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Original Article

In vitro and *in vivo* evaluation of efficacy and safety of photoprotective formulations containing antioxidant extracts

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ABSTRACT

Chronic exposure to solar radiation could contribute to premature skin aging and skin cancer. Skin presents its own antioxidant defense, however when defenses are out of balance, reactive oxygen species could damage biological structures. In the present work, an oil-in-water photoprotective emulsion was developed and *Bauhinia microstachya* var. *massambabensis* Vaz, Fabaceae, extracts at 1% (obtained by extraction with different solvents) were added to this emulsion. *In vitro* and *in vivo* efficacy and safety of the formulations were evaluated. Spectrophotometric methods and *in vivo* Colipa test were performed to evaluated efficacy of the formulations, through sun protection factor (SPF) determination and UVA protection factor assessment. To the *in vitro* safety assessment HET-CAM, CAM-TBS and Red Blood Cell tests were performed. Results showed that both extracts contributed to a higher *in vivo* photoprotection (SPF 18) when compared to the formulation without extract (SPF 13), this result could be attributed to the antioxidant activity of the plant extracts that act by capturing reactive oxygen species. Concerning safety, all formulations were considered non-irritant according to *in vitro* tests. Formulations containing extracts could be considered efficient and safe for cosmetic use since they presented higher sun protection factor and passed the toxicity tests.

Introduction

Skin is the outer covering of human body conferring protection against ultraviolet (UV) radiation (Bouwstra et al., 2007; Bolzinger et al., 2012). However, chronic exposure to UV radiation leads to many side effects to the skin, such as premature aging, skin cancer and reduction of immune response capability. These health problems are directly related to the formation of reactive oxygen species (ROS) by UV radiation (Jain and Jain, 2010; Gilbert et al., 2013).

Even presenting antioxidant defense mechanisms, skin could be affected by ROS; when defense mechanisms are out of balance, oxidative stress could damage cellular membranes, proteins, carbohydrates and nucleic acids promoting their oxidation (Finkel and Holbrook, 2000; Gálvez, 2010). On the other hand, ROS play *in vivo* positive functions related to energy production, phagocytosis, cell growth regulation and intercellular signaling (Gutteridge and Halliwell, 2000; Rouanet et al., 2010).

Inorganic and organic sunscreens are added to photoprotective formulations since they act protecting the skin against UV radiation; however, recently there has been much research about the use of antioxidants extracted from plants. These natural antioxidants usually come from a diet rich in fruits and vegetables or they are carried in creams and topically applied (Podda and Grundmann-Kollmann, 2001). Plant extracts with antioxidant properties raise great interest in the phytocosmetic field as they present molecules that could inactivate ROS restoring skin homeostasis thus preventing erythema and premature aging of the skin (Calderon-Montano et al., 2011; Mansur et al., 2012). Barradas and coworkers (2014) developed nanoemulsions containing plant oil, sweet fennel oil, to be applied topically; the researchers verified that sweet fennel oil presented antioxidant properties, probably

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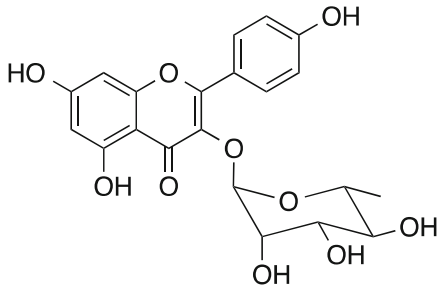
due to the presence of flavonoids and terpenoids that promote high radical scavenging activity.

In a previous work from our group, it was studied the antioxidant effect of different plant extracts from the genus *Bauhinia* (Mansur et al., 2012). *B. microstachya* var. *massambabensis* Vaz, Fabaceae, is restricted to arid zones and it is found only in Rio de Janeiro State, Brazil.

Our study has demonstrated a higher antioxidant activity of *Bauhinia* leaf extract when compared to the *Gingko biloba* standard extract (EGb 761) and Trolox® (a vitamin E water-soluble analog). *Bauhinia* plant extracts present high amounts of flavonoid glycosides, including galloyl derivatives, as well as methyl gallate and gallic acid-like substances that are acknowledged as potent antioxidants thus being a source for the study of different pharmacologic activities and did not show phototoxicity according to the Minimal Inhibitory Concentration Method (MIC), where no zone of inhibition in the growth of in the *Saccharomyces cerevisiae* was verified (Mansur et al., 2012).

