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Article

Development and characterization of photoprotective nanoemulsions containing Babassu (*Orbignya phalerata* Mart.) lipophilic extract

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Oil-in-water photoprotective nanoemulsions (NEs) were developed using Babassu (BBS) lipophilic extract, nonionic surfactants, and low concentrations of organic sunscreens by ultrasonic processing. BBS extract was chosen due to its suitable physicochemical properties (acidity index, peroxide index, refraction index, and relative density) and predominance of saturated fatty acids, identified by gas chromatography-mass spectrometry (GC-MS), which promote biological activities and high oxidative stability. NEs were characterized by mean droplet size, morphology, polydispersity index (PdI), pH, and organoleptic properties, and the physical stability of the NEs was evaluated for 120 days at room temperature. The sun protection factor (SPF) was determined, and the photostability and *in vitro* cytotoxicity assays were performed for NEs. All NEs remained stable for 120 days, with a droplet size <150 nm and a monomodal distribution profile. The pH values were compatible with the skin's pH. NE3 showed a spherical morphology, with a mean droplet size of 125.15 ± 0.16 nm and PdI of 0.145 ± 0.032. NE3 containing BBS extract and sunscreens presented an SPF of 35.5 ± 3.0, was photostable after 6 h of radiation and was non-cytotoxic to fibroblast cells. Thus, NE3 could be considered a promising formulation for developing synergic plant-extract sunscreen photoprotective products for the market.

Keywords: *Orbignya phalerata*. Babassu extract. Sunscreens. Nanoemulsions. *In vitro* SPF.

INTRODUCTION

Babassu (BBS) is the name given to oilseed palm trees belonging to the *Palmae* family and members of the *Orbignya* and *Attalea* genera (de Morais *et al*., 2021; Silva *et al*., 2020). BBS is one of Brazil's most valuable national species, found mainly in the north and northeast regions of the country (Bauer *et al*., 2019; Campos *et al*., 2017). The oil extracted from the fruit of BBS is considered the main extractive product, widely applied in the food, biofuels, and cosmetics industries (Bauer *et al.,* 2019; Ferreira, Faza, Le Hyaric, 2012; Vieira *et al*., 2017).

In recent years, BBS lipophilic extract (*Orbignya phalerata* Martius) has been pointed out by many researchers as an important emollient active in cosmetic formulations, mainly for topical use to treat skin diseases (Rosário *et al*., 2021; Silva *et al*., 2020; Vieira *et al*., 2017). BBS oil has important bioactive compounds based on saturated fatty acids, mainly lauric (44.0-46.0%), myristic

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(15.0-20.0%) and oleic (12.0-18.0%) acids (Bauer *et al.*, 2019; de Oliveira *et al.*, 2019; Santos *et al.*, 2021), in addition to containing small amounts of phospholipids and tocopherols (Vieira *et al.*, 2017). These components are responsible for biological properties including anti-inflammation (Reis *et al.*, 2017; Santos *et al.*, 2020) and healing (Fernandes *et al.*, 2021), as well as antibacterial (Barroqueiro *et al.*, 2016; Nobre *et al.*, 2018), and antioxidant activities (Bauer *et al.*, 2019; Santos *et al.*, 2021). Thus, the BBS extract can be an important raw material for developing ecological cosmetic products.

In the last decades, nanotechnology has provided great advances, especially in the pharmaceutical and cosmetics industries, allowing the encapsulation of actives to solve problems associated with solubility and stability (Mansur *et al.*, 2020; Santos *et al.*, 2021; Schuenck-Rodrigues *et al.*, 2020). In this context, oil-in-water nanoemulsions (NEs) are considered important carrier systems for lipophilic substances, including drugs, plant extracts and sunscreens (Bajerski *et al.*, 2016; Cerqueira-Coutinho *et al.*, 2015). NEs are colloidal systems generally of translucent aspect, with droplet sizes varying from 10-200 nm, mainly prepared by high-energy methods (Dammak *et al.*, 2020; Mansur *et al.*, 2020). Lately, NEs based on BBS extract have been produced for food and some cosmetic formulations (Rocha-Filho *et al.*, 2017; Santos *et al.*, 2021).

Commercial sunscreens are widely used to prevent the harmful effects of ultraviolet (UV) radiation (Batista *et al.*, 2022). Exposure to UV radiation generates reactive oxygen species (ROS) on the skin surface that could lead to premature skin aging, burns, and ultimately, skin cancer (Mansur *et al.*, 2020). Some plant extracts, such as BBS lipophilic extract, could act on capturing ROS, thus reducing skin erythema and then indirectly improving *in vivo* SPF (Cerqueira-Coutinho *et al.*, 2015). Using BBS extract in the formulations could be a useful strategy for reducing the content of synthetic sunscreens since these organic molecules in high concentrations can promote some skin irritation (Cerqueira-Coutinho *et al.*, 2015; Mansur *et al.*, 2016). The synergy between commercial organic sunscreens and plant extracts has shown satisfactory results in preventing photocarcinogenesis and skin photoaging (Mansur *et al.*, 2016). Therefore, the development of NEs containing an emollient ingredient such as BBS extract and low concentrations of organic sunscreens would be interesting for producing a novel photoprotective product with moisturizing potential.

Thus, this work aimed to develop and characterize NEs containing BBS lipophilic extract and organic sunscreens for broad-spectrum protection and to evaluate the physical stability, safety, and efficacy of the NEs for application in photoprotective products.

MATERIAL AND METHODS

Material

Babassu (BBS) lipophilic extract was obtained directly from rural producers in the State of Maranhão, Brazil (2°31'48.0" S and 44°18'10.0" W). BBS extract samples were obtained by crushing selected almonds and cooking the mass to obtain the oil, which was filtered and stored (Vieira *et al.*, 2017). The other ingredients were the nonionic surfactants polyoxyethylene sorbitan monooleate (purchased from Mapric, Brazil) which presents 20 moles of ethylene oxide, and sorbitan monostearate (purchased from Mapric, Brazil); the organic sunscreens for broad-spectrum protection, benzophenone-3 (UVA/UVB sunscreen purchased from Fagron, Brazil), diethylamino hydroxybenzoyl hexyl benzoate (UVA sunscreen purchased from Basf, Brazil), octocrylene, and octyl methoxycinnamate (both UVB sunscreen purchased from Fagron, Brazil); and a blend of phenoxyethanol and methylisothiazolinone (purchased from PharmaSpecial, Brazil) used as a preservative.

