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Original article

In vitro photoprotective effects of *Marcetia taxifolia* ethanolic extract and its potential for sunscreen formulations

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ABSTRACT

The species *Marcetia taxifolia* (A. St.-Hil.) DC., Melastomataceae, which is endemic of the rupestrian fields of northeastern Brazil, contains a significant amount of flavonoids. In this work, the potential of the ethanolic extract of *M. taxifolia* as the active principle in a sunscreen photoprotection (UV-A and UV-B) formulation was investigated. The Liquid Chromatography High Performance-Diode Array Detector quantification (quercetin), total flavonoid content, antioxidant activity through 2.2-diphenyl-1- picrylhydrazil method, photoprotective activity against UV-B and UV-A radiation *in vitro* (spectrophotometric method) and potential for eye irritation using the methodology of the hen egg test-chorioallantoic membrane were performed in the extract. After that, the formulations were prepared using different concentrations of active ethanolic extract (5, 10, 20 and 30%) and the evaluation of the sun protection factor was carried out using the same methodology used for the crude extract. The crude extract showed UV-A photoprotection and low eye irritation in the hen egg test-chorioallantoic membrane test. All formulations containing *M. taxifolia* extract had ≥ 6 sun protection factor. Its shows the possibility to use this extracts as a sunscreen in pharmaceutical preparations.

Introduction

The ultraviolet radiation (UVR) is divided into three distinct regions: ultraviolet A (UV-A, 315-400 nm), ultraviolet B (UV-B, 280-315 nm) and ultraviolet C (UV-C, 100-280 nm) (Polonini et al., 2011). Reaching the skin, with cumulative action, UVR causes a complex process associated with morphological and chemical reactions. DNA is an important macro molecule that absorbs UVR and hence can mutate, what in the future can result in malignant transformation of the cell skin cancer (Balogh et al., 2011). The effective protection against UVR is available as preparations for topical use containing solar filters, known as sunscreens. The efficacy of such products is dependent on their capacity to absorb radiant energy. The effectiveness of a sunscreen is measured as a function of their sun protection factor (SPF). Thus, the necessity to provide high SPF and screening efficiency against both ultraviolet A and ultraviolet B wavelengths is evident (Vilela et al., 2011).

Flavonoids compounds are widely distributed in the plant kingdom and possess a biological action, especially antimicrobial, antioxidant and photoprotective activities (Pietta, 2000; Lacombe et al., 2010). The demand for active flavovoid-rich extracts has become an important component for the discovery of new molecules active to human photoprotection. That is due to its structural similarity to chemical filters which makes it susceptible to radiation absorption in the ultraviolet region (Agati et al., 2013). Plant extracts rich in flavonoids are capable of absorbing ultraviolet light, usually two maximum peaks of ultraviolet absorption in the UV-B and UV-A regions, what results in the possibility for the use of these extracts in the development of sunscreen formulations (Bobin et al., 1995).

The Melastomataceae family contains around 170 genera and 4,600 species distributed in tropical and subtropical areas of the world (Reis et al., 2005). They are especially abundant in Brazil, occurring in forests, savannahs and rupestrian fields. Several populations of the *Marcetia* genus are found in rocky fields in the Minas Gerais (Serra do Espinhaço) and Bahia (Chapada Diamantina) states. These fields are a set of elevations around 1,200 m with high irradiation, high winds, drought and a rocky soil quartz nature with small depth and low fertility (Giulietti et al., 1987). The genus *Marcetia* currently comprehends 29 species, with approximately 90% inhabiting Bahia (Brazil). The species *Marcetia taxifolia* (A. St. Hil.) DC. is represented by shrub and subshrub between 15 and 300 cm in height, small leaves, opposite and sessile or shortly petiole (Martins, 1989).

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The previous investigation made in our laboratory in the *Marcetia taxifolia* show the antimicrobial activity and the presence of flavonoids using HPLC-DAD analysis (Leite et al., 2012). In this work the potential of photoprotective activity of the ethanolic extract from the same plant in a sunscreen formulation is investigated.