The main substances and their molecular structures identified in the phytochemical fractionation of *Bauhinia* ethyl acetate extract are the flavonoids kaempferol-3-*O*-rhamnoside (**1**) and astragalin-2″,6″-di-*O*-digallate (**2**) (Mansur et al., 2012). Other authors also identified these flavonoids in *Bauhinia* active plant extracts (Menezes et al., 2004; Silva et al., 2007).



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Flavonoids generally occur in plants as glycosylated derivatives that participate in photosynthesis (Pietta, 2000). These phenolic compounds confer protection to the plants since they capture ROS, protecting them from oxidation, which is generated by UV radiation from the sun. For this reason, these compounds could be topically applied on humans with the same intention of capturing ROS, thus inhibiting lipid peroxidation, which is responsible for skin aging and skin cancer (Zuanazzi and Montanha, 2004; Laguerre et al., 2007).

In recent years, natural substances have been increasingly incorporated into dermocosmetic formulations; it is a worldwide tendency to add value to products especially because of the great commercial appeal and increased acceptance by customers. Besides, the development of photoprotective formulations using less synthetic sunscreens is one of the objectives in photoprotection research (Gilaberte and González, 2010; Hayes et al., 2011). However, the safety of these formulas must be evaluated before their availability to customers; thus the irritant potential of photoprotective formulations should be assessed through *in vitro* and *in vivo* tests to guarantee the presence of safer products in the market, reducing risks to costumers (Anvisa, 2012).

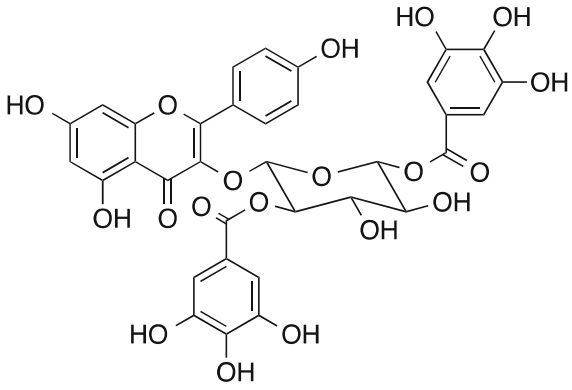
Many *in vitro* tests are available to evaluate toxicity of cosmetic products; some of which are destined to evaluate ocular toxicity, *e.g.* Hen's Egg Chorioallantoic Membrane (HET-CAM) test, Chorioallantoic Membrane-Trypan Blue Staining (CAM-TBS) test, Red Blood Cell (RBC) test and Bovine Corneal Opacity and Permeability (BCOP) test (Anvisa, 2012). These tests aim to evaluate the safety of facial cosmetic products, since the product could be easily in contact with the eye mucous membrane generating irritation. Moreover, toxicity tests could be also performed to evaluate primary cutaneous irritation, which is necessary when it comes to topical formulations.

The aim of the present study was to develop photoprotective oil-in-water (O/W) emulsions containing the sunscreens benzophenone-3 (BZF-3), octylmethoxycinnamate (OMC) and octocrylene (OCT) and *Bauhinia microstachya* var. *massambabensis* Vaz leaf extracts – in water and acetone (WAc) and in ethanol treated with activated carbon (EtOH-AC) – and evaluate the *in vivo* efficacy and safety of these formulations.

Materials and methods

Chemicals

All reagents were of analytical grade (Sigma, Merck). Sunscreens used in the formulations: BZF-3, purchased from Galena (Brazil); OMC, purchased from Spectrum (Brazil) and OCT, purchased from DEG (Brazil).