Physicochemical characterization and fatty acid composition

BBS samples were evaluated according to the physicochemical parameters described by the analytical protocol of Instituto Adolfo Lutz (2008). Briefly, the acidity index was determined from the titration of the sample with a standard alkali solution and is expressed in milligrams of KOH needed to neutralize the free acids of 1.0 g of oil; the peroxides index was calculated from the release of iodine from potassium iodide and expressed in milliequivalents of active oxygen/kg of oil; the refractive

index at 40°C was obtained using an Abbé refractometer; and the relative density was determined in a 10 mL glass pycnometer at 25°C. All experiments were performed in triplicate (n=3), and values were expressed as mean ± SD (standard deviation).

The fatty acid composition of the BBS samples was determined by gas chromatography-mass spectrometry (GC-MS) by analysis of fatty acid methyl esters (FAME), according to the method described by Silva *et al.* (2020). To prepare FAME, 50.0 g of BBS samples previously dried in an oven at 110°C for 4 h were added in methanolic solution to 1.3 mol L^{-1} KOH, and the mixture was kept under magnetic stirring for 1 h. Then, the ester-glycerin mix was transferred to a separatory funnel and rested for 24 h.

FAME was analyzed using a Shimadzu GC-17A gas chromatograph coupled to a Shimadzu QP5050A mass spectrometer equipped with a DB17HT capillary column (30 m length, 0.25 mm i.d., 0.15 µm film thickness). The injection volume was 1 µL. The temperature program was set up from 50°C to 250°C with 4°C/min; both the injector and detector temperatures were 300°C, and helium (He) was used as carrier gas. The mass spectra were compared with the Wiley 229 library (Santos *et al.*, 2013).

Preparation of the NEs

NEs were prepared by ultrasound (US) processor UP100H (Hielscher, Germany) with 100 watts and 30 kHz, with a 50% amplitude and constant ultrasound homogenization (Cycle 1). The aqueous phase was prepared by mixing polyoxyethylene sorbitan monooleate, sorbitan monostearate, preservative, and distilled water. The oil phase was prepared by mixing the organic sunscreens with BBS extract. Table I shows the ingredients used to prepare NEs and their respective concentrations, and Figure 1 shows the production of NE3.

Three NEs were prepared: NE1, containing BBS extract without sunscreens; NE2 containing only sunscreens; and NE3 containing BBS extract and sunscreens. The NEs were stored in the dark at 25°C.

TABLE I - Composition of the NEs

Ingredients	Function	Formulation		
		NE1	NE2	NE3
Oil phase (wt%)				
OMC	UVB sunscreen	-	8.0	8.0
OCT	UVB sunscreen	-	6.0	6.0
DHHB	UVA sunscreen	-	3.0	3.0
BZF-3	UVA and UVB sunscreen	-	3.0	3.0
BBS lipophilic extract	Emollient/moisturizer	5.0	-	5.0
Aqueous phase (wt%)				
Sorbitan monostearate	Surfactant	15.0	15.0	15.0
Polyoxyethylene sorbitan monooleate	Surfactant	3.0	3.0	3.0
Phenoxyethanol and methylisothiazolinone	Preservative	0.3	0.3	0.3
Distilled water	Vehicle	Qs 100 wt%	Qs 100 wt%	Qs 100 wt%

NEs = nanoemulsions (NE1 = containing BBS extract without sunscreens; NE2 = containing only sunscreens; NE3 = containing BBS extract and sunscreens); BZF-3 = Benzofenone-3; DHHB = Diethylamino hydroxybenzoyl hexyl benzoate; OCT = Octocrylene; OMC = Octyl methoxycinnamate; Qs = quantity sufficient.

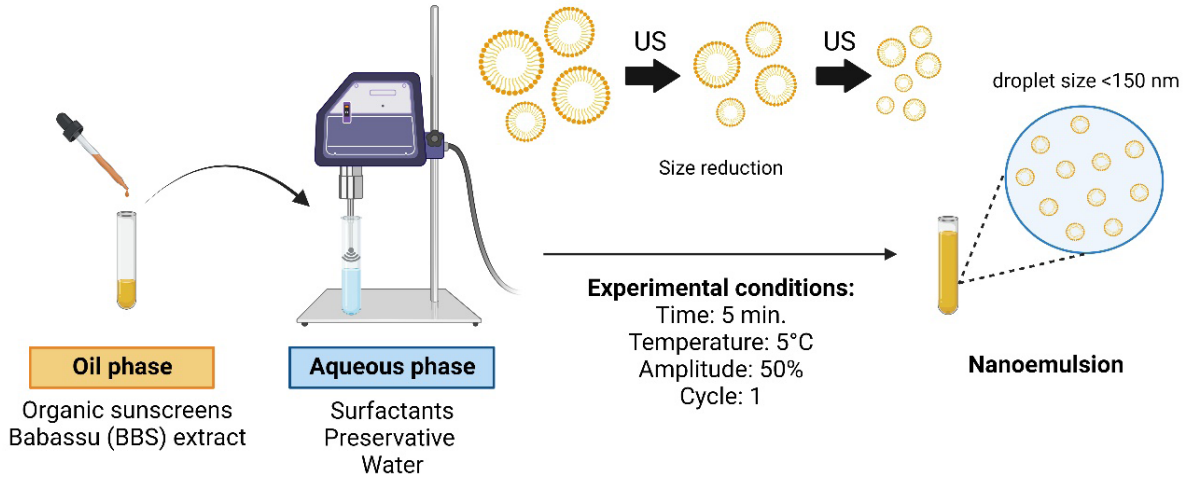

FIGURE 1 – Production of NE3.

Characterization of the NEs

pH measurements and organoleptic properties

The pH was measured using a pH electrode (Model 922, Bante Instruments, USA) in the NEs formulations. The pH meter was previously calibrated with standard solutions of pHs 4.0 and 10.0 (Hanna Instruments, USA). Measurements were performed in triplicate at 25°C. The organoleptic properties, such as aspect, color, homogeneity, and phase separation were also evaluated.

Determination of mean droplet size and polydispersity index (PdI)

The mean droplet size of the NEs and the polydispersity index (PdI) were determined by dynamic light scattering (DLS) using a Zetasizer Nano® Model S90 (Malvern Instruments, UK). Mean size and PdI measurements were performed by diluting 20 µL of the samples in 1 mL of distilled water. Measurements were performed in triplicate at 25°C.

Zeta potential (ZP) assessment

The zeta potential (ZP) of the NEs was measured by electrophoretic light scattering (ELS) using a NanoBrook® ZetaPALS analyzer (Brookhaven Instruments, UK). The samples were diluted in double-distilled water (ratio 1: 100), and the ZP measurements were performed at 25 °C, with a total of 10 runs.

