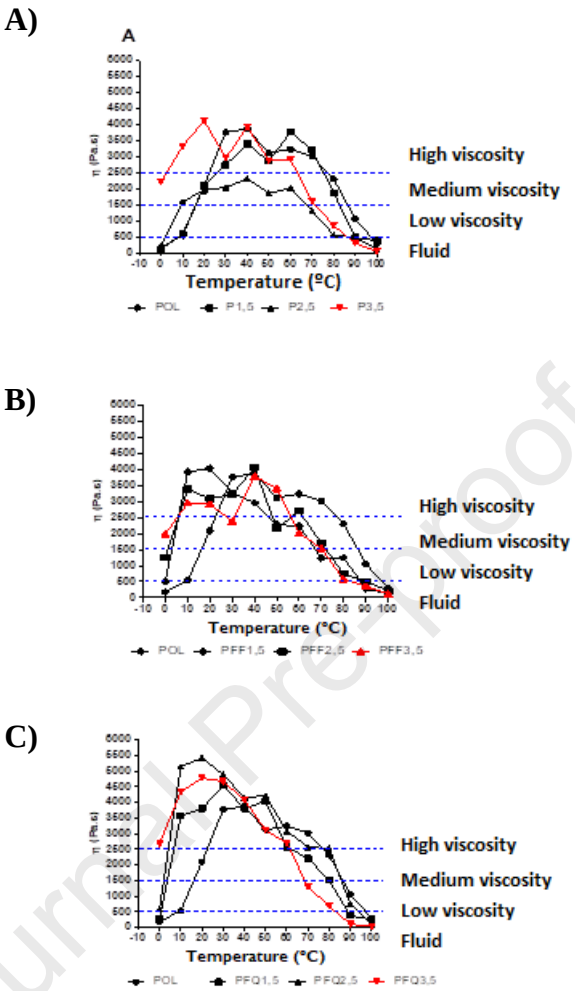

Figure 5. Characterization of the viscosities of the formulations as a function of temperature. **A)**: POL: Poloxamer 407 gel base; P1.5, P2.5 and P3.5: Poloxamer / HERP gel base 1.5%, 2.5% and 3.5%, respectively; **B)**: POL: Poloxamer 407 gel base; PFF1.5, PFF2.5 and PFF3.5: Poloxamer / TiO2 gel base 3% / HERP 1.5%, 2.5% and 3.5%, respectively. **C)**: POL: Poloxamer 407 gel base; PFQ1.5, PFQ2.5 and PFQ3.5: Poloxamer gel base / octyl methoxycinnamate 7.5% / HERP 1.5%, 2.5% and 3.5%, respectively.

Figure 6 shows the rheological properties of the control formulation of poloxamer 407 and the gel containing different concentrations of HERP, at temperatures ranging from 0ºC to 100ºC. The results show that the POL (**Figure 6A**) formulation presents values of G''> G' in the temperature range between 0ºC and 5ºC, depicting behaviour typical of a viscous liquid. With the increase in temperature, the value of the G' module exceeds that of G'', with a phase change (20ºC), with the formulation having a semi-solid characteristic. Figure 6 demonstrates that, with the addition of different concentrations of HERP, the transition temperature changes to lower temperatures. In **Figure 6B**, it can be seen that when 1.5% HERP (P1.5) was added, the transition temperature to the gel phase was shifted to approximately 5ºC.