Materials And Methods

Plant material

Marcetia taxifolia (A. St.-Hil.) DC., Melastomataceae, was collected in Rio-de-Contas (Chapada Diamantina, state of Bahia, Brazil). Voucher specimens were deposited in the Herbarium of the Department of Biology of the State University of Feira de Santana (HUEFS) with the following number: 191053.

Preparation of plant extracts

The dried powdered leaves of *M. taxifolia* (1500 g) were subjected to thorough maceration in 95% ethanol. The extractions were carried out at intervals of 72 h. The ethanolic extract was filtered throught a vacuum filter and the filtrate was defatted using hexane. The ethanolic portions were dried and used for the studies.

HPLC-DAD quantification

A Purospher 100 RP-18 (250 mm × 4.6 mm i.d., 5 μm) column (Merck) was used. The mobile phase was composed by solvent (A) H_2O/H_3PO_4 0.1% and solvent (B) MeOH. The solvent gradient was programmed as A (75-0%) and B (25-100%) for 20 min, then 100% B for 4 min, then 75% A and 25% B for 10 min. A flow rate of 1.0 μl/min was used in a 30 °C oven, and 20 μl of each sample was injected. The eluate was monitored at a detection wavelength of 360 nm. Precisely, weighed sample were dissolved in methanol (10 mg/μl). The method was validated by an external calibration curve using standard solutions of quercetin, prepared in methanol in five different concentrations, ranging from 0.1 to 1.5 mg/μl. The quercetin standard solutions were injected three times, and the curve was constructed on Microsoft Office Excel© using the average of the area. The accuracy was expressed as the agreement between the experimentally measured value and the set reference value. The precision and accuracy were calculated according to the formula, respectively: RSD (%) = (SD × 100)/C, where RSD (%) is the precision, SD is the standard deviation and C is the mean concentrations calculated; Accuracy (%) = (Cexp × 100)/TC, where Cexp is the total concentration of quercetin from the extract and TC is the theoretical concentration of the standard reference. The detection limit (LOD) and quantification limit (LOQ) were estimated by the slope and the mean standard deviation of the concentrations used to construct the analytical curve.

Total flavonoid content (TFC)

The TFC was determined by using a colorimetric method as previously described with a few modifications (Gursoy, 2009). The data were expressed as μg of quercetin equivalents (Que) per 10 mg of ethanolic extract weight.

DPPH Free radical scavenging assay

The free radical scavenging activity was measured using the 2.2-diphenyl-1- picrylhydrazil (DPPH) assay (Mensor et al., 2001). The absorbance values were measured at 518 nm and converted into the percent antioxidant activity (AA) using the following formula: AA% = [(absorbance of the control − absorbance of the sample)/absorbance of the control] × 100. The IC_{50} values were calculated from a linear regression of the data using the GraphPad Prism® 5.0 program.

Sun protection factor (SPF) of the crude extract

The ethanolic extract was dissolved in ethanol to a final concentration of 12.5, 25, 50, 125 and 250 μg/μl. The SPF model used in this study was according to the methodology described by Mansur et al. (1986). The sample absorbances were measured in UV-B wavelength range (290-320 nm), with 5-nm increments and three determinations were made at each point. The SPF was calculated by applying the Mansur equation: SPF = CF x $\sum_{290}^{320}$ EE (λ) x I(λ) x abs (λ), where: CF (correction factor) = 10; EE (λ) is the erythemal efficiency spectrum; I(λ) is the solar intensity spectrum; abs (λ) is the absorbance of the solution. The values of EE (λ) x I(λ) are constant according Sayre et al. (1979).

UV-A blocking activity of the crude extract

A 1 mg/ml hydroalcoholic solution of *trans*-resveratrol was prepared. Petri dishes of 4.5 cm diameter were filled with 5 μl of this solution. Each Petri dish was covered with a 0.04-g evenly spread layer of dry ethanolic extract of *M. taxifolia* to be tested for their UV blocking activity. Petri dishes with clean lids were used as control. The petri dishes were placed inside the UV-A chamber and were irradiated with a radiation intensity of about 830 W/m^2 (between 320 and 400 nm) for a period of time (0-120 min). In predetermined intervals (20 min) a sample was collected and diluted at 1:10. The absorbance was measured spectrophotometrically (306 nm) using a UV-VIS Varian® (Cary 100 BIO) (Kerrilee et al., 2009). The absorbance of a sample totally protected from light was also measured in predetermined intervals to assure that the degradation was induced by UV-A light; *trans* isomer, when exposed to light, becomes the *cis* making constant absorbance (Kerrilee et al., 2009). This experiment was performed in triplicate.