Morphology evaluation by transmission electron microscopy (TEM)

The droplet morphology of NE3 (containing BBS extract and sunscreens) was evaluated using a Jeol 1200 EX transmission electron microscope (TEM), with a voltage of 80 kV and a capture system equipped with a digital camera (Megaview III). The samples were diluted in distilled water (1:10 v/v), and 10 µL of the solution was deposited on a copper grid (200 mesh) with Formvar® coating. For negative staining, 10 µL of 5% uranyl acetate solution was added, and the excess was removed with filter paper for further observation under the microscope after 24 h. The average particle size was estimated using ImageJ software.

Stability assays

In the stability study the droplet size distribution, mean droplet size, PdI, and pH of the NEs (NE2 and NE3) were analyzed. The measurements were done in triplicate at 25°C immediately after processing at the initial time (T0) and after 7 (T7), 14 (T14), 21 (T21), 30 (T30), 60 (T60), 90 (T90), and 120 (T120) days. The organoleptic properties were also analyzed. Measurements were performed in triplicate.

Efficacy assays: Sun protection factor (SPF) assessment

In vitro SPF measurements were performed using a UV transmittance analyzer (Labsphere® UV-2000 S) and quartz plates with an area of 25 cm² covered by Transpore™ tape on one surface to simulate the skin surface. Approximately 50 mg (2.0 mg/cm²) of each formulation was applied to the plate support and spread manually in sequential movements to obtain a uniform film. Glycerin was spread on the support as a reference for 100% transmission (Castro *et al*., 2021; Cerqueira-Coutinho *et al*., 2015). The UV-2000S measured nine points on the plate, varying the wavelength from 250 to 450 nm. The readings were performed in triplicate at times T0 and T30. SPF, UVA/UVB ratio, and critical wavelength (λ_c) for each formulation were determined.

Photostability assay

NE2 and NE3 formulations were evaluated for photostability using a solar light simulator (SSL) (Oriel 91192-1000, Newport Corp., USA) and a UV transmittance analyzer (Labsphere® UV-2000 S). The samples (500 mg) were applied on quartz plates covered by Transpore™ tape and irradiated at the initial time (T0) and after 2 h (T2), 4 h (T4) and 6 h (T6) in SSL, with a radiation intensity of 104.0 J/s.m² (UVA) and 8.1 J/s.m² (UVB). Three plates were stored without light and without being irradiated for negative control. After irradiation, samples were analyzed in the UV transmittance analyzer. The protocol used in this study mimics the characteristics of a sunny summer day in Rio de Janeiro (Brazil) at 12:00 h (22°54'23" S and 43°10'21" W) (Hossy *et al*., 2013). The tests were performed in triplicate, and the mean ± SD was assessed.

In vitro cytotoxicity assays with MTT

In vitro cytotoxicity of the formulations was evaluated by the cell viability assay based on the reduction of the salt 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) in human fibroblast (HFB) cells. This assay is commonly used to assess the safety of pharmaceutical formulations since the cell types investigated are quite sensitive to the toxic effects of topical formulations. Cells were grown in 96-well plates with Dulbecco's modified eagle medium (DMEM), containing 4% v/v l-glutamine, 4% v/v penicillin-streptomycin, 4% v/v sodium pyruvate, and 10 % v/v bovine fetal serum (BFS), and kept in an oven with a 5% CO_2 atmosphere at 37°C. After reaching cell confluence, the medium was removed, and the formulations NE1, NE2 and NE3 were added at concentrations of 10.0, 5.0, 2.5, 1.25, 0.62, 0.31 and 0.16 mg/mL in the novel culture medium. Furthermore, the cytotoxic potential of BBS extract and sunscreens was also evaluated.

After 24 h, the 5 mg/mL MTT solution was added to each well, and the plates were incubated for 4 h. After MTT reduction, the formed formazan crystals were dissolved in 200 µL of dimethyl sulfoxide (DMSO), and the absorbance in each well was determined in a microplate spectrophotometer (Multiskan™ FC, Thermo Fisher Scientific) at a wavelength of 570 nm. Then, the CC_{50} (50% cytotoxic concentration) and LC_{50} (50% lethal concentration) of fibroblast cells for each formulation (NE1, NE2 and NE3) were calculated by linear regression analysis. The absorbance values were obtained in triplicate, and the means and standard deviations were assessed.

Statistical analysis

Statistical analysis was assessed by one-way analysis of variance (ANOVA) followed by Tukey's test using Origin® Pro 9.0 software (OriginLab, USA). All experimental data were presented as mean ± SD (standard error), and $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Characterization of BBS lipophilic extract

Physicochemical parameters

The physicochemical properties of BBS lipophilic extract were investigated to assess oil quality after

extraction and storage. Table II shows the main physicochemical parameters evaluated in comparison with the Codex Alimentarius (2019), an international standard with recommendations relating to food and drugs.

TABLE II - Physicochemical parameters of Babassu (BBS) lipophilic extract

Physicochemical parameters	BBS (this work)	Codex Alimentarius (2019)
Acidity index (mg KOH/g)	0.624 ± 0.002	Up to 4.0
Peroxides index (meq O_2/kg)	2.025 ± 0.016	Up to 15.0
Refractive index (nD^{40}) at 40ºC	1.449 ± 0.001	1.448–1.451
Relative density (g mL^{-1}) at 25 °C	0.918 ± 0.002	0.914–0.917

Mean ± SD (n=3)

According to Table II, the acidity value of the BBS extract was 0.624 ± 0.002 mg KOH/g, which is lower than the limit (up to 4.0 mg KOH/g) established by the Codex Alimentarius (2019). The acid index is a quality parameter of vegetable oils and can indicate deterioration due to free fatty acids resulting from the hydrolysis of triacylglycerols (Silva *et al.*, 2020). These reactions can be accelerated by heat or exposure to light, with hydrolytic rancidity being the leading indicator of oil degradation (Bauer *et al.*, 2020). Thus, to maintain the desirable organoleptic characteristics, such as taste, color, and odor, the oil must have a low acidity content to guarantee quality.

Another indication of the quality of oils and fats is the determination of the peroxides index (Bauer *et al.*, 2020). Peroxides analysis evaluates the process of oxidative rancidity by determining peroxides and hydroperoxides as primary auto-oxidation products, indicating oil deterioration (Reis *et al.*, 2017). The result obtained for the peroxides index suggests that the BBS extract was in perfect condition, not having developed a rancidity process. In the absence of specific legislation for determining the quality of vegetable oils for application in cosmetic products, the comparison made with the Codex Alimentarius (2019) shows that the peroxides index value found, 2.025 ± 0.016 meq O_2/kg for the BBS extract, is within the limit of 15.0 meq O_2/kg.