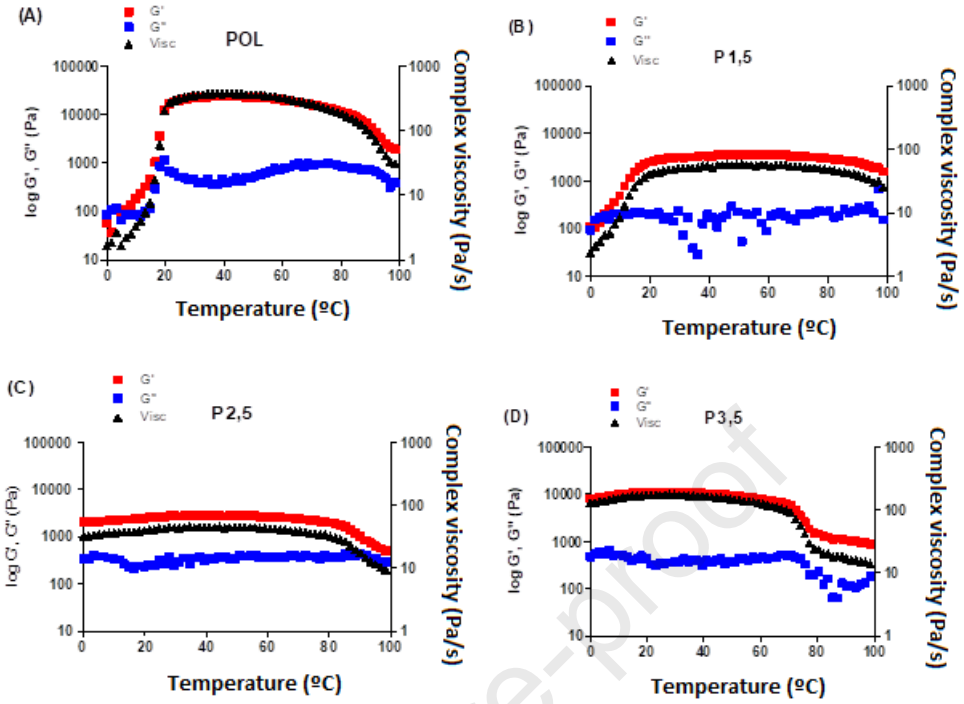

Figure 6. Effect of the HERP concentration on the rheological properties of the formulations, evaluated in the temperature range from 0ºC to 100ºC. **(A):** POL (Poloxamer 407 gel base); **(B)**: P1.5: Poloxamer / HERP 1.5% gel base; **(C)**: P2.5: Poloxamer / HERP 2.5% gel base; **(D)**: P3.5: Poloxamer / HERP 3.5% gel base. G': elasticity storage module; G'': viscosity loss module; Visc: Complex viscosity.

When the concentration of HERP in the formulation was 2.5% (P2.5) and 3.5% (P3.5) (**Figures 6C** and **6D**, respectively), the transition temperature is shifted to temperatures below of 0ºC since, at this temperature, both formulations presented values of G' > G", typical of elastic behaviour [40,41]. In addition, the increase in the concentration of HERP in the formulations results in an increase in the values of the elastic modulus G' as a function of temperature (**Figure 6**). The results prove that the addition of HERP, in different concentrations, promotes a change in the poloxamer 407 sol-gel transition temperature range which, according to rheological data in the literature, is between 25ºC and 32ºC [42-44].

van Hemelrijck and Müller-Goymann (2012) [24] reported a decrease in the transition temperature of the poloxamer when it was associated with the lipophilic active 5-aminolevulinic acid. In addition, other studies associate the influence of additives (such as co-solvents) on the gelation temperature of the poloxamer to the decrease in the gelation enthalpy of the polymer [45,46]. In addition, at the same temperature, it was observed that the viscosity decreased by P1.5 and P2.5 in relation to POL. This behavior is probably due to the decrease in the poloxamer molecules in the formulation. For each 1% of HERP added to the formulation, there is a deficit of 0.3% of poloxamer in the final concentration. As the poloxamer molecule is responsible for the micellar structure of the formulation, which

generates a higher viscosity, the decrease in this polymer generates a lower viscosity. In contrast, the increase in hydrophobic substance in the formulation increases the micellar organization, promoting an increase in viscosity [26]. This statement explains the behavior of increasing viscosity at P3.5. **Figure 7** shows the POL and POL rheological properties containing 3.5% HERP associated with the physical filter (PFF3.5) and the chemical filter (PFQ3.5), at temperatures ranging from 0ºC to 100ºC. As observed in the characterization of formulations containing only HERP (P3.5), the formulations containing the HERP associated with the physical filter (PFF3.5) and the chemical (PFQ3.5) shifted the gel's transition temperature to temperatures below 0ºC, because the module G' presents higher values than the module G'' in all studied temperature range.