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Plant leaf extracts

Bauhinia microstachya var. *massambabensis* Vaz, Fabaceae, leaves were collected from the botanical garden of the Department of Botany and Pharmacognosy (Federal University of Rio de Janeiro) and the leaf extracts were obtained according to the Management Council for Brazililan Genetic Patrimony (CGEN), resolutions n° 28 and n° 29, 2007. The specimen was deposited under the number 30813 in the *Herbarium* of the Biology Institute of the Federal University of Rio de Janeiro, a CGEN-accredited *Herbarium* RFA. Extracts were obtained according to Mansur and coworkers (2012) – in water and acetone (WAc) and in ethanol submitted to treatment with activated carbon to provide a less colored ethanol extract (EtOH-AC). These extracts were chosen since they are more suitable to cosmetic application than the other extracts obtained by the group in a previous work (Mansur et al., 2012). Sunscreens used in the formulations: BZF-3, purchased from Galena (Brazil); OMC, purchased from Spectrum (Brazil) and OCT, purchased from DEG (Brazil).

Development of the formulations

The ingredients used to the development of the formulations are listed in Table 1. It was developed four O/W emulsions: with

Table 1
Composition of the formulations A, B, C, D and E.

Ingredients	Formulations				
	A	B	C	D	E
Oil phase (wt%)					
BZF-3	5	5	5	–	–
OCT	5	5	5	–	–
OMC	5	5	5	–	–
Cetostearyl alcohol ethoxylate	3	3	3	3	3
Stearic acid	8	8	8	8	8
Isoctyl stearate	7	7	7	7	7
Glyceryl monostearate	3	3	3	3	3
Propylparaben	0.1	0.1	0.1	0.1	0.1
Dimethicone copolyol	9.5	9.5	9.5	9.5	9.5
Cyclomethicone	11	11	11	11	11
Aqueous phase (wt%)					
Aminomethyl propanol 95%	0.3	0.3	0.3	0.3	0.3
Glycerin	5	5	5	5	5
Imidazolidinyl urea	0.2	0.2	0.2	0.2	0.2
Methylparaben	0.1	0.1	0.1	0.1	0.1
Hydroxypropyl starch phosphate	1	1	1	1	1
EtOH-AC extract	–	**1** grams	–	**1** grams	–
WAc extract	–	–	**1** grams	–	**1** grams
Purified water	Qs[a] 100 wt%	Qs[a] 100 wt%	Qs[a] 100 wt%	Qs[a] 100 wt%	Qs[a] 100 wt%

[a] Qsf: quantity sufficient.

the mixture of the three sunscreens, BZF-3, OMC and OCT (formulation A); with the same mixture of sunscreens and 1 wt% of EtOH-AC extract (formulation B) and with the same mixture of sunscreens and 1 wt% of WAc extract (formulation C). In addition, it was also prepared O/W emulsions containing only one extract, without sunscreens: formulation D with 1 wt% of EtOH-AC extract and formulation E with 1 wt% of WAc extract to verify if the *Bauhinia* extracts alone (without sunscreens) presented sun protection (*in vitro* SPF assessment).

The standard O/W emulsion (without sunscreens or extracts) was a typical white-colored formulation with pH of 6.0 that was produced by the classical emulsification method where the oil phase is heated and poured into the aqueous phase at the same temperature (around 70 °C) under slow stirring until complete homogenization; the emulsifier enable the interaction of both phases. To formulation A, B and C, sunscreens were solubilized in the oil phase before the mixture with the aqueous phase and to formulations B, C, D and E the respective extracts were added after the phases' mixture, under slow stirring when the temperature was at 40 °C to prevent the extracts' degradation by heat.

In vitro *SPF and UVA Protection Factor (UVA-PF) assessment*

In vitro SPF evaluation is usually performed to estimate the *in vivo* SPF. To the *in vitro* SPF assessment formulations A, B, C, D and E were evaluated.

Samples were diluted in ethanol at a final concentration of 2 μl/ml and analyzed by UV spectrophotometry (Jasco V-630) from 290 to 320 nm, with intervals of 5 nm, according to Mansur's method (1986) (Mansur et al., 1986). Mansur's method is simple and easily reproducible, the SPF determination which is the correlation between the erythemogenic effect (EE) and the radiation intensity at each wavelength (*I*) (Table 2) and are adjusted according to Eq. (1). Where the correction factor (CF) is 10, EE (λ) is the erythemogenic effect of radiation on wavelength λ, *I*(λ) is the intensity of solar light with wavelength λ and abs (λ) is the sample (2 μl/ml in ethanol) spectrophotometric absorbance value at wavelength λ.

$$\text{Spectrophotometric SPF} = \text{CF}\sum_{290}^{320}\text{EE}(\lambda)I(\lambda)\text{abs}(\lambda) \quad (1)$$

Table 2
Correlation between the erythemogenic effect (EE) and the radiation intensity at each wavelength (*I*) (Mansur et al., 1986).