Regarding the refractive index analysis, the value found of 1.449 ± 0.002 nD^{40} was within the interval 1.448–1.451 nD^{40} of the Codex Alimentarius (2019). The refractive index varies for each type of oil and is mainly related to the composition of the fatty acids that make up the triglycerides, such as the chain size, molecular mass, and degree of unsaturation of the bonds (Bauer *et al.*, 2020). As BBS oil has a high content of saturated fatty acids, a refractive index value within the established standard was expected. As well as the refractive index, density analysis is an identity parameter of vegetable oils and can serve as a quality control (Silva *et al.*, 2020). For the BBS extract, the relative density value of 0.918 ± 0.002 g mL^{-1} was slightly above the Codex Alimentarius (2019) (0.914–0.917 g mL^{-1}), suggesting that the residues from the extraction may have influenced the increase in density in samples of unrefined BBS.

Fatty acid composition

The fatty acid composition of BBS lipophilic extract was determined by GC-MS from the analysis of FAME obtained from saponification and esterification of BBS samples. Table III shows the percentages of the primary fatty acids identified in BBS samples.

TABLE III - Fatty acid composition of Babassu (BBS) lipophilic extract

Fatty acids	Retention time (min)	Fatty acids (%)	Codex Alimentarius (2019)
Caprylic (C8:0)	11.48 ± 0.01	0.64	2.6-7.3
Capric (C10:0)	9.15 ± 0.05	2.42	1.2-7.6
Lauric (C12:0)	23.82 ± 0.08	52.64	40.0-55.0
Myristic (C14:0)	31.24 ± 0.02	15.42	11.0-27.0
Palmitic (C16:0)	35.68 ± 0.14	8.68	5.2-11.0
Stearic (C18:0)	39.23 ± 0.02	3.02	1.8-7.4
Oleic (C18:1)	38.25 ± 0.03	15.76	9.0-20.0
Linoleic (C18:2)	37.52 ± 0.04	1.42	1.4-6.6
∑Saturated	-	82.82	61.90-115.4
∑Unsaturated	-	17.18	1.4-20.0
Total	-	100.00	-

BBS lipophilic extract showed higher concentrations of saturated fatty acids (82.92%), including lauric (52.64%) and myristic (15.42%) acids, identified as medium-chain (C12:0) and long-chain (C14:0) fatty acids, respectively (Ferreira, Faza, Le Hyaric, 2012; Santos *et al*., 2013). Saturated fatty acids provide high oxidative stability and long shelf life for the oil (de Araujo Silva *et al*., 2023). On the other hand, significant amounts of oleic acid (15.76%) were identified among the unsaturated fatty acids. Except for caprylic acid (C8:0), all percentages of fatty acids were within the range of values established by the Codex Alimentarius (2019) for the quality standard of BBS oil from *Orbignya* sp. Furthermore, the fatty acid composition of BBS oil may vary according to its geographic region and seasonal factors, such as the time of year when the almonds are collected (greater or lesser incidence of rainfall, for example). Using multivariate analysis, Santos *et al.* (2013) demonstrated that the fatty acid composition of BBS oil varied according to its geographic location within the State of Maranhão (Brazil).

The predominance of lauric acid in oleaginous species of the genera *Orbignya* and *Attalea* may explain their performance in cosmetic formulations, mainly due to their emollient, emulsifying, and stabilizing properties (Silva *et al*., 2020; Vieira *et al*., 2017). Studies show that high concentrations of lauric fat obtained from coconut can increase skin hydration due to its emollient and humectant power, which also improves the spreadability of the formulation on the skin (Paramita *et al*., 2022; Vieira *et al*., 2017). Moreover, recent research has shown that lauric acid also confers biological properties: anti-inflammatory, antiviral, and promoting healing activity (Fernandes *et al*., 2021; Santos *et al*., 2020; Silva *et al*., 2020). Thus, BBS lipophilic extract consisting predominantly of lauric acid proved suitable for this study's photoprotective formulations.

Characterization of the NEs

Table IV shows the results of the physicochemical characterization of the NEs. All NEs had small mean droplet sizes (<150 nm) and low polydispersity (PdI), which confirms the stability of the NEs. NE1 had the smallest mean drop size (58.23 ± 1.18 nm) compared to the NE2 (112.24 ± 1.08 nm) and NE3 (125.15 ± 0.16 nm) formulations. This effect is mainly due to the increase in the oil phase in the NE2 and NE3 systems from the addition of BBS extract and sunscreens, thus increasing

the mean droplet size in the photoprotective NEs. All formulations showed significant differences in the mean droplet size ($p < 0.05$). Furthermore, the low PdI value of NEs may indicate a greater uniformity of droplet size distribution, suggesting suitable droplet homogeneity (Mansur *et al.*, 2020).

TABLE IV - Physicochemical characterization of NEs: mean size, PdI, ZP, pH, and aspect

Formulation	Mean size (nm) ± SD	PdI ± SD	ZP (mV) ± SD	pH ± SD	Aspect
NE1	58.23 ± 1.18	0.095 ± 0.021	-28.7 ± 0.8	5.51 ± 0.06	Translucent, homogenous, no phase separation
NE2	112.24 ± 1.08	0.185 ± 0.084	-25.5 ± 1.2	5.52 ± 0.12	Yellow, homogenous, no phase separation
NE3	125.15 ± 0.16	0.145 ± 0.032	-26.4 ± 1.4	6.12 ± 0.03	Yellow, homogenous, no phase separation

NEs = nanoemulsions (NE1 = containing BBS extract without sunscreens; NE2 = containing only sunscreens; NE3 = containing BBS extract and sunscreens); PdI = polydispersity index; ZP = zeta potential. Mean values of three determinations ± standard deviation (SD).

The ZP values of the NE1, NE2 and NE3 formulations were -28.7 ± 0.8 mV, -25.5 ± 1.2 mV and -26.4 ± 1.4 mV, respectively. In general, nanoemulsified systems have small negatively charged droplets, which can contribute to the stability of the dispersion. Previous studies show that NEs with ZP values around -30 mV can generate greater repulsion between the droplets, thus avoiding coalescence and dispersion breakage (Marques *et al.*, 2018; Schuenck-Rodrigues *et al.*, 2020). The statistical analysis did not indicate a significant difference between the NEs ($p > 0.05$).