In vitro eye irritation tests (HET-CAM)

The irritating potential of the crude extract was performed using HET-CAM (hen's egg test-chorioallantoic membrane) test according to ICCVAM (ICCVAM, 2010). The assay employs the CAM of a 10-day-old fertilized hen's egg. The CAM, a membrane which surrounds the developing chick embryo, is highly vascularized (Bagley et al., 1991), and is regarded as being insensitive to pain (Dannhardt et al., 1996). For this test, the ethanolic extract of *M taxifolia* was dissolved in distilled water at concentrations of 250, 125, 62.5 μg/μl. The fertilized chicken eggs were obtained from commercial sources. On the 10th day of incubation, the egg shell was removed around the chamber air, showing the shell membrane. After CAM was exposed, 300 μl of the extracts was applied. The positive controls (NaOH 0.1 M), and a negative control (distilled water) were performed to demonstrate the validity of the test. For each concentration and controls, three eggs were used. After the application of the extracts, the membrane and blood vessels were examined for 5 min. The time of appearance, measured in seconds, of each irritant effect (haemorrhage, lysis and coagulation) was recorded.

The following formula is used to generate an irritation score (IS):

$$IS = \frac{(301 - H) \times 5}{(300)} + \frac{(301 - L) \times 7}{300} + \frac{(301 - C) \times 9}{300}$$

Where H = the time taken to start the hemorrhage reactions; L = time taken to start of vessel lysis; C = time taken to start coagulation.

Table 1
Polowax lotion composition for the incorporation of *Marcetia* extract.

Phase	Raw material	Amount	Properties
1	Methylparaben	0,15%	Preservative
	Glycerine	5%	Moist
	Deionized water	q.s.p. 100 g	Carrier
2	Propylparaben	0,05%	Preservative
	Liquid Vaseline	3%	Lubricant
	Octyl stearate	4%	Emollient
	Polowax	5%	Basis and Auto-Emulsifier
3	Imidazolidinyl urea	0,1%	Preservative

Table 2
Composition of the sunscreen formulation.

Phase	Raw material	Amount	Properties
1	Methylparaben	0,15%	Preservative
	Glycerine	5%	Moist
	Deionized water	q.s.p. 100 g	Carrier
2	Propylparaben	0,05%	Preservative
	Vaseline liquid	3%	Lubricant
	Octyl stearate	4%	Emollient
	Polowax	5%	Basis Auto Emulsifier
	Benzophenone-3	3%	Sunscreen UVA/UVB
3	Imidazolidinyl urea	0,1%	Preservative

The average score was calculated for each extract, and the extracts were classified into four categories: non-irritant (IS > 1), low irritant (1 ≤ IS < 5), Moderate irritant (5 ≤ IS < 9) and irritant (IS ≥ 9) (Debbasch et al., 2005).

Preparation of sunscreen formulations

The pharmaceutical base for incorporating *Marcetia* extract and sunscreen formula were adapted from the Pharmaceutical Medical Form (Batistuzzo et al., 2006) as described in Tables 1 and 2, respectively.

The vehicle, polowax lotion, used for the incorporation of herbal extracts, was prepared by heating the phase 1 and 2 at 75 °C, separately (Proença et al., 2009). After that, phase 2 was poured into phase 1 under constant agitation, ending with the addition of phase 3 and the mixing temperature below 40 °C. The formula consisted of a standard sunscreen emulsion of O/W containing benzophenone-3 as chemical filter, in according to Table 2. The emulsion was prepared with the same method described for preparing the O/W emulsion without benzophenone-3. The extract was incorporated to the lotion polowax varying in their concentrations of 5, 10, 20 and 30%. Once formulated, the pH was measured and tuned with triethanolamine to pH 6.0 to 7.0, which pH is desirable for sunscreen according to Ribeiro (2010).