The formulations P3.5 (**Figure 6B**) and PFF3.5 (**Figure 7C**) showed similar values of modulus of elasticity (G') and complex viscosity over the entire temperature range, suggesting that the addition of the physical carbon dioxide filter titanium did not modify the rheological behavior of the gel containing 3.5% HERP. However, when associated with the octyl methoxycinnamate chemical filter (**Figure 7D**), the values of the modulus of elasticity were higher (> 10,000 Pa) in the temperature range between 0ºC and 80ºC. As discussed in the apparent viscosity results (**Figure 5**), the lipophilicity of the octyl methoxycinnamate may have increased the hydrophobic interactions between the PPO blocks, favoring a greater packaging of the micelles, thus promoting high values of elasticity storage module [25].

In the present study, the rheological characterizations depicted in **Figures 6** and **7** also demonstrated that, at high temperatures, there is a sudden loss of viscosity, represented by the decrease in the values of the elasticity module. It is suggested that the unfavorable interaction between water and the PEO (hydrophobic) group at higher temperatures results in the shrinking of the micellar crowns. As a consequence, there is a decrease in the micellar volume, thus disorganizing the structure of the polymeric network [47].

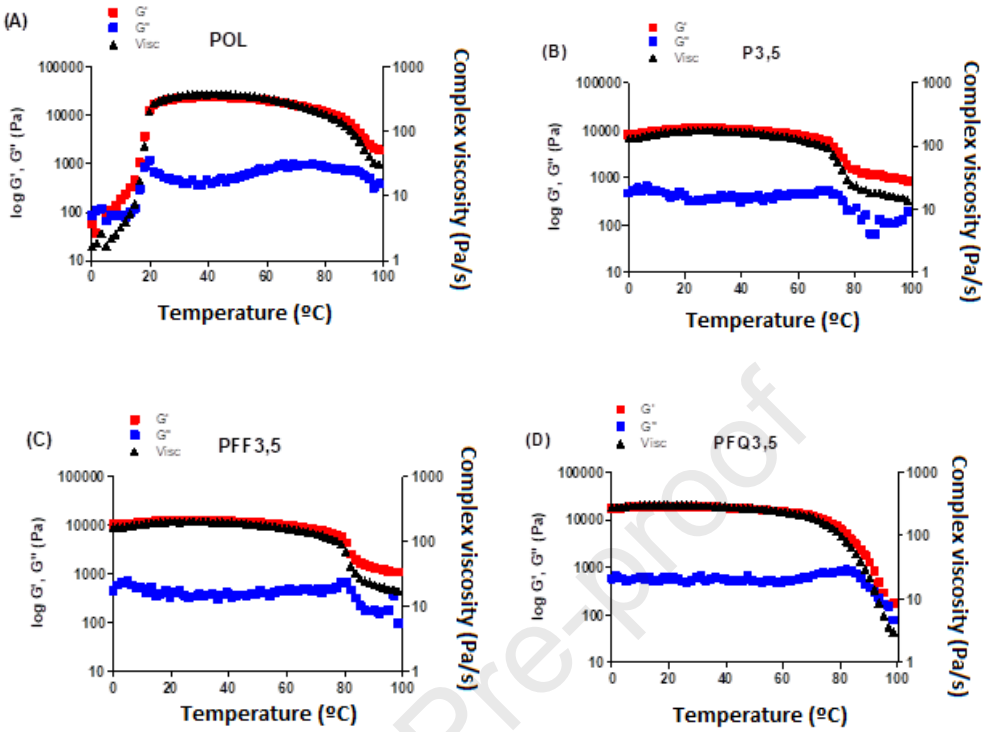

Figure 7. Effect of the association of 3.5% HERP with the physical filter (titanium dioxide) at 3% and the chemical filter (octyl methoxycinnamate) at 7.5% on the rheological properties of the formulations, evaluated in the temperature range from 0ºC to 100ºC. **(A)**: POL (Poloxamer 407 gel base); **(B)**: P3.5: Poloxamer / HERP 3.5% gel base; **(C)**: PFF3.5: Poloxamer gel base / 3.5% HERP / 3% TiO2; **(D)**: PFQ3.5: Poloxamer gel base / 3.5% HERP / 7.5% octyl methoxycinnamate. G': elasticity storage module; G'': viscosity loss module; Visc: Complex viscosity.