The pH values obtained for the NEs varied between 5.5–6.1, which is considered compatible with the human skin pH of approximately 5.0–6.0 (Marques *et al.*, 2018). Regarding the organoleptic properties, NE1 was translucent, bright, and homogeneous, and there was no phase separation. In comparison, NE2 and NE3 showed yellow color (due to the addition of sunscreens), were homogeneous, and did not present phase separation.

TEM analysis was performed to confirm the size and droplet morphology of NEs. The morphology and droplet size dispersion of NE3 are shown in Figure 2. As shown in Figure 2, the droplets exhibited a spherical morphology with a narrow size dispersion. The histogram showed a mean droplet size of 145.8 ± 16.5 nm, which correlated to the DLS results. Although the droplet sizes analyzed by TEM and DLS are correlated, the techniques are complementary; thus, size dispersion, size distribution, and mean droplet size could be evaluated by two different methods.

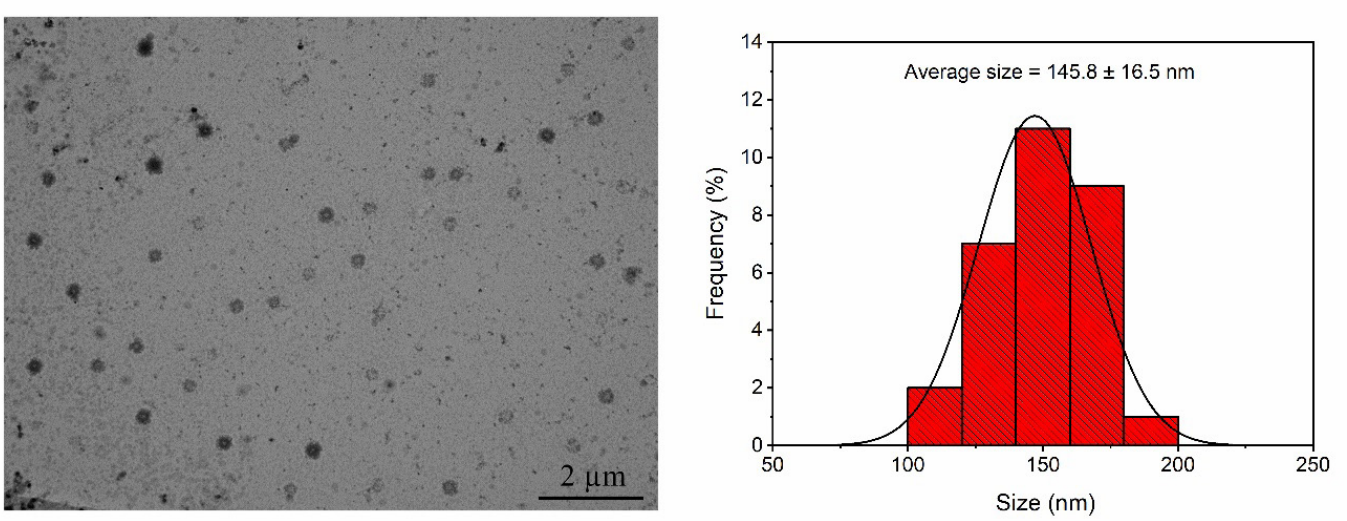

FIGURE 2 – TEM image and histogram of particle size distribution for NE3.

It is important to point out that a variation in mean particle size comparing DLS and TEM is often common due to the principle associated with each technique. While in DLS, a liquid sample is analyzed under the incidence of light; in TEM, the particle size is provided after applying an electron beam to the solid sample's surface (Klang *et al.*, 2012). Thus, the average particle size of colloidal systems, such as nanoemulsions, analyzed by TEM can be mainly influenced by the drying process of the sample, which can easily spread under the grid. In the case of nanoemulsions containing vegetable oils or sunscreens, a relationship between the negative contrast agent and the droplet surface can also influence its size and morphology (Salvia-Trujillo *et al.*, 2013). Additionally, the images obtained by TEM do not reflect the sample as a whole since a very restricted area of the investigated sample can remain intact and can be displayed. Furthermore, the images processed by the software do not represent all the samples, as only a small area of the grids was evaluated (Castro *et al.*, 2021).

Stability assays

NE2 and NE3 have been subjected to long-term stability studies. The study was carried out 24 hours after the processing of the NEs and after 7, 14, 21, 30, 60, 90 and 120 days at room temperature. The samples were evaluated in terms of mean droplet size and distribution, PdI, pH, and organoleptic properties.

In terms of organoleptic properties, the photoprotective NEs remained homogeneous, yellow colored, bright, and with a slight odor of the BBS extract. No formulation showed precipitate or visual breakdown of the emulsified structure.

Table V shows the results of mean droplet size, PdI, and pH evaluated during 120 days. The results showed small changes in the mean droplet size for NE2 and NE3; however, these values were not statistically significant ($p > 0.05$). Figure 3 shows the droplet size distribution profile for NE2 and NE3. Both formulations showed a monomodal size distribution, with only one peak. The addition of BBS extract may have contributed to the maintenance of the stability of NE2 and NE3 since the NE1 formulation had the smallest droplet size (Table IV). The PdI values also remained below 0.2, suggesting uniformity in droplet size (Mansur *et al.*, 2020). Furthermore, the pH values did not change, remaining around 5.5 and 6.1 for NE2 and NE3, respectively. The pH of the NEs was maintained within the cutaneous pH range (Marques *et al.*, 2018).

TABLE V - Mean size (nm), PdI, and pH of NEs observed along the stability period studied

Time (days)	NE2			NE3		
	Mean size ± SD	PdI ± SD	pH ± SD	Mean size ± SD	PdI ± SD	pH ± SD
0	112.24 ± 1.08	0.185 ± 0.084	5.52 ± 0.06	125.15 ± 0.16	0.145 ± 0.032	6.12 ± 0.08
7	114.46 ± 1.16	0.144 ± 0.043	5.53 ± 0.09	128.46 ± 1.34	0.164 ± 0.016	6.11 ± 0.04
14	118.35 ± 1.91	0.192 ± 0.042	5.52 ± 0.11	131.24 ± 1.94	0.192 ± 0.038	6.12 ± 0.11
21	122.18 ± 1.64	0.123 ± 0.035	5.51 ± 0.06	134.38 ± 2.35	0.213 ± 0.052	6.13 ± 0.17
30	125.12 ± 2.23	0.114 ± 0.014	5.54 ± 0.04	136.05 ± 2.64	0.216 ± 0.024	6.14 ± 0.14
60	128.64 ± 2.24	0.265 ± 0.068	5.53 ± 0.01	139.68 ± 3.38	0.246 ± 0.049	6.12 ± 0.11
90	134.53 ± 2.11	0.232 ± 0.024	5.55 ± 0.12	142.19 ± 3.63	0.132 ± 0.034	6.15 ± 0.12
120	136.13 ± 2.46	0.264 ± 0.038	5.56 ± 0.13	145.24 ± 2.52	0.152 ± 0.046	6.16 ± 0.16

NEs = nanoemulsions (NE2 = containing only sunscreens; NE3 = containing BBS extract and sunscreens); PdI = polydispersity index; ZP = zeta potential. Mean values of three determinations ± standard deviation (SD).