Determination of the SPF of sunscreens

For the determination of the SPF of the formulations extracts (5, 10, 20 and 30%), they were dissolved in ethanol to a final concentration of 0.2, 2.0, 5.0, 10, 15, 20, 30 and 50 mg/μl to evaluate the profile of the sunscreen formulations prepared. The methodology used for SPF evaluation of these formulations was the same used for the crude extract. The absorbance readings were performed in triplicate.

Statistical analysis

The analyses were performed in triplicate, and the results expressed as mean ± standard deviation (SD). Differences were considered significant when $p < 0.05$. Multiple comparisons between more than two groups were performed with one-way ANOVA supplemented with Tukey's test. The data obtained were analyzed using the Graph Pad Prism® version 5.0.

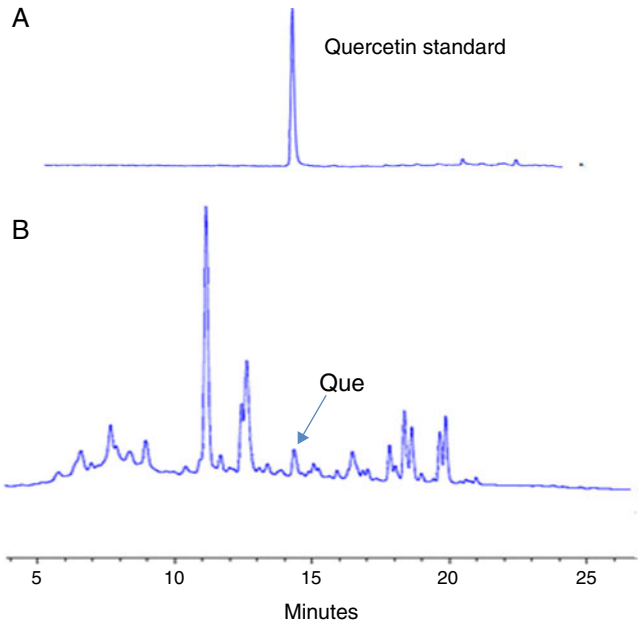

Fig. 1. HPLC chromatogram of the ethanolic extract of *Marcetia taxifolia* (A): flavonoid glycosylated region (Rt: 10–15 min), flavonoid non-glycosilated region (Rt: 16-20 min); HPLC chromatogram (B) of the quercetin standard (Rt: 14.35 min).

Results and Discussion

HPLC quantification

The crude extract of *Marcetia taxifolia* was analyzed through HPLC-DAD (Fig. 1A), what shows the presence of the non-glycosylated (peaks between Rt 10 to 15 min) and glycosylated (peaks between Rt 16 to 20 min) flavonoids. The UV-spectra of the peak at Tr 14.34 min was similar to the flavonol quercetin. Thus, the quercetin was identified from the retention time (Rt) comparison using standard (Fig. 1B). Due to the good photoprotective action of quercetin, this compound was HPLC-quantified in the *M. taxifolia*.

The linearity was confirmed by preparing standard solutions of quercetin solutions in methanol at five concentrations. Calibration curve was plotted and determined using the standards data: for quercetin, y= 30000000x + 14619 and R^2 =0.9987, which were linear for specified concentration ranges. The detection limit was 0.0145 mg/μl and quantification limit was 0.048 mg/μl for quercetin. The quantification results show that the amounts of quercetin present in the ethanolic extract was high above the detection and quantification limits, further emphasizing the reliability of the method. The precision and accuracy of the measurements were within allowable values; the accuracy did not allow values that exceeded 2.09%. The concentration of quercetin present in ethanolic extract from *M. taxifolia* was 1.12 mg/μl.

Total flavonoid content and antioxidant activity

The flavonoid content of *M. taxifolia* extract calculated as the quercetin equivalence shows 168 ± 0.35 μg/μl of quercetin, which is equivalent to 10 mg of each ethanolic extract weight. The antiradical activity of the antioxidant is show in the Table 3. These results show association between antioxidative activities and flavonoid compounds in *M. taxifolia*, EC_{50}: 5,132 ± 1,078 (R^2 = 0.853).

Table 3
Antioxidant activity of the ethanolic extract obtained from *Marcetia taxifolia*.

