Photoprotective activity and HPLC-MS-ESI-IT profile of flavonoids from the barks of *Hymenaea martiana* Hayne (Fabaceae): development of topical formulations containing the hydroalcoholic extract

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ABSTRACT

The development of photoprotective products has been highly focused on natural ingredients, and exposure to ultraviolet (UV) radiation is also related to the production of reactive oxygen species (ROS), which are extremely damaging to tissues. *Hymenaea martiana* Hayne has flavonoids related to its pharmacological activities, but a flavonoid profile of this species has not been reported yet. In this context, this work aimed to perform high-performance liquid chromatography-mass spectrometry-electrospray ionization-ion trap (HPLC-MS-ESI-IT) to characterize the flavonoids from *Hymenaea martiana* bark extract and develop a cosmetic formulation with photoprotective action. Total phenolic compounds, total flavonoids, sun protection factor (SPF) and antioxidant activity of the crude extract were determined. An HPLC-ESI-IT profile of flavonoids was developed. Photoprotective gel formulations were developed with the extract of *H. martiana*. The highest content of phenolic compounds was found in the crude extract, followed by the ethyl acetate fraction. The total flavonoid content in the samples demonstrated that these are the major phenolic compounds. The crude extract showed significant antioxidant and photoprotective activity. A flavonoid profile was developed, and bioactive flavonoids reported in the species were identified. In the development of the formulations, it was evidenced that the addition of the crude extract to the chemical filter increased the antioxidant activity and FPS, suggesting a synergistic effect in the photoprotection. Formulation F4 was considered a promising product. These results show the cosmetic potential of *Hymenaea martiana*, thus justifying the development of a cosmetic formulation with photoprotective activity.

INTRODUCTION

Medicinal plants have played an important role in therapeutics since antiquity, but their value as a clinical alternative has been gaining prominence in public policies in several countries around the world, including in Brazil. This country has standardized the methods of analysis, mainly in relation to some criteria, such as safety, efficacy and quality of drugs and cosmetics with active vegetable raw material. The development and production of photoprotective products has been highly focused on natural active ingredients, and consumers have shown preference for using natural raw material, mainly vegetable derivatives [1]. Natural products with antioxidant activity have been highlighted, due to the fewer undesirable effects in relation to the synthetic additives [2]. Following this trend, the development of photoprotective formulations currently aims at the inclusion of...
natural products and plant extracts in the formulations [3], mainly raw materials with antioxidant activity [4]. Ultraviolet (UV) radiation is a range of the electromagnetic spectrum that lies between 100 and 400 nm and is divided into UVA (320–400 nm), UVB (290–320 nm) and UVC (100–290 nm) [5]. In addition to sunburn and skin cancer, exposure to UV radiation is also related to early cutaneous aging, which can be related to the production of Reactive Oxygen Species (ROS), which are extremely damaging to tissues [6].

As a result, natural chemical compounds with antioxidant and photoprotective activities, such as phenolic compounds and flavonoids, have shown important pharmaceutical and cosmetic potential, due to their biological actions already reported. The main pharmacological activities associated with flavonoids include antioxidant activity [7], immunomodulatory, anti-inflammatory, bactericidal, antiviral, hepatoprotective and gastroprotective activity [8]. Other activities that have been studied are the antimicrobial activity [9] and photoprotective activity of flavonoids [10].

Among the medicinal plants found in the Caatinga biome, stands out the *Hymenaea martiana* Hayne, popularly known in the Brazilian Northeast as ‘jatobá’. Jatobá has traditionally been used in the form of food, building material and traditional medicine, being used in the form of alcoholic extract, for the treatment of anemia, gastritis, inflammation, rheumatism, and also as antinociceptive and analgesic [11–13]. Some substances that have been related to the pharmacological activities are flavonoids, being astilbin the major component of the bark extract [7, 11, 14–17]. Due to the presence of these chemical compounds whose absorption spectrum presents with two peaks maximum between 240–280 nm and another at 300–550 nm, *Hymenaea martiana* could then be associated with the development of photoprotective preparations [18].

In this context, this work aimed to perform a phytochemical study, with the characterization of flavonoids by high-performance liquid chromatography-mass spectrometry-electrospray ionization-ion trap (HPLC-MS-ESI-IT) from *Hymenaea martiana* bark extract, and to develop a cosmetic formulation with photoprotective action.