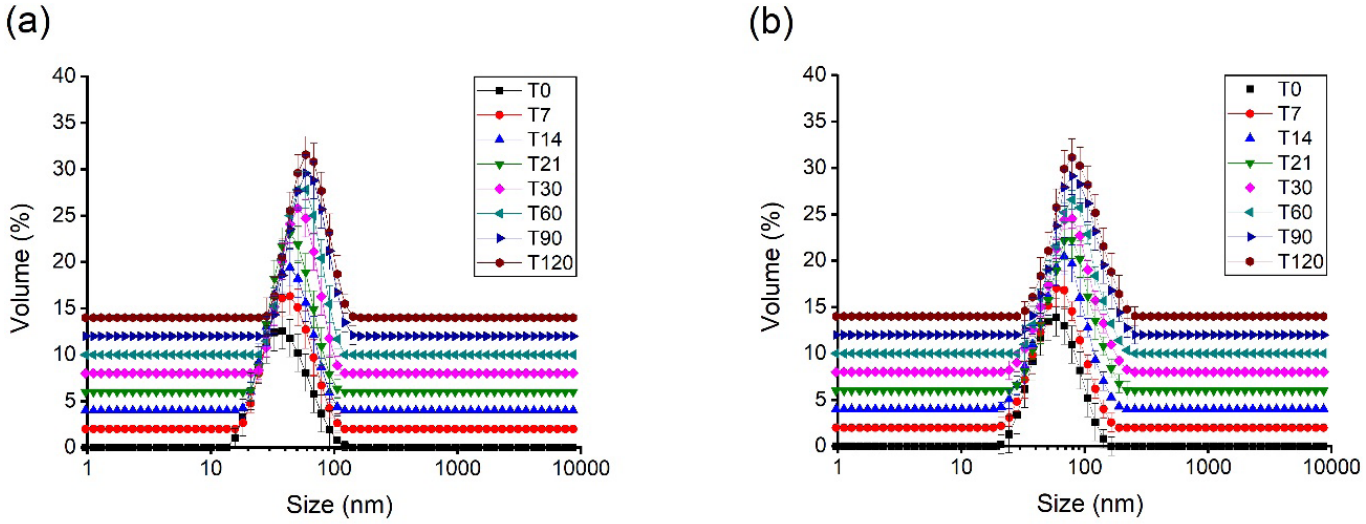

FIGURE 3 – Droplet size distribution of the NEs: (a) NE2 and (b) NE3. Error bars indicate the standard deviation (SD) for the triplicates.

As observed for mean droplet size, PdI, and pH measurements, no large variations were observed over time. These data are a suitable indicator of the physical stability of NEs and can be correlated with the structural stability previously presented (Santos *et al*., 2021).

Efficacy assay: Sun protection factor (SPF) assessment

The SPF measurements were performed after NE preparation (T0) and after 30 days (T30) to verify the stability of the sunscreens in the formulations. As there

was no significant variation in SPF values over time, the results shown in Figure 4 were related to time T30. Previous studies have shown that the storage time of photoprotective formulations based on plant extracts also did not interfere with *in vitro* SPF efficacy (Mansur *et al.*, 2020; Silva *et al.*, 2020).

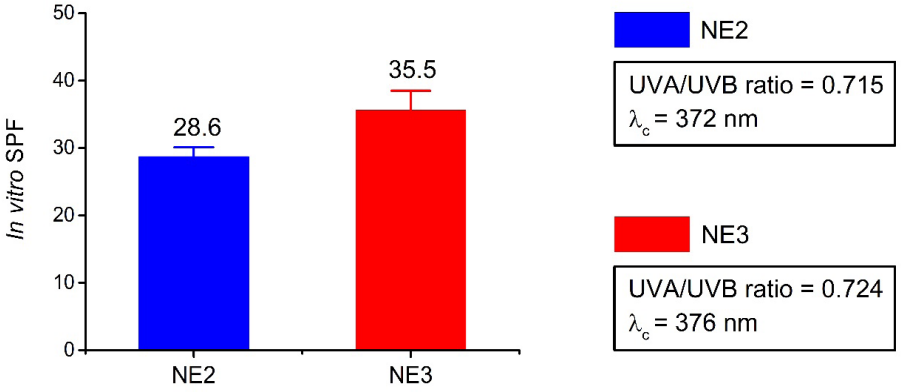

FIGURE 4 – *In vitro* sun protection factor (SPF), UVA/UVB ratio, and critical wavelength (λ_c) were measured for NEs after 30 days of storage at room temperature. Error bars indicate the standard deviation (SD) for the triplicates.

SPF values obtained for NE2 and NE3 were 28.6 ± 1.5 and 35.5 ± 3.0, respectively (Figure 4). These results were statistically different ($p<0.05$). NE1, containing only the BBS extract in the oil phase, showed an insignificant SPF value (<2.0). Although the BBS extract does not absorb UV radiation, its presence in the NE3 formulation seems to have acted synergistically with commercial sunscreens, increasing the SPF value. This effect was observed in previous studies, where adding plant extracts in formulations based on sunscreens enhanced photoprotective efficacy, mainly due to plant species containing antioxidant compounds that could act improving SPF (Cerqueira-Coutinho *et al.*, 2015; Mansur *et al.*, 2016). In the study by Mansur *et al.* (2016), the antioxidant potential of plant extracts was revealed to have a greater effect on the photoprotective potential of the formulations, since the plant species could act by capturing ROS produced by UV radiation that is responsible for skin erythema, thus increasing the SPF *in vivo*.