λ (nm)	EE (λ) × *I* (λ)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180

To the *in vitro* UVA-PF assessment it was used a UV transmittance analyzer (Labsphere® UV-2000S) and quartz plates with an area of 25 cm^2. The plates were covered by Transpore™ tape on one surface and an amount of 50 mg (2 mg/cm^2) of each formulation was applied with a micropipette on this surface and manually spread with circular movements in order to obtain a homogeneous film. Glycerin was used as reference for 100% of transmission (Labsphere, 2008). After drying for 15 min in dark chamber, samples were analyzed. Both assays were performed in triplicate and the mean ± standard deviation (SD) was assessed.

Photostability assay

Formulations A, B and C were evaluated in the photostability assay. The assay was performed using a solar simulator (Oriel 91192-1000), a UV spectrophotometry (Jasco V-630) and a UV transmittance analyzer (Labsphere® UV-2000S). To the SPF assessment (Mansur's method), the amount of 250 mg of each formulation was applied to 30 mm diameter Petri dishes with 1 ml of ethanol forming a homogeneous film. The plates dried in a dark chamber for 60 min to allow ethanol to evaporate. For UVA-PF assessment, the samples were applied in quartz plates covered by Transpore™ tape. All plates and Petri dishes were irradiated for 90 min in the solar simulator with radiation intensity of 315 $J/m^2/s$ (UVA) and 3.35 $J/m^2/s$ (UVB). Three plates and three Petri dishes were stored in the absence of light without being irradiated, as a negative control. After irradiation, samples were diluted in ethanol and analyzed by UV spectrophotometry according to Mansur's method (SPF) or analyzed in the UV transmittance analyzer

(UVA-PF). The test was performed in triplicate and the mean ± SD was assessed.

In vivo *SPF assessment*

To the *in vivo* SPF assessment formulations A, B and C were evaluated. This assay was carried out according to the Brazilian Ethics Committee for Clinical Trials (number: 120/10/2011). The methodology employed was based on the International Sun Protection Factor Test Method developed by Colipa and published as a guideline in 2006 (Colipa, 2006).

The SPF value is defined as the ratio between the ultraviolet energy required to produce a minimal erythemal dose (MED) on protected skin and the ultraviolet energy required to produce a MED on unprotected skin according to Eq. (2).

$$\text{SPF} = \frac{\text{MED of protected skin}}{\text{MED of unprotected skin}} \quad (2)$$

Ten women between the ages of 18 and 42 years with skin phototypes I, II or III (Fitzpatrick, 1975) were selected as volunteers after being informed about the study protocols and agreed to participate giving their written consent. The back of each volunteer was exposed to ultraviolet radiation using a multiport (model 601) ultraviolet solar simulator. The exposure time was changed according to skin phototype. On the second day the MED (without sunscreen) was assessed for each volunteer and then the formulations B, C and D were applied in the amount of 2 mg/cm^2 to other areas on the back of the volunteer. After the application, the products were left to dry for 15 min before being irradiated. Areas of 5 × 6 cm were irradiated in six points with increasing UV radiation doses. The SPF for each formulation was assessed as a mean ± SD of the SPF values obtained for the volunteers.

Safety of the photoprotective formulations and extracts: irritant potential evaluation

Formulations A, B and C were evaluated regarding their safety (Anvisa, 2012). In addition, to verify the safety of the free extracts (without being incorporated into the O/W emulsion), they were vehiculated at 1 wt% in phosphate buffer (PB) pH 7.4. Three different methods were performed: HET-CAM test, CAM-TBS test and RBC test.