**Materials and methods**

**Plant material**

The barks of *H. martiana* were collected in the city of Petrolina, Pernambuco, Brazil, in July 2015, and were identified by a botanist of Herbarium Vale do São Francisco (HVASF), at the Federal University of São Francisco Valley, with voucher specimen n° 6444, coordinates 09°11’04.30” S, 040°18’05.40” W, 357 m high. The barks were dried at 40°C for 72 h in air circulation oven (Ethiktechno®, Model TD 420), and pulverized using a mill (Quimis®). All procedures for access to genetic patrimony and associated traditional knowledge were carried out and the project was registered in SisGen (Register #A3E4538).

**Extract preparation**

For the preparation of the crude extract, 300 g of the barks were submitted to extraction in the National Institute of Semi-arid (INSA, Campina Grande, Paraíba, Brazil), using the Accelerated Solvent Extraction (ASE) Thermo Scientific Dionex® ASE 350, equipped with a stainless-steel cell extractor hermetically sealed. For this, the dried and powdered plant material had its granulometrically homogenized using sieves of mesh 14 and 35, then this material together with diatomaceous earth (2:1) was distributed in 15 extraction cells with 100 mL capacity for 20 g of vegetal material each. The extraction was done with 99.5% ethanol, with a temperature of 40°C, for 15 min, flow of 5 mL/min and average pressure of 1500 psi., with two extractions per cell.

After the extraction process, the solvent was concentrated in a Thermo Scientific Rocket Evaporator at 40°C. The residual solvent was eliminated in an oven (Ethiktechno®) at 45°C for 24 h, obtaining 67 g of the crude ethanolic extract (Hm-CEE).

The crude ethanolic extract (10 g) was solubilized in a mixture of methanol and water (3:7 v/v), which was submitted to a liquid-liquid partition in a separation flask, with manual shaking, extensively, with hexane, chloroform and ethyl acetate. After this process, the solutions were concentrated for solvent evaporation under vacuum at 50°C, obtaining 1.24 g of the hexane fraction, 0.18 g of the chloroform fraction, and 7.90 g of the ethyl acetate fraction.

**Determination of total phenolic compounds**

The total phenol content was measured by the colorimetric method using Folin-Ciocalteu reagent (Sigma®) and gallic acid (Sigma®) as standard, based on the method described by [19]. For this, an aliquot (40 μL) of the diluted extract and fractions was added to 3.16 mL of distilled water and 200 μL of the Folin-Ciocalteu reagent and mixed immediately. The
mixture was allowed to stand for 6 min and then 600 μL of stock solution of Na₂CO₃ (200 g/L) were added and mixed. The final solutions were left to stand at 20°C for 2 h. At the end of the process, the absorbance of each solution was determined in a spectrophotometer (Quimis®) at 756 nm against the blank (all components except the sample under analysis) and the results were plotted on a plot correlating the absorbance of the sample with concentration. The results were expressed as milligrams of gallic acid equivalent (mg GAE)/g of dry weight of plant extract. The standard curve was obtained using gallic acid as reference (50–1000 mg/L, R² = 0.9923). All assays were developed in triplicate.

**Determination of total flavonoids**

The total flavonoid content was measured for the samples with significant values for the total phenolic compounds, by the colorimetric method by metallic complexation [20]. A standard solution and the extract and fraction solutions (1 mg/mL) in ethanol 99% were prepared, and 0.2 mL of aluminum chloride 2.5% alcoholic solution and 3.80 mL of ethanol. The solutions could stand at room temperature for 30 min. The absorbance of each solution was obtained at 408 nm in a spectrophotometer (Quimis®) against the blank (all solvents except the sample). The total flavonoids were expressed as milligrams of catechin equivalents per gram of sample (mg EC/g), using a standard curve with catechin as reference (2.5–20 μg/mL, R² = 0.9937). All assays were carried out in triplicate.