	250*	125	50	25	10	5
Extract	95.31	93.97	93.43	74.19	35.52	20.25
BHT**	94.64	94.07	86.58	57.16	30.20	18.38
AA***	97.03	96.62	96.98	96.57	74.32	24.60

* μg/ml
** Butylated hydroxytoluene
*** Ascorbic acid

Sun protection factor (SPF) of the crude extract

The ethanolic extract of *M. taxifolia* had the sunscreen activity evaluated with the method developed by Mansur et al., 1986. The results of the determinations of *in vitro* SPF values are shown in Fig. 2. According to the Brazilian law, RDC 30 from June 1, 2012 (Anvisa, 2012a), only SPF greater than or equal to 6 is suitable for use in cosmetic products with photoprotective activity. The *M. taxifolia* extracts with different concentrations (250 and 125 μg/μl) had satisfactory sunscreen activity (15.52 and 8.35, respectively), higher than the minimum required by Anvisa. The SPF values of the extracts tested were concentration-dependent, the increase in concentration resulted in an increment of the SPF. This activity can be attributed to the flavonoids found in species of the *Marcetia* family.

The plant extracts rich in flavonoids which are efficient in absorbing ultraviolet light, usually show two maximum peaks of ultraviolet absorption, one between 240-280 nm and another 300-550 nm (Bobin et al., 1995). Several classes of natural compounds were examined for their antioxidant and photoprotective activity. Recent studies (Polonini et al., 2011) reviewed the importance of natural sunscreens in the compositions of commercial sunscreens and therefore analyzed their role in the prevention of skin cancer. Photoprotective properties have been evaluated in extracts of a great variety of plants, extracts *Chrysanthemum ramosum* and *Crocus sativus* L. proved to be good weather reducing agent-induced erythema as compared to benzophenone 4 (Hu and Wang, 1998). Violante et al. (2009) have developed studies on the species *Macrosiphonia velame*, *Oxalis hirsutissima* and *Lafoensia pacari*, which these species showed absorption in the UV in λmax. 318 nm, 324 nm and 356 nm. The phytochemical studies described the presence of flavonoids, and other constituents as tannins and alkaloids, which are substances described with a photoprotective action. The *Calophyllum inophyllum* extract is rich in various natural sunscreen compounds, such as biflavonoids, etocotrienols tocoferols, and broad absorbance in the UV, as well as antioxidant activity (Said et al., 2007). Studies using the dry extract from the leaves of *Encholirium spectabile*, Bromeliaceae, described a potent antioxidant activity and significant photoprotective activity correlated with flavonoid compounds present (Oliveira et al., 2013).

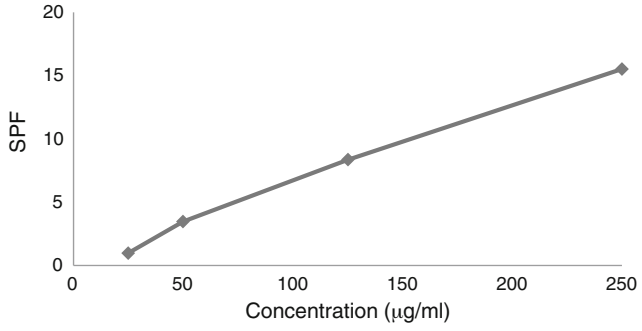

Fig. 2. Sun Protection Factor (SPF) of the ethanolic extract of *Marcetia taxifolia*.

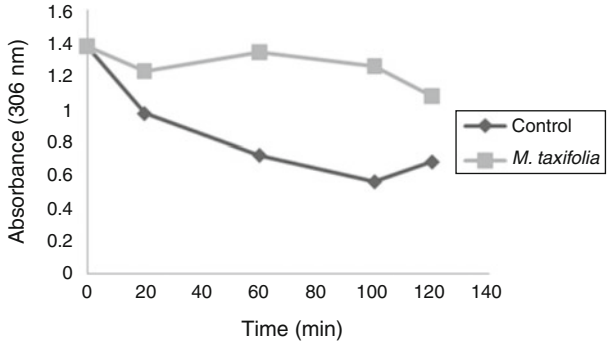

Fig. 3. Evaluation of the photoprotective potential against UVA radiation by the *Marcetia* ethanolic extract.