Hen's Egg Chorioallantoic Membrane (HET-CAM) test

Chicken embryos have been widely used as an alternative to the *in vivo* ocular irritation test. For this test, fresh fertile Leghorn eggs weighing 50–60 g were placed in an automatic rotation incubator and kept at 37.5 ± 0.5 °C with relative humidity of 62.5 ± 7.5% during 10 days. On the tenth day, the egg shell around the air chamber was removed using an odontological saw, exposing the shell membrane that was moistened with saline solution 0.9%. With the aid of tweezers the shell membrane was removed exposing the chorioallantoic membrane (CAM). Visual analysis was performed to verify if the CAM was suitable to the test then 300 μl of each formulation was placed on the CAM surface. After 20 s, the formulation was removed with saline solution. The CAM was observed under a magnifying glass for 5 min to determine the incidence of any irritant effects in the CAM blood vessels (hyperemia, hemorrhage or coagulation) (Luepke and Kemper, 1986; Worth and Balls, 2001; Liebsch and Spielman, 2002). The irritant effects were classified by scores (1, 3, 5, 7, or 9) according to the time they were observed: less than 30 s (hyperemia: 5; hemorrhage: 7; clot formation/opacity: 9); between 30 and 120 s (hyperemia: 3; hemorrhage: 5; clot formation/opacity: 7); or between 120 and 300 s (hyperemia: 1; hemorrhage: 3; clot formation/opacity: 5). If an effect was not observed after 5 min, it was scored as zero.

Each formulation was classified according to the scores mean value of four eggs: 0–4.99 corresponding to non-irritant/slightly irritant (NOI/SLI); 5.00–8.99 corresponding to moderately irritant (MOI); and 9.00–21.00 corresponding to severely irritant (SEI). As a negative control four eggs were submitted to the same procedure, but no formulation was added.

Chorioallantoic Membrane-Trypan Blue Staining (CAM-TBS) test

CAM-TBS is a quantitative method for the evaluation of the formulations' toxicity (Invitox, 1996; Lagarto et al., 2006). The CAM-TBS test uses trypan blue as an indicator of chorioallantoic membrane (CAM) injury and shows a good correlation with the *in vivo* Draize eye irritation test (Liebsch and Spielman, 2002; Scott et al., 2010). The methodology is similar to the HET-CAM and after the removal of the cosmetic formulation, 500 μl of a phosphate saline buffer and 0.1% of trypan blue staining (TBS) were added to the CAM in the area limited by a 18 mm diameter silicone ring. The excess of TBS was rinsed off with distilled water, and the CAM area that was limited by the silicone ring was removed with scissors and put into 5 ml of formamide and then agitated and centrifuged. The absorbance of the supernatant was measured by spectrophotometry at 595 nm. The quantification of the trypan blue staining that entered the cells could be correlated to the injury caused by the formulation to the CAM.

Each formulation was classified according to the mean value of four eggs based on the HET-CAM scores: 0–4.99 corresponding to NOI/SLI; 5.00–8.99 corresponding to MOI; and 9.00–21.00 corresponding to SEI. The score (d) for each formulation was assessed using Eq. (3) (Lagarto et al., 2006). As a negative control four eggs were submitted to the same procedure, but no formulation was added.

$$\text{TBS concentration} = d \times \frac{5}{1000} \times 10^9 \ \text{nmol} \quad (3)$$

Red blood cell (RBC) test

RBC test enables the quantification and evaluation of the side effects caused by surfactants added to many cosmetic products, such as shampoo, shower gel and emulsions in the red blood cells plasmatic membrane and consequently the hemoglobin release (hemolysis) and the hemoglobin denaturation index, both quantified by spectrophotometry. The relationship between hemolysis and hemoglobin oxidation provides a parameter to the characterization of the *in vitro* irritant effects of the surfactants (Alves et al., 2008).