**In vitro antioxidant and photoprotective activity**

**Determination of total antioxidant capacity (TAC)**

The Total Antioxidant Capacity was determined by the phosphomolybdenum method [21]. A volume of 0.1 mL of sample solutions (1 mg/mL) were added to 1 mL of reagent solution (sulfuric acid 600 mmol/L, sodium phosphate 28 mmol/L and ammonium molybdate 4 mmol/L). The tubes were incubated at 95°C for 90 min, and then the absorbance was measured at 695 nm against the blank. Ascorbic acid was used as reference and the Total Antioxidant Capacity was expressed as equivalents of ascorbic acid. The TAC (%) was calculated using the following formula: TAC (%) = [(Aₒ − Aₛ)/(Aₒ − Aₐ) × 100], where Aₒ is the absorbance of the sample, Aₛ is the absorbance of the blank, and Aₐ is the absorbance of ascorbic acid. All assays were carried out in triplicate.

**Inhibition of 2,2-azino-bis-(3 ethylbenzothiazoline)-6-sulfonic acid (ABTS⁺) radical**

The inhibition of ABTS⁺ radical (Sigma®) by the samples was assayed according to a method previously described [22]. The ABTS⁺ solution 7 mmol/L was prepared by adding potassium persulfate 140 mmol/L and incubating the mixture away from light at room temperature for 12–16 h (time required for radical formation) before its use. The ABTS⁺ solution was standardized with the dilution with ethanol to an absorbance of 0.7 (± 0.02) at 732 nm. The absorbance of the samples was measured using 30 μL of the sample solutions (1 mg/mL), added to 3 mL of the standardized ABTS⁺ solution 7 mmol/L, at different time points (6, 15, 30, 45, 60 and 120 min) at 734 nm. The inhibition of the oxidation was calculated and plotted as a function of reference antioxidant concentration (Trolox) and expressed as Trolox Equivalent Antioxidant Capacity (TEAC, μmol/L).

**DPPH free radical scavenging assay**

The free radical scavenging activity was measured using the 2,2-diphenyl-1-picrylhydrazil (DPPH) assay with adaptations [23]. Samples were diluted in methanol to an initial concentration of 5 mg/mL, followed by serial dilution to 0.078 mg/mL. Then, 40 μL of each dilution was transferred to the 96 well plate and 250 μL of the 0.008% DPPH methanolic solution was added. The absorbance of each sample was measured at 517 nm in a spectrophotometer, 25 min after the addition of DPPH solution to the sample solutions. The Antioxidant Activity (AA) was calculated using the following formula: AA% = [(Aₒ − Aₐ)/Aₒ]×100, where Aₒ is the absorbance of the control, and Aₐ is absorbance of the sample. All assays were carried out in triplicate.

**In vitro photoprotective activity**

The photoprotective activity was evaluated spectrophotometrically by the method of diluted solutions. Samples were dried in an oven at 40°C for 60 min, and then dilutions were prepared (100 mg/L). A spectrophotometer (Quimis®) was used, with quartz cuvettes with 1 cm optical path for the acquisition of the spectra and ethanol as blank. For maximum absorption wavelength (λmax) determination, spectrophotometric scanning of the crude extract was performed at wavelengths between 260 and 400 nm, with intervals of 5 nm. Calculations of the Sun Protection Factor (SPF) were made considering the intervals λ determined using the following formula: SPF = CF × AA × EE × SA, where CF is Correction Factor,
AA is Amount of absorbance at 290–320 nm, Erythemogenic Effect of Radiation (λ), and SA is Spectrophotometric reading of sample absorbance (λ). The values of EE×I are constant and previously determined [24, 25]. The synthetic filter benzophenone-3 (10mg/L) and the standard flavonoid quercetin (10mg/L) were used as positive controls.

**Characterization of flavonoids by HPLC-ESI-IT-MS**

The HPLC-ESI-IT-MS analysis was performed in NPPNS-Ribeirão Preto, with the ethyl acetate fraction of the Hm-CEE. This analysis was done in an HPLC (Shimadzu Prominence®), equipped with two binary pumps (LC-20AD), degassing unit (DGU-20A), automatic sampler (SIL-20AHT), oven (CTO-20A), communication module (CBM-20A), -M20A) and a Luna-Phenomenex reverse phase column, 250mm × 4.6mm, 5 μm). The mobile phase was composed of a mixture of solvent A (0.1% formic acid in ultra-purified water) and solvent B (0.1% formic acid in methanol), following a concentration gradient, flow rate of 1 mL/min.