BBS may contain varying amounts of bioactive compounds based on saturated fatty acids, phenolic compounds, and tocopherols. These bioactive compounds are responsible for the anti-inflammatory, antiviral, and promotion of healing activity biological properties (Bauer *et al.*, 2019; Santos *et al.*, 2021; Vieira *et al.*, 2017). In the study by Bauer *et al.* (2019), although in low amounts, the presence of phenolic compounds (phenolic acids and flavonoids) and tocopherols (α, β, and γ-tocopherol) in BBS oil samples obtained by artisanal extraction were associated with their antioxidant activity. Furthermore, Santos *et al.* (2021) verified through the method of capture of the 2,2-diphenyl-1-picryl-hydrazyl (DPPH•) radical that BBS lipophilic extract in nanoemulsified form can exhibit greater antioxidant potential than its isolated lipophilic extract. The authors reported that the emulsification process could make BBS lipophilic extract more dispersible, increasing the oil droplets' surface areas, thus allowing the antioxidant components to be more available to come in contact with the free radical DPPH•. Thus, it can be suggested that the antioxidant components present in the BBS extract may act more effectively from the nanoemulsified system, thus influencing the value of *in vitro* SPF of the formulation.

The results of this study are also in agreement with the research of Silva *et al.* (2020) who evaluated the in vitro SPF of a cosmetic emulsion based on BBS oil and organic sunscreens and found that the formulation exhibited an SPF of around 40.0, which could be classified as a high SPF formulation. Concerning the results

obtained in this study, it showed that the application of NEs in photoprotection has several advantages, including an increase in SPF, due to a decrease in the contact surface area and retention of the formulation on the skin, as well as an increase in photostability and the protection spectrum of formulations containing sunscreens. Furthermore, NEs based on plant extracts containing low amounts of sunscreens have better sensory properties, which may be safer and more accepted by consumers (Cerqueira-Coutinho *et al.*, 2015; Mansur *et al.*, 2016).

According to COLIPA (2009) and FDA (2011), a photoprotective product with a λ_c of 370 nm or higher is considered a broad-spectrum product. NE2 and NE3 formulations showed λ_c greater than 370 nm **(**Figure 4**)**, thus are considered broad-spectrum products, providing high protection against UVA and UVB radiation (Mansur *et al.*, 2016). This property may be related to the synergistic interaction of different sunscreens and their respective UV wavelengths. In this study, the organic sunscreens used cover the primary ranges of wavelengths associated with UVB (290 to 320 nm) and UVA (320 to 400 nm) radiation, such as OMC (λ_{max} = 310 nm), OCT (λ_{max} = 302 nm), DHHB (λ_{max} = 354 nm), and BZF-3 (λ_{max1} = 210 nm and λ_{max2} = 257 nm) (Palm, O'Donoghue, 2007). Furthermore, according to the Boots star rating system (which classifies a product according to the UVA/UVB ratio) NE2 and NE3 had a UVA/UVB ratio greater than 0.7 (Figure 4), so they could be considered products with suitable UVA photoprotection, since the higher the ratio, the better protection against UVA (Wang, Stanfield, Osterwalder, 2008).

In addition to the photoprotective efficacy, BBS extract has a high potential for skin hydration, shown by studies of *in vivo* skin biometrics (Rocha-Filho *et al.*, 2017; Vieira *et al.*, 2017). The lipid constitution of fatty acids and the emollient properties of the BBS extract provide protection and hydration to the skin, which is very useful for treating skin diseases such as psoriasis and atopic dermatitis (Vieira *et al.*, 2017). Furthermore, it can be a suitable candidate for treating conditions that cause changes in the stratum corneum, such as cutaneous xerosis caused by leprosy (Rosário *et al.*, 2021; Silva *et al.*, 2020). Thus, developing NEs containing BBS extract and low concentrations of sunscreens could be an interesting strategy for the pharmaceutical industry, aiming to manufacture novel products with moisturizing and photoprotective properties. In addition to reducing the concentration of sunscreens in the formulation, adding BBS extract would be an alternative for treating diseases caused by the harmful effects of solar radiation, such as skin irritation, burns, and cancer.

Photostability assay

The photostability of NE2 and NE3 was evaluated after irradiation for 2 h, according to the FDA guideline for the reapplication of a photoprotective product. However, to evaluate the long-term exposure to solar radiation, the formulations were irradiated for 4 h and 6 h. Figure 5 shows the *in vitro* SPF results of the NE2 and NE3 formulations after 6 h of simulated solar irradiation.

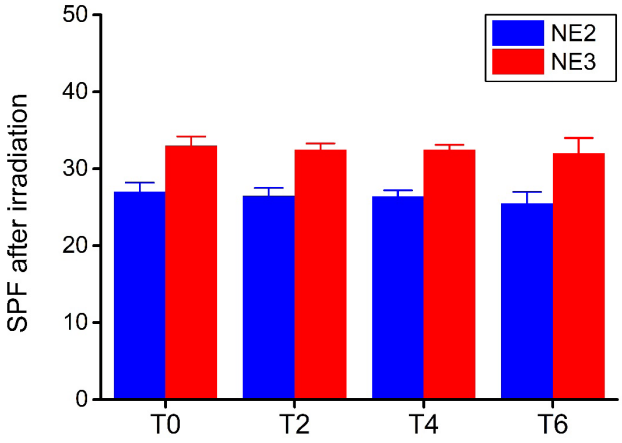

FIGURE 5 – *In vitro* sun protection factor (SPF) of NE2 and NE3 formulations after 6 h of solar irradiation. Error bars indicate the standard deviation (SD) for the triplicates.

According to photostability data, NE2 and NE3 formulations remained photostable during 6 h of irradiation and no statistical difference was obtained for T0, T2, T4, and T6 for each group ($p>0.05$). On the other hand, after 6 h of irradiation, the NE3 formulation showed the highest SPF value, suggesting that the BBS extract may have contributed to the maintenance of the photostability of the sunscreens. There was a statistically significant difference ($p<0.05$) between the SPF values of the NE2 and NE3 formulations over time.

In the study by Cerqueira-Coutinho *et al.* (2015), the photostability of photoprotective NEs was improved in

the presence of antioxidant compounds from pomegranate extract. They observed that NEs were photostable after 4 h exposure to simulated solar radiation, with no significant differences in FPS. The authors concluded that the plant extract increased sunscreens' photostability. In another study, keratin particles incorporated in photoprotective formulations based on organic sunscreens (OMC and BZF-3) contributed to high stability during 6 h of solar radiation since the SPF values were higher than the control formulation (Teixeira *et al.*, 2019). Thus, adding plant extracts and natural compounds in formulations based on sunscreens can increase the formulation's long-term photostability.

Furthermore, evaluating the photostability of sunscreens becomes an important analysis that must be considered during the development of photoprotective formulations, as it directly influences the effectiveness of the sunscreen and prevents the formation of toxic products on the skin.

In vitro cytotoxicity assays

Cytotoxicity analyses were performed to evaluate the safety of photoprotective formulations containing BBS extract and sunscreens. To estimate the cytotoxicity of the samples, the relative viabilities of fibroblast cells were measured using the MTT assay. Figure 6 shows the percentage of cell viability in response to the dose applied in fibroblastic cells.