UV-A blocking activity of the crude extract

The plant extracts shown had the ability to protect a photolabile solution (*trans*-resveratrol) from UVA radiation. When *trans*-resveratrol is exposed to UVA radiation, it undergoes degradation which can be seen by the absorbance decreases during the time intervals (Kerrilee, 2009). It was found that during the control board of exposition to UVA radiation, there was photobleaching of 50.73% *trans*-resveratrol. The plate was coated with *M. taxifolia* extract and photobleaching was less pronounced; the end of the 120 min exposure conversion of *trans*-resveratrol to *cis*-resveratrol was 9.48%. This study showed that the extract from the *M. taxifolia* is capable of preventing UV-A radiation from permeating across a Petri dish lid (Fig. 3). This study of *M. taxifolia* showed its good ability of photoprotection against UV-A.

In vitro eye irritation tests (HET-CAM)

The HET-CAM test showed that *M. taxifolia* (250 and 125 μg/μl) contend low irritation potential: 1.93. These results indicated that the ethanol extract of *M. taxifolia* is suitable for use in cosmetics (Table 4). Reports on the toxicological evaluations of species of the *Marcetia* genera were not found in the literature.

In addition to the protection against UV-B radiation, products being currently researched also have photoprotective activity against UV-A rays. Initially, it was believed that UV-A radiation did not cause damage to the skin. However, studies have showed that this process contributes to radiation and promotes the appearance of photo-aging skin tumors. The UV-A radiation penetrates more deeply into the dermal layers of the skin, unlike UV-B which is absorbed into the skin epidermis. UV-A generates more oxidative stress than does UV-B, and at levels found in sunlight, it is ten times more efficient than is UV-B in causing lipid peroxidation leading to plasma membrane damage (Damiani, 2006). Some studies have demonstrated that sunscreen photo-instability is primarily a problem concerning the UV-A region (Maier et al., 2001; Hojerova et al., 2011).

Table 4
Classification of extracts according to the score of the phenomena.

	Concentration (μg/ml)	Irritation Score (IS)	Classification Irritancy
M. taxifolia	250	1.93	low irritant
	125	1.08	low irritant
	62,5	0.14	non-irritant
NaOH 0,1 M*	0.1 M	12.6	irritant
Distilled water**		0.0	non-irritant

* Positive control
** Negative control

Table 5
Dilution (mg/μl) and SPF for the formulations with the *Marcetia taxifolia* ethanolic.

MT 5%	MT 10%	MT 20%	MT 30%	Benzophenone 5%
0.2 mg/ml				
1.241 ± 0.119^{d}	1.796 ± 0.0800^{c}	2.231 ± 0.0494^{c}	2.860 ± 0.0655^{b}	4.823 ± 0.0907^{a}
2.0 mg/ml				
6.397 ± 0.0626^{e}	7.336 ± 0.2748^{d}	13.360 ± 0.0625^{c}	14.530 ± 0.0598^{b}	38.530 ± 0.3256^{a}
5.0 mg/ml				
10.200 ± 0.3160^{e}	15.810 ± 0.2379^{d}	17.170 ± 0.0511^{c}	27.460 ± 01150^{b}	38.800 ± 0.01464^{a}
10.0 mg/ml				
25.430 ± 0.1806^{e}	28.480 ± 0.0265^{d}	30.54 ± 0.0505^{c}	33.780 ± 0754^{b}	38.730 ± 0.01201^{a}
15.0 mg/ml				
32.620 ± 0.1931^{e}	33.150 ± 0.0775^{d}	34.590 ± 0.3471^{c}	35.340 ± 0987^{b}	39.100 ± 0.0808^{a}
20.0 mg/ml				
32.730 ± 0.1561^{d}	33.30 ± 0.1103^{c}	33.730 ± 0.0657^{b}	33.890 ± 0887^{b}	39.20 ± 0.0624^{a}
30.0 mg/ml				
33.250 ± 0.1402^{e}	34.140 ± 0.0340^{d}	24.610 ± 0.0209^{c}	36.550 ± 0417^{b}	42.370 ± 0.2960^{a}
50.0 mg/ml				
37.310 ± 0.1708^{c}	38.430 ± 0.0377^{b}	42.550 ± 0.2215^{a}	43.110 ± 0.0800^{a}	39.840 ± 0.2343^{a}

The level of significative was $p < 0.05$. The equal letters represent statistically equal values for the same dilution.