The chromatograph was coupled to an Amazon SL ion trap mass spectrometer (Bruker Daltonics®), equipped with an electrospray ionizer and ion trap analyzer, under the following conditions: 3500V capillary; 500V end plate; nebulizer 60 psi; gas flow 10.0 L/min and gas temperature 330°C.

The results were analyzed using the GNPS website on-line database (Global Natural Product Social Molecular Networking) (GNPS, 2017). The data obtained in the chromatograph coupled to the mass spectrometer were converted to the mzXML format directly into the software Data Analysis 4.2 (Bruker Daltonics®), and submitted to dereplication analysis at GNPS website, and the substances were considered as identified in the sample if the mass spectra obtained at least six combining ions and cosine score above 0.5 [26]. Molecular formulas and classifications of substances were obtained from PubChem [27].

**Preparation of photoprotective formulations**

The base gel used was composed of Carbopol® 940 (Table 1). This base was prepared by dispersing the gelling agent (Carbopol®) in water with methylparaben preservative, along with a humidifying agent (propylene glycol). Thereafter, it could stand for 24 h to facilitate preparation of the gel in the dispersion of Carbopol® in water. Subsequently, the alkalinizing agent (triethanolamine) was added and mixed until a pH range of 5.0–5.5 was obtained obtaining a clear gel.

From the prepared gel base, the chemical filter and Hm-CEE were incorporated in different concentrations (Table 2). The chemical filter used was a water-soluble UVA/B filter composed of benzophenone-3 (Fagron®). The formulations were evaluated for photoprotective activities with adaptations in the concentration of the sample solution to 1mg/mL, using the gel base as white, and antioxidant activity.

The SPF of the prepared formulations was determined using the previously described methodology, with adjustments in concentration of the sample solution to 1mg/mL [24].

The formulations were submitted to physicochemical quality control tests, as appearance, color, odor, pH, consistency by extensibility and centrifugation resistance test.

The appearance, homogeneity, and organoleptic characteristics were evaluated by macroscopic analyses. The pH value (MS Tecnopon®, model mPA-210, Brazil) was determined by inserting the electrode directly into the aqueous dilution 1:10 (w/v) of the samples. The determination of consistency by extensibility was performed as proposed by [28], using 0.3 g of the sample placed between two glass plates, 10 × 20 cm and 0.5 cm thick, laid on a graph paper. The diameters of the formulations were measured after the addition of weights, every three minutes, on the top plate. The average extensibility was calculated in cm² by multiplying the square of the diameter by π/4. Centrifugation test was performed 24 h after preparation of the formulations at 3000 rpm (Fanen, model 206 BL, Brazil) for 30 min at room temperature.

For the preliminary stability study, the freezing/defrosting cycle method was used [29]. The formulations were submitted to physicochemical tests (appearance, color, odor, pH, consistency by extensibility and centrifugation resistance test), before (T0) and after

| Component               | Concentration (%) |
|-------------------------|-------------------|
| Carbopol® 940           | 1.00              |
| Methylparaben           | 0.10              |
| Propylene glycol        | 7.00              |
| EDTA                    | 0.10              |
| Triethanolamine         | Until pH = 5.0 to 5.5 |
| Purified Water          | q.s.p.            |

q.s.p.: Quantity sufficient for preparation.
six freezing/defrosting cycles (12th day, T12). Each cycle corresponds to alternate 24 h at high temperatures of −5°C ± 2°C and 24 h at low temperatures of 45°C ± 2°C [30].

### Statistical analysis

All determinations were performed in triplicate and the results are shown as mean values with standard deviation (±SD). Values were considered significantly different when \( p < 0.05 \). For the correlation analysis, one-way analysis of variance (ANOVA), followed by the Tukey’s test and the Student’s \( t \) test, the software Statistica® version 10.0 (StatSoft®, EUA), and Microsoft Excel® 2017 were used.

### Results and discussion

#### Total phenolic compounds and total flavonoids

The total phenolic content of the crude extract and fractions is presented in Table 3. The highest content of phenolic compounds was found in Hm-CEE, followed by the ethyl acetate fraction and the values found in the present study is lower to that found in another study with the bark of this species [31]. The content of flavonoid compounds found were significant in this study, and these results can indicate that flavonoids are the major phenolic compounds present in the extract and ethyl acetate fraction of *H. martiana*. The difference between the results found in this study and the previous values reported in the literature can be justified by the different extraction methods, and the volumes and dilutions in the methods for the determination of phenolic compounds and flavonoids used [31].