The texture profile analysis was originally proposed by Jones *et al*. (1996) [48] as a suitable method to characterize the mechanical properties of semi-solid products. Such properties determine the ability to spread on the skin, ease of removal of the product from the packaging and its bioadhesion. The hardness expresses the applicability of the gel in the desired location and the adhesiveness is related to the retention of the product on the skin [17,49]. The mechanical properties of poloxamer 407 gel and formulations containing 3.5% HERP associated or not with physical and chemical filters are shown in **Figure 8**.

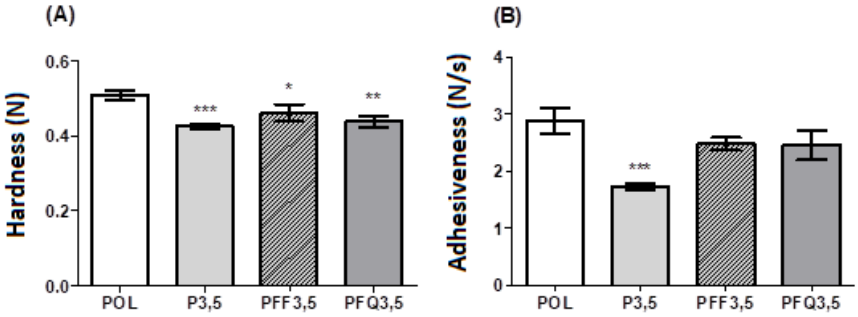

Figure 8. Hardness (N) (panel A) and adhesiveness (N/s) (panel B) of formulations at room temperature (20º-25ºC). POL (Poloxamer 407 gel base); P3.5: Poloxamer / HERP 3.5% gel base; PFF3.5: Poloxamer gel base / 3.5% HERP / 3% TiO2 and PFQ3.5: Poloxamer gel base / 3.5% HERP / 7.5% octyl methoxycinnamate. Significant differences compared to the formulation without assets (POL) were expressed as: * p <0.05; ** p <0.01; and *** p <0.001 (ANOVA followed by Tukey's post hoc test).

As seen in **Figure 8**A, the hardness values of formulations containing 3.5% HERP (P3.5), associated with the physical filter (PFF3.5) and the chemical filter (PFQ3.5), were significantly lower ($p < 0.001$; $p < 0.05$ and $p < 0.01$, respectively) in relation to the poloxamer base (POL). However, when compared to each other, the formulations P3.5, PFF3.5 and PFQ3.5 did not present hardness values that were statistically different ($p > 0.05$). Tests developed with poloxamer 407 demonstrate that the increase in the concentration of the polymer results in a higher micellar concentration, making the polymeric complex more resistant [50,51].

When compared to formulations containing filters, the POL formulation has a higher concentration of poloxamer 407, which may have resulted in a higher hardness value. This result has a desirable characteristic for formulations developed with the extract, since the lower the hardness values, the better the product will be able to spread on the skin, which would also facilitate the removal of the product from the packaging [49,52].

Adhesiveness is defined as the work required to overcome the attractive forces between the sample surface and the probe surface of the texturometer [48]. The P3.5 formulation showed a lower adhesion value ($p < 0.001$), when compared to the POL, PFF3.5 and PFQ3.5 formulations (**Figure 8B**). High adhesion values indicate greater product adhesion when applied onto the skin and, consequently, longer skin retention [53].

The *in vitro* SPF values of formulations containing only HERP or associated with physical and chemical filters are shown in **Table 7**. Formulation P3.5 revealed a SPF of 2.75, which does not offer photoprotection since the minimum value accepted for photoprotectors is 6.0 [54]. When HERP was associated with physical and chemical filters, an increase in the SPF value was observed. The PFF3.5 showed an SPF value of ~ 20. The highest SPF value was observed for PFQ3.5 with an SPF of ~ 30. The *in vitro* SPF determination test predicts the photoprotective potential of product assets before

they are tested *in vivo* [55]. The evaluation of the *in vitro* SPF of natural products is based on the absorption characteristics in the UV spectrum and the concentration of its antioxidant compounds in the extract [4-6]. The low SPF value of the formulation containing only HERP corroborates the statement above and may therefore be related to the low concentration of UV-absorbing molecules. However, the antioxidant activity of the extract may justify its presence in photoprotective formulations.

Table 7. SPF values of formulations containing HERP associated or not with chemical and physical sunscreens.