Sheep RBC were isolated by *g* force. For hemolysis analysis, solutions of 1 mg/ml of each formulation in PBS were added to vials in order to create a range of increasing concentrations to enable linear regression. Their volume was filled up to 975 μl with PBS and it was added 25 μl of RBC suspension with a well-known concentration of oxyhemoglobin. The resulting suspension was incubated for 10 min at room temperature under slow stirring. After 10 min the samples were centrifuged at 10,000 × *g* for 1 min. The absorbance of the supernatant was determined by spectrophotometry at 540 nm (for oxyhemoglobin) and at 575 nm (for deoxyhemoglobin). For a 0% hemolysis control, 25 μl of RBCs was added to 975 μl of PBS, and for a 100% hemolysis control, 25 μl of RBC was added to 975 μl of distilled water. The denaturation index obtained are comparable to the *in vivo* ocular irritant effect: >100 corresponding to NOI; >10 corresponding to SLI; >1 corresponding to MOI; >0.1 corresponding to irritant (I); and <0.1 corresponding to SEI. The test was performed in triplicate.

Table 3
In vitro assessment of the SPF and the UVA-PF of the formulations A, B, C, D and E before and after irradiation for formulations that presented photoprotective activity.

Formulations	SPF[a]	SPF after irradiation[a]	UVA-PF[a]	UVA-PF after irradiation[a]
A	17.0 ± 0.25	16.6 ± 0.22	2.63 ± 0.17	2.50 ± 0.16
B	17.8 ± 0.77	16.4 ± 0.01	2.50 ± 0.10	2.40 ± 0.20
C	16.9 ± 0.62	16.6 ± 0.13	2.45 ± 0.10	2.40 ± 0.20
D	0.70 ± 0.06	They were not considered photoprotective		
E	0.68 ± 0.02	formulations since SPF in too low		

[a] Mean ± SD.

Statistical analysis

Experimental data are presented as the mean ± SD with at least three determinations for independent experiments. All data were analyzed by paired and unpaired *t*-tests using Origin 8.5.1 (OriginLab, USA) software and $p < 0.05$ was considered to be statistically significant.

Results and discussion

Development of the formulations

The formulations A, B, C, D and E were successfully obtained. All formulations presented a visual aspect and texture suitable to be applied topically.

In vitro *assessment of SPF and UVA-PF before and after irradiation (photostability assay)*

Table 3 shows the results of the *in vitro* SPF for formulations A, B, C, D and E.

Formulations containing only sunscreens (A) and sunscreens and extract (B and C) presented *in vitro* SPF suitable to photoprotective products. Formulations D and E did not present SPF, for this reason they were not considered photoprotective formulations, thus they were not evaluated in terms of UVA-PF and photostability (Santos et al., 1999; Freitas et al., 2001; Monteiro et al., 2012; Mota et al., 2013).

Even not contributing with the *in vitro* SPF, both extracts present phenolic compounds that could act preventing UV-induced damage by other mechanisms, *e.g.* capturing and inactivating ROS (Greul et al., 2002).

The addition of EtOH-AC and WAc *Bauhinia* extracts did not influence sunscreens' photostability since there was no statistical difference among *in vitro* SPF and UVA-PF ($p > 0.05$). On the other hand, extracts did not contribute to sunscreens photodegradation when exposed to UV radiation.

The photodegradation of sunscreens generates ROS that can damage skin structures (Butt and Christensen, 2000) and an antioxidant plant extract added to the formulation could act by capturing these reactive species and increasing sunscreens photostability (Jarzycka et al., 2013; Cerqueira-Coutinho et al., 2015). Cerqueira-Coutinho and co-workers (2015) found out that the addition of pomegranate extract to a photoprotective formulation increased sunscreens photostability, since the plant extract presented high antioxidant activity according to DPPH• (2,2-diphenyl-1-picrylhydrazyl) assay (described as EC50 – half maximal effective concentration).

In our previous study (Mansur et al., 2012), DPPH• free radical assay was performed to evaluate the antioxidant potential of the isolated compounds. DPPH• free radical assay does not take into account the formulation where the compound was vehiculated. This as a limitation of the DPPH• assay since the active substance must be solubilized in a solvent, commonly an organic one. The measurements are performed in a spectrophotometer and for this reason the sample must be completely solubilized. The other components present in the formulation such as waxes and other oily materials that constitute the emulsion where the extracts were vehiculated, could interfere in the measurements and for this reason for this test, the active is evaluated alone (Blois, 2002; Boonne and Yotsawimonwat, 2011; Faudale et al., 2008; Oktay et al., 2003; Piccaglia and Marotti, 2001; Ruberto et al., 2000; Salama et al., 2013). The ability to scavenge DPPH• radical was measured by the discoloration of the solutions prepared in this experiment, using spectrophotometry. The greater the antioxidant activity the higher the intensity of solution discoloration (Mensor et al., 2008).