Flavonoids were described as the major compounds of the crude extract and the ethyl acetate fraction of *H. martiana* previously [14, 32]. Flavonoids have, among other biological activities, a characteristic antioxidant activity, which is related to their capacity to eliminate free radicals donating electrons or hydrogen atoms or cations of metal chelate compounds [33]. The complex chemical structure of flavonoids, as well as the diversity of their molecules, makes the structure–activity relationship more complicated than that of phenolic acids. Some of the structural characteristics and the nature of the substitutions in rings B and C can determine the antioxidant and photoprotective activity of flavonoids. The degree of hydroxylation and the positions of the hydroxyl groups on ring B, in particular a ring B ortho-dihydroxy structure in the positions 3’ and 4’ (known as the ‘catechol’ group), results in increased activity as greater radical stability is conferred by relocation [33]. Dihydroxyflavonoids that present the catechol group in ring B have already been described in *H. martiana*. Astilbin and taxifolin were previously described as substances related to the bioactivities of the species [7, 14, 32, 34]. Other important compounds found in the species include engelitin, eucrifin and daucosterol [14, 34].

#### In vitro antioxidant activity

Table 4 presents the antioxidant activity of the crude extract and fractions of *H. martiana in vitro*. These data demonstrate the high antioxidant capacity (TEAC), analyzed by the ABTS+ radical inhibition method, with 83.75±11.96% and 98.98±1.06% for crude extract and the ethyl acetate fraction, respectively. The ethyl acetate fraction had the highest total antioxidant capacity (phosphomolybdenum method), with 85.97±6.30%. The crude extract showed the highest scavenging activity *in vitro* in the the DPPH radical assay, with 91.4%. It was observed that the fractionation potentiated the antioxidant power in the ABTS+ assay and the phosphomolybdenum method and was supported...
by the DPPH radical scavenging assay. The significant antioxidant activity of the crude extract and the ethyl acetate fraction points to the antioxidant potential of the species being studied.

In vitro SPF determination

The in vitro photoprotective effect of the crude extract was determined by the Mansur method [24] and a significant absorption in the UVB/UVA regions was observed, in the spectrophotometric scanning for the crude extract and the positive controls, quercetin and the synthetic filter, suggesting a possible photoprotective activity (Figure 1).

Aiming the total protection of the skin, which is not achieved by several photoprotective formulations containing only chemical synthetic sunscreens, plant extracts rich in antioxidant compounds are being widely used. This can be explained by the presence of phenolic compounds, which can provide protection against UV rays and neutralize free radicals after sun exposure. For this reason, studies have investigated antioxidant substances that absorb ultraviolet radiation in the UVA range (320–400 nm) and UVB range (290–320 nm), and thus can be used as natural sunscreen.

The application of the photoprotective potential of natural products in sunscreens formulations is a trend [3, 35, 36], including polyphenols, especially flavonoids [10, 35, 37]. Some studies have presented the photoprotective activity of this class of chemical compounds, pointing to the beneficial potential of natural substances in photoprotective and cosmetic formulations [35–41].

Due to their photochemical properties, phenolic compounds such as flavonoids are great candidates for natural photoprotective and antioxidant substances. As Figure 1 presents, the photoprotective potential of the H. martiana extracts showed relevant values. When compared to the positive control (quercetin and benzophenone-3), the UV spectra of the crude extract of H. martiana evidenced an important absorption in the UVB/UVA regions, suggesting a possible photoprotective activity.

Probably due to the high concentrations of phenolic compounds and expressive antioxidant capacity, the crude extract presented significant values for SPF, with 12.43 ± 1.25 in the concentration of 100 mg/L, value like the SPF presented for the flavonoid standard quercetin, which showed an SPF of 12.12 ± 0.02 in the concentration of 10 mg/L, while the synthetic filter benzophenone-3 obtained 8.45 ± 0.06. These values can show the photochemical potential for the

Table 4. In vitro antioxidant activity of the crude extract and fractions obtained from the barks of H. martiana.