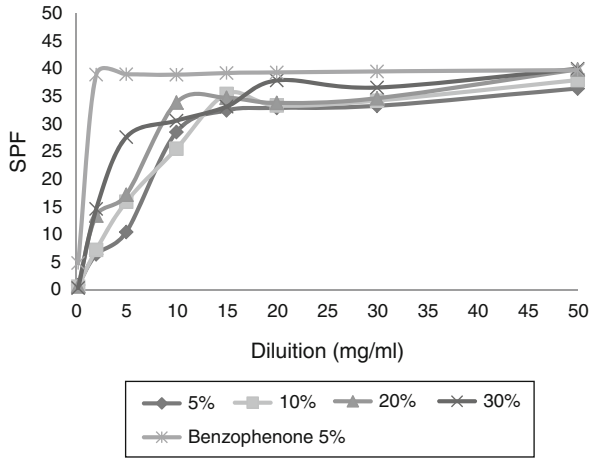

Fig. 4. Profile of sunscreen formulations with the *Marcetia taxifolia* ethanolic extract.

Currently, the pharmaceutical and cosmetic industries, along with government regulatory and quality control of products, have used alternative methodologies for assessing the potential for eye irritation. In this perspective, the National Health Surveillance Agency (Anvisa) has adopted the Guideline for the Safety Evaluation of Cosmetic Products (Anvisa, 2012b). The *in vitro* HET-CAM, which provides information on the eye-irritating properties, is an essential part of the hazard identification of chemicals and products. The HET-CAM basically assesses vascular changes and generates results which, in turn, compared to the animal model, have a great relationship with phenomena observed in conjunctiva, even if they are, as a rule, lighter weight than changes detected in other systems such as, for example, those induced in isolated organs (Nóbrega et al., 2008)

Determination of the SPF of sunscreens

Tukey's multiple-comparison test was used to evaluate whether the SFP in the dilutions was the same, thereby forming a UV-B sunscreen profile for formulations of *M. taxifolia* extract (Fig. 4).

The SFP evaluation show good results for formulations content *M. taxifolia* extracts (5, 10, 20 and 30%) which were observed SFP ≥ 6 dilution from 2 mg/μl (Table 5). For the species investigated, it was observed that the dilution of 50 mg/μl was close to the SFP standard of benzophenone 5%, in the fixed protective factor of 30 - 50, which indicates that, according to the RDC 30/12, the formulations may be suitable for very sensitive skin sunburn, because of their high sun protection, as demonstrated in the *in vitro* methodology.

Analyzing statistically the SPF values is possible noted that the formulations with *M. taxifolia* extract 10% and 20% (Table 5) showed the same SPF values for the dilution of 0.2 mg/μl. The formulations at 20% and 30% at a concentration of 20 mg/μl were also statistically equal. The same is true for the concentration of 50 mg/μl and the standard formulation of 5% of benzophenone.

Conclusion

The ethanolic extract of *M. taxifolia* show good antioxidant and photoprotective activity against UV-B and UV-A radiation. These effects can be attributed to flavonoid derivatives present in the plant including quercetin. Sunscreens were developed and SPF was evaluated. The formulations containing *M. taxifolia* ethanolic extract showed SPF values near a chemical filter frequently used in the pharmaceutical industry, benzophenone-3. The *M. taxifolia* has great a potential for use as an active ingredient for sunscreens.

Authors' Contributions

SCCC helped with the measurement of the *in vitro* antioxidant activity, total flavonoid and HPLC analysis. She also participated of the preparation of the formulations, interpretation of data and statistical analysis, evaluation of the *in vitro* photoprotective activity of sunscreens and the evaluation stability tests. CBD was in charge of the evaluation of the *in vitro* photoprotective activity of sunscreens. MBB helped with the measurement of the *in vitro* Eye Irritation Tests (HET-CAM) and carried out the interpretation of data and statistical analysis. CRCB conducted studies of the HPLC and the development of the sunscreen formulation. AB participated in the coordination of the project. All authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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