In vivo *SPF assessment*

Formulation A, without plant extract presented the lowest SPF, 13.48 ± 1.99 while formulations B and C, both containing plant extract, presented SPF of 18.98 ± 3.30 and 17.90 ± 3.35, respectively (Fig. 1). No statistical differences were obtained for comparisons between the SPF of formulations B and C ($p > 0.05$). On the other hand, the SPF of formulation A was statically lower than the SPF of formulations B and C ($p < 0.05$).

All formulations presented a suitable SPF but the extracts contributed to the enhancement of *in vitro* SPF. The higher *in vivo* SPF of formulations B and C (with antioxidant extracts) when compared to formulation A (without extract) could be attributed to the scavenging activity of the antioxidant molecules present in the plant extracts that act by capturing ROS.

All formulations presented a suitable SPF but the extracts contributed to the enhancement of *in vivo* SPF. The higher *in vivo* SPF of

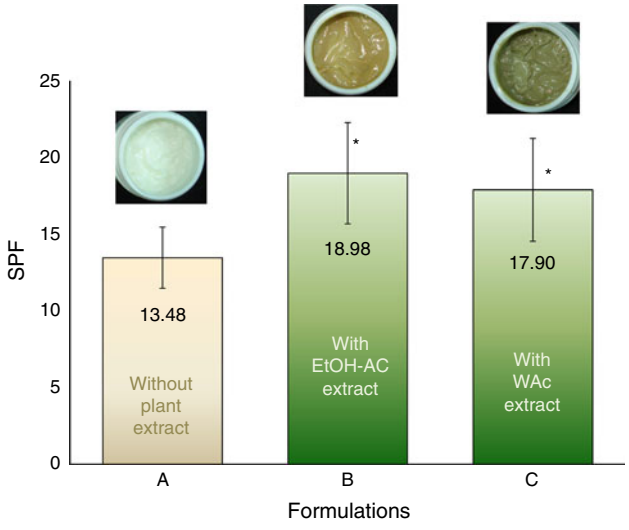

Fig. 1. Comparison among SPF of the formulations containing plant extract and the formulation containing sunscreens only. * Significantly different from formulation A ($p < 0.05$).

Table 4
HET-CAM, CAM-TBS and RBC results for formulations and pure extracts (1 wt% in PB pH 7.4) with their classification based on the irritant effects and denaturation index.

Formulations	HET-CAM	CAM-TBS	RBC	Classification
	Score[a]	Score[a]	Lisys/denaturation index	
A	1.92 ± 0.38	4.59 ± 0.24	>100	NOI/SLI
B	2.13 ± 0.88	4.53 ± 0.42	>100	NOI/SLI
EtOH-AC 1 wt% in PB	2.25 ± 1.06	3.09 ± 0.38	>100	NOI/SLI
C	2.0 ± 0.71	4.48 ± 0.31	>100	NOI/SLI
WAc 1 wt% in PB	0	1.07 ± 0.64	>100	NOI/SLI

[a] Mean ± SD.

formulations B and C (with antioxidant extracts) when compared to formulation A (without extract) could be attributed to the scavenging activity of the antioxidant molecules present in the plant extracts that act by capturing ROS. It is suggested that the solvent used in the extraction (WAc and EtOH-AC) did not influence on the antioxidant activity.

The plant extracts evaluated in this work did not absorb in the UV part of the spectrum. However, the role of the plant extract in the formulation is to indirectly enhance *in vivo* SPF. ROS are produced by UV radiation and are responsible to the skin erythema appearance, consequently when ROS are inactivated by antioxidant molecules, *in vivo* SPF tends to rise as it is assessed based on the appearance of skin erythema (Gilaberte and González, 2010). For this reason it is suggested that the higher photoprotective capacity of the formulations containing the extracts revealed the antioxidant potential of the extracts, already confirmed in our previous work (Mansur et al., 2012).