| Sample                  | DPPH (%) ± S.D. | ABTS* (% TEAC) ± S.D. | Phosphomolybdenum (%TAA) ± S.D. |
|-------------------------|-----------------|-----------------------|---------------------------------|
| Crude ethanolic extract | 91.47 ± 0.03    | 83.75 ± 1.96          | 46.97 ± 0.04                     |
| Ethyl acetate fraction  | 90.77 ± 0.02    | 98.98 ± 1.06          | 85.97 ± 0.30                     |
| Hexane fraction         | 21.94 ± 0.06    | 9.54 ± 3.83           | 23.99 ± 1.94                     |
| Chloroform fraction     | 31.29 ± 1.07    | 23.56 ± 0.52          | 33.93 ± 3.19                     |
| Ascorbic acid           | 5.45 ± 0.11     | –                     | –                               |

S.D., standard deviation. TEAC, Trolox equivalent antioxidant capacity; TAA, total antioxidant capacity.
extract, and these results were above the levels required for sunscreens in Brazil, according to the Brazilian National Health Surveillance Agency [42], which requires that the minimum SPF value of a photoprotective formulation should be 6.0. These values were also higher than that recommended by the United States Food and Drug Administration (FDA), which considers as a sunscreen a formulation with an FPS value greater than 2.0 [43]. Therefore, these samples presented adequate values for the future development of photoprotectors, with an important antioxidant activity.

The content of phenolic compounds was correlated to antioxidant activity (DPPH radical sequestration) and SPF, and the Pearson ($\rho$) and $R^2$ coefficients were calculated. The results indicate the strong correlation between the antioxidant activity and the total phenolic compounds content of the analyzed samples ($R^2 = 0.995; \rho=0.997$). Several studies point to a strong relationship between the presence of phenolic compounds and antioxidant activity in medicinal plants and fruits [44, 45]. A strong correlation between the phenolic compounds content and the photoprotective activity (SPF) was also presented in the analyzed samples ($R^2 = 0.954; \rho=0.977$), important data for the future development of photoprotectors from plant extracts that present phenolic compounds as chemical markers, as is the case of the species in study.

**Characterization of flavonoids by HPLC-MS-ESI-IT**

For a more in-depth study on the chemical composition of *H. martiana*, a metabolomic study by an HPLC-MS-ESI-IT analysis was performed to identify the main flavonoids of the species. Metabolomic studies are characterized by the separation of complex matrix components, using chromatographic and spectroscopic methods, as well as bioactivity studies. Hyphenated techniques stand out for this type of study, based on the comparison of the information obtained with databases, resulting in the detection of compounds already known or even in the structural elucidation of new compounds. Several natural compounds have been identified using the metabolomic study as flavonoid glycosides, isoflavonoids and flavonoid derivatives [26, 34, 46, 47].

The flavonoid profiling was developed by HPLC-MS method using the GNPS website as an online database, and 18 known flavonoids were detected (Figure 2, Table 5), among two flavanonols, six flavonols, four isoflavones, five flavones and one flavan-3-ol.

All compounds identified showed biological activities in the literature, including antioxidant and photoprotective activities. The dihydroflavonoid astilbin was previously detected, which was previously identified as the major component of *Hymenaea martiana* [14, 34]. This flavonoid has several pharmacological activities already reported, as anti-inflammatory [14], anti-ischemic [48], antinociceptive and antidematogenic [32] and antioxidant [49].

Among the flavonoids identified in the ethyl acetate fraction, only quercetin has photoprotective activity reported in the literature [10]. It also has antioxidant activity and anti-inflammatory activity reported [50]. This flavonoid was tested in this study and presented important absorption in the UVA/UVB region, and significant photoprotection (SPF of 12.12 ± 0.02) in a low concentration (10mg/L). These data show the importance of the identification of this flavonoid in *H. martiana*.

The antioxidant activity has already been reported among the other identified flavonoids, such as taxifolin [51], isoquercetin [52], quercetin-7-O-rhamnoside [53], kaempferol-7-O-α-rhamnoside [54], quercitrin [55], ononin [56], glycitin [57], sissotrin [58], amentoflavone [59], baicalin [52], isovitexin [60], apigenin-C-hexosyl [61] and nobiletin [62]. Thus, the identified flavonoids demonstrate the bioactive potential of the bioactive extract from the barks of *Hymenaea martiana*.