FORMULATIONS	MEAN FPS (±SD)
P3.5	2.75 (± 0.47)
PFF3.5*	21.24 (± 1.3)
PFQ3.5	32.50 (± 0.32)

P3.5: Poloxamer / HERP 3.5% gel base; PFF3.5: Poloxamer gel base / 3.5% HERP / 3% TiO2 and PFQ3.5: Poloxamer gel base / 3.5% HERP / 7.5% octyl methoxycinnamate. * Absorbance obtained by diffuse reflectance.

Other studies have evaluated the SPF *in vitro* of formulations containing plant extracts rich in antioxidant compounds and the results are similar to ours. Cefali *et al*. (2019) [71] produced nanoemulsions containing a blend of extracts obtained from *Ginkgo biloba* L., *Dimorphandra mollis* Beth, *Ruta graveolens* and *Vitis vinifera* L., showing an *in vitro* SPF of about 2.94 [5]. Using the spectrophotometric assay, Gregoris et at. (2011) reported that to reach a SPF of 20, a concentration below 8% of most of all propolis' polyphenols would be enough [56]. Among the identified polyphenols in propolis, caffeic acid showed a significant effect requiring only 4% to reach SPF of 20. Our formulation containing 3.5% HERP obtained SPF of ~ 1.0. Therefore, the method of determining SPF *in vitro* by absorption does not seem to be adequate for the response on the effectiveness of natural products in the photoprotection process.

For a product to be applied onto the skin for cancer prophylaxis, its safety and absence of skin irritation must be ensured. The irritation potential of a cosmetic product depends on a series of variables, such as components used in the formulation, concentration of the components, absorption, amount applied, skin condition, mode and frequency of application. Therefore, in order to ensure the absence of skin irritation when using these products, the skin compatibility study, which represents the first contact of the finished product in humans.

For the clinical study, formulations containing the highest HERP concentration (3.5%) were selected associated or not to chemical and physical filters, in comparison to plain hydrogels i.e. without HERP but having none, one or both filters (Table 8). As seen in **Table 8**, all formulations developed with poloxamer 407 associated or not with HERP and chemical and physical filters showed maximum skin

compatibility (grade 0 of reaction) since irritation reactions were not observed after 24 hours of contact under occlusion.

Table 8. Skin compatibility of the volunteers after 24 hours of application of the studied formulations by means of Patch Test occlusive contact test (ALERGOCHAMBERS - ProDermatho) (n = 20).

VOLUNTEER	WF	POL	P3.5	PFF3.5	PFQ3.5	POLFF	POLFQ	POLFFQ
1	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-

Caption: Negative Result (-). = Grade: 0: **WF**; without formulation; **POL**: Poloxamer 407 gel base**; P3.5**: Poloxamer / HERP gel base 3.5%, **PFF3.5**: Poloxamer / TiO2 gel base 3% / HERP 3.5%; **POLFF**: Poloxamer gel base / Physical filter (3% titanium dioxide); **POLFQ**: Poloxamer gel base / Chemical filter (7.5% octyl methoxycinnamate); **POLFFQ**: Poloxamer gel base / Physical filter (3% titanium dioxide) and Chemical filter (7.5% octyl methoxycinnamate).

4. Conclusions

Our study showed that the poloxamer 407-based gel was successfully formulated with HERP at a concentration of 3.5%, ensuring the stability of the formulation under the tested conditions. From the texture analyses and rheological behaviour, we confirmed that the stable formulations presented viscosity and mechanical characteristics that favor the application of the product on the skin, as well as its retention on its surface. At product storage and application temperatures (between 20 and 40 ºC) poloxamer 407-based formulations exhibited an elastic behaviour, with no change in consistency. Stable formulations containing 3.5% HERP associated or not with chemical and/or physical filters showed skin compatibility in humans. The results of *in vitro* SPF show that HERP alone in a

poloxamer gel does not have scattering effect, which suggests that the spectrophotometric method is the only method suitable for the assessment of photoprotective potential of natural compounds.

Informed Consent Statement

Skin compatibility testing was carried out after approval of the research project by the Research Ethics Committee - CEP of Tiradentes University with opinion number: 1.427.187 and was carried out in accordance with the rules and guidelines of CNS Resolution No. 466/12. All volunteers were informed about the research objective and their agreement was obtained and formalized by reading and signing the Informed Consent Form.

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