In vitro cytotoxicity tests showed that the BBS lipophilic extract had no cytotoxic effect against fibroblastic cells at any concentration tested. Furthermore, at a low concentration, the BBS extract showed no statistical difference from the positive control ($p>0.05$). This result was in agreement with the study by Santos *et al.* (2020), who evaluated the *in vitro* and *in vivo* healing effects of BBS oil and observed that BBS oil was not cytotoxic to L929 fibroblast cell lines and murine peritoneal macrophages. Thus, BBS lipophilic extract-based formulations are safe for application in topical products.

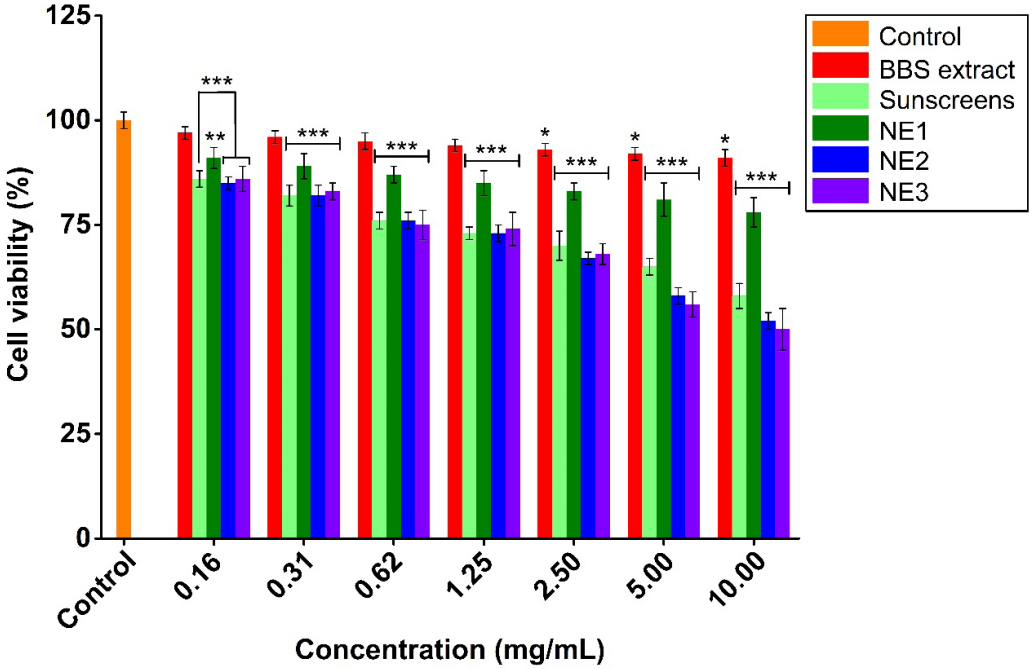

FIGURE 6 – Cytotoxicity analysis of nanoemulsions (NE1, NE2, and NE3), BBS extract, and sunscreens using fibroblastic cells (HFB) with MTT assay. Each bar represents the mean value of triplicates (n=3), and the error bars represent the standard error. Data were analyzed using one-way ANOVA with Tukey's test considering *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to the control group.

A similar effect was observed for the sunscreen mixture, indicating low toxicity at all tested concentrations ($p<0.001$). Similar high cell viability was observed for the NE1 and NE2 formulations, with CC_{50} values of 33.12 ± 0.45 mg/mL and 9.33 ± 0.21 mg/mL, respectively. Furthermore, the LC_{50} in fibroblastic cells was 29.64 ± 0.02 and 8.06 ± 0.04 mg/mL for NE1 and NE2 formulations, respectively. Although these results demonstrate low cytotoxicity, a decrease in cell viability at high concentrations for the NE2 formulation could be due to the presence of sunscreens. The NE3 formulation containing BBS extract and sunscreens maintained its low toxicity in fibroblasts, with a CC_{50} of 8.71 ± 0.32 mg/mL and LC_{50} of 7.55 ± 0.15 mg/mL. Statistical analysis shows that only NE2 and NE3 formulations do not show significant differences ($p>0.001$).

Different formulations based on sunscreens and plant extracts have been developed and shown low *in vitro* toxicity. A recent study performed by Chu *et al.* (2022a) evaluated the cytotoxicity of sunscreen formulations containing a tocotrienol-rich fraction. The photoprotective formulations did not show toxicity against normal human dermal fibroblast cells (NHDF) at 2 and 4 mg/mL concentrations. In another study, the authors prepared a photoprotective formulation containing kenaf seed oil (*Hibiscus cannabinus* L.). They evaluated the cytotoxicity against normal human epidermal keratinocyte cells (NHEK) (Chu *et al.*, 2022b). The authors reported high cell viability (above 100%), mainly related to the presence of antioxidants in the formulation that induce skin cell proliferation.

Thus, plant extracts such as BBS, incorporated into photoprotector formulations, can be an important source of phytonutrients including vitamins, antioxidants, fatty acids, and anticancer compounds, which play vital roles against oxidative stress for dermatological and cosmetic purposes.

CONCLUSIONS

Stable photoprotective formulations were produced based on oil-in-water nanoemulsions (NEs) containing Babassu (BBS) lipophilic extract and organic sunscreens. The physicochemical properties (acidity index, peroxide index, refractive index, and relative density) of BBS samples remained within the standards of the legislation, and the fatty acid profile revealed the predominance of saturated fatty acids (lauric and myristic acids), responsible for its high oxidative stability during storage; therefore, they were considered acceptable for the development of photoprotective formulations. The stability tests showed that NEs have a homogeneous droplet distribution profile, nanometer size, and pH compatible with the skin's pH. Nanoemulsion 3 (NE3), containing BBS extract and sunscreens, presented a yellow color, homogeneous aspect, shiny appearance, and typical BBS oil odor. The *in vitro* SPF value for this formulation was 35.5 ± 3.0, higher than the control formulation, thus providing a high potential for protection against UVA and UVB radiation. The results suggested that the antioxidant compounds (such as phenolics compounds and tocopherols) present in the BBS extract play a fundamental role in the photoprotection properties, acting synergistically with the sunscreens, thus increasing the SPF. Furthermore, the NE3 formulation was photostable for 6 h and showed no cytotoxicity to fibroblast cells. Therefore, it can be suggested that using BBS extract in photoprotective formulations may be a suitable candidate for application in cosmetic products, as it is safe and effectively protects against UV radiation.

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