From the flavonoids identified in this study, only astilbin, taxifolin [14, 34] and quercitrin [34] have been previously described in the species. Therefore, this is the first report of the identification of all other flavonoids described in Table 5, evidencing the contribution of this work for the phytochemical study of *Hymenaea martiana*.

**Preparation of photoprotective formulations**

The significance of these results supports the antioxidant and photoprotective potential of *H. martiana*. Since no patents were found with photoprotective formulations with the genus *Hymenaea* or with the species studied in the available data, the results obtained in this study support the development of a novel formulation with photoprotective action from the barks of *Hymenaea martiana*.

Four test formulations were obtained using the Carbopol® gel base, the UVAB chemical filter and the Hm-EEB. The formulations presented visual aspects and homogeneity suitable for cosmetic formulations before and after the freezing/defrosting cycles.

The formulations had pH values between 5.5 and 6.0 (Figure 3) after the manipulation and after the
In addition, the formulations did not present any changes or phase separations after the centrifugation test over the entire period of this study (T0 and T12). The extensibility of the formulations was calculated (Figure 3), showing important data about the efficacy and sensory aspects. A photoprotective gel should have a suitable extensibility, contributing for the formation of a film on the skin. In this way, the formulation can guarantee the specified Sun Protection Factor (SPF). The results presented by the proposed formulations are above the values found for emollients used in commercial emulsions [28], demonstrating that the gels developed with the extract of *H. martiana* achieved an adequate extensibility for photoprotective formulations.

Thus, the results for the quality control and preliminary stability suggest that the formulations showed adequate quality control properties, even after thermal stress.

The antioxidant and photoprotective activities of the formulations were evaluated (Table 6). The results showed that the formulation F1, which contains only the chemical UVAB filter, did not present significant antioxidant activity. The formulation F2, composed of 5% of Hm-BSE, presented 77.55 ± 0.02% AS (DPPH method), 26.60 ± 1.90% TEAC (ABTS method) and 15.00 ± 0.55% TAA (Phosphomolybdenum method), demonstrating that the replacement of the chemical filter by crude extract interfered with the antioxidant activity, causing a significant increase. The F3 formulation, composed of 5% of the UVAB chemical filter and 5% of the Hm-BSE, presented 29.52 ± 0.11% AS, 4.60 ± 0.10% TEAC and 6.55 ± 0.09% TAA, demonstrating lower values in relation to the formulation containing only the crude extract. The F4 formulation, composed...
of 10% of the UVAB chemical filter and 10% of the crude extract, presented 57.88 ± 0.10% AS, 34.60 ± 1.20% TEAC and 12.92 ± 0.12% TAA, evidencing that the increase in the concentration of the sunscreens increased the antioxidant activity of the formulation F4 in relation to the formulation F3. The results show that the gel with photoprotective activity containing only the chemical filter UVAB (formulation F1), which is already commercialized, did not present antioxidant activity in the tested methods, and that the addition of the crude extract under study greatly increased this activity. The addition of the crude extract, therefore, may provide an advantage for the formulations.

However, it can be observed that the addition of the UVAB chemical filters and the crude extract at 5% concentration (F3 formulation) caused a drop in the antioxidant activity. This result may suggest a chemical interaction between the chemical filter and the crude extract, which could be justified by the pH of the crude extract of *H. martiana* bark, which was considered mildly acidic in a previous study [63]. The acidic pH could interfere with the electrons in the chemical structure of the solar filters, causing the product to absorb UV radiation at different wavelengths [63]. It is noteworthy that this interference was not found in photoprotective activity.

On the other hand, increasing the amount of the crude extract to 10% resulted in an increase in both antioxidant activity and photoprotective activity, indicating that this concentration positively affected the activities evaluated. Thus, pH monitoring in preliminary stability studies is more important for monitoring this possible interaction [30, 64].

Therefore, the results presented for the antioxidant activity of the formulations tested may add greater value to the development of the photoprotective formulation, since it may provide a decrease in the use of synthetic additives with antioxidant action in the formulation, which could bring fewer undesirable effects [2, 64].