In addition, one of the goals of the photoprotection research is to decrease the concentration of synthetic sunscreens added in photoprotective formulations since these organic molecules in high concentrations could promote skin irritation (Morabito et al., 2011; Sambandan and Ratner, 2011; Chen et al., 2012; Jansen et al., 2013). For this reason, plant extracts could be added to photoprotective formulations enabling a reduction in sunscreens' concentration but keeping the same *in vivo* SPF.

Studies related to oral uptake or topical application of plant derivatives having antioxidant components showed that these substances could protect the skin against ROS, avoiding premature skin aging and free radical-related diseases. For example, studies have demonstrated that regular application of an emulsion containing hyperforin reduced free radical formation and stabilized the lipids that constitute the skin barrier (Meinke et al., 2012, 2013; Haag et al., 2014). In the *in vitro* experiment, SPF results for formulations A, B and C were not statically different; all formulations presented a SPF around 17. However, when compared to the *in vitro* experiment, the *in vivo* SPF for formulation A was the lowest one (13.48 ± 1.99). It could occur since *in vivo* testing often presents biological factors that have to be taken into consideration. Even presenting a good *in vitro* correlation, only the *in vivo* test is accepted to register a photoprotective formulation in a health care regulatory agency for commercial purposes. *In vitro* SPF tests are usually performed as a screening to select the best formulations and then the next step is *in vivo* testing (Vergnanini et al., 1999).

Hen's Egg Chorioallantoic Membrane (HET-CAM) test and Chorioallantoic Membrane-Trypan Blue Staining (CAM-TBS) test

The results of HET-CAM and CAM-TBS tests are showed in Table 4. According to the classification by scores, for both tests the formulations were classified as non-irritant/slightly irritant (NOI/SLI), suggesting that they are safe to be applied on the skin.

WAc extract vehiculated at 1 wt% in phosphate buffer (PB) pH 7.4 showed the lowest score in HET-CAM and CAM-TBS tests, indicating that the extract alone (without being vehiculated in a cosmetic formulation) is not a potential irritant.

Both tests are related to ocular irritation, which can be associated to cosmetic application on the face, near to the ocular mucous membrane.

HET-CAM and CAM-TBS classification requires a comparison with RBC assay, to confirm that the formulation is not toxic since the aim is to obtain data related to vascularization (HET-CAM) and cytotoxicity (RBC) to evaluate ocular irritation potential. Since skin irritation starts with vascular alterations (Hyperemia) and HET-CAM alterations is based upon vascular effects on the CAM, the non observation of these effects may suggest that formulation possess also a lower skin irritation potential.

Nascimento and co-workers (2012) also found that sunscreens encapsulated in polymeric nanocapsules did not present irritant potential, which corroborate our results.

Red blood cell (RBC) test

The results to the RBC test are showed in Table 4. All formulations were classified as non-irritant/slightly irritant (NOI/SLI), which confirm the previous *in vitro* safety tests (HET-CAM and CAM-TBS).

Conclusions

Bauhinia microstachya var. *massambabensis* leaf extracts (EtOH-AC and WAc) were successfully incorporated into O/W emulsions containing sunscreens. The photoprotective formulations containing extracts were photostable after irradiation in the solar simulator, thus the extracts did not contribute to sunscreen's photodegradation when exposed to UV radiation. Concerning the efficacy tests, all formulations presented a suitable SPF and both plant extracts contributed to a higher photoprotective effect, as an enhancement in the *in vivo* SPF, when the extracts were added to the formulations. It is suggested that plant extracts acted by capturing ROS, thus minimizing erythema and collaborating indirectly to *in vivo* SPF enhancement. Both formulations could be considered safe for cosmetic use since they passed the toxicity tests, demonstrating that they are not irritant to the eyes and skin.

Authors' contributions

MCPPRM (MSc student), contributed to the development of the formulations, efficacy and safety testing; CCC and ABV, efficacy testing; RSS, and OAFP, safety testing; AACL. Photostability studies, SGL and GGL, plant collection, drying and preparation of plant extracts; ERJ and EPS, development of the formulations.

Conflicts of interest

The authors declare no conflicts of interest.

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