The evaluation of the photoprotective activity of the formulations tested is shown in Figure 4. The formulation containing only the crude extract presented an FPS value greater than 6.0, demonstrating the photoprotective potential of the extract under study. The formulations containing the chemical filter and the crude extract (formulations F3 and F4) showed a considerable increase in the FPS values, in relation to the formulations containing the filters tested alone (formulations F1 and F2), which may suggest a synergistic effect between the chemical filter and the crude extract in the photoprotective activity.
The formulation F4 presented the best result for the photoprotective activity, with significant antioxidant activity. It can be considered a promising product, since the addition of natural sunscreens to the chemical filters is considered an alternative to increase the safety and efficacy of photoprotectors [2, 10, 41].

Conclusions

The results presented in this study showed that the crude extract of H. martiana contains phenolic compounds, mainly the flavonoids, major class of the chemical composition of the extract. Several flavonoids were identified in the species, and the flavonoids astilbin, taxifolin and quercitrin were identified in the extract, corroborating previous studies. The knowledge about the antioxidant and photoprotective activity can add therapeutic and cosmetic value to this species, with a strongly positive correlation between the phenolic compounds and the evaluated activities. Formulations containing the crude extract were prepared, presenting relevant antioxidant and photoprotective activity. The results showed a synergistic effect between the crude extract and benzophenone-3, bringing promising results for the development of a formulation with photoprotective action. The results obtained in this study up to the present moment show the relevant antioxidant and photoprotective activity of the crude extract, demonstrating the cosmetic potential of Hymenaea martiana, thus justifying the development of medicinal or cosmetic formulations associated with oxidative stress and skin care.

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Data availability statement

All data that support the findings reported in this study are available from the corresponding author upon reasonable request.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

[1] Iha SM, Migliato KF, Vellosa JCR, et al. Estudo fitoquímico de goiaba (Psidium guajava L.) com potencial antioxidante para o desenvolvimento de formulação fitocosmética. Rev Bras Farmacogn. 2008;18(3):387–393. http://dx.doi.org/10.1590/S0102-695X2008000300013
[2] Krishnaiah D, Sarbatly R, Nithyanandam R. A review of the antioxidant potential of medicinal plant species. Food Bioprod Process. 2011;89(3):217–223.
[3] Oliveira-Júnior RG, Almeida JRGS. Prospecção tecnológica de fotoprotetores derivados de produtos naturais. GEINTEC. 2012;3:32–40.
[4] Polonini HC, Lima LL, Gonçalves KM, et al. Photoprotective activity of resveratrol analogues. Bioorg Med Chem. 2013;21(4):964–968.
[5] Nascimento CS, Nunes LCC, Lima AAN, et al. Incremento do FPS em formulação de protetor solar utilizando extratos de própolis verde e vermelha. Braz J Pharm. 2009;90:334–339. http://rbfarma.org.br/files/pag_334a339_incremento_fps_257_90-4.pdf
[6] Velasco MVR, Balogh TS, Pedriali CA, et al. Associação da rutina com p-metoxicinamato de octila e benzofenona-3: avaliação in vitro da eficácia fotoprotetora por espectrofotometria de refletância. Lat Am J Pharm. 2008;27:23–27. http://hdl.handle.net/10915/7576
[7] Almeida JRGS, Silva MEGC, Guimarães AL, et al. HPLC-DAD analysis and antioxidant activity of Hymenaea martiana Hayne (Fabaceae). J Chem Pharm Res. 2012;4:1160–1166. https://pdfs.semanticscholar.org/93e6/ace6ae819bcb19c45156e24c001eeb8c6d2e.pdf
[8] Guardia T, Rotelli AE, Juarez AO, et al. Anti-inflammatory properties of plant flavonoids, effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. II Farmaco. 2001;56(9):683–687.

[9] Oliveira-Júnior RG, Araújo CS, Santana CRR, et al. Phytochemical screening, antioxidant and antibacterial activity of extracts from the flowers of Neogloazia variegata (Bromeliaceae). J Chem Pharm Res. 2012;4:4489–4494. http://www.jocpkr.com/articles/phvtochemical-screening-antioxidant-and-antibacterial-activity-of-extracts-from-the-flowers-of-neogloazia-variegata-br.pdf

[10] Alencar-Filho JMT, Sampaio PA, Pereira ECV, et al. Levantamento etnobotânico de plantas medicinais comercializadas por raizeiros em uma feira livre no município de Patos – PB. Biofar Especial. 2012;2012;39–48. http://sites.uemp.br/biofar/download/v-especial-2012/LEVANTAMENTO%20ETNOBOTANICO%20DE%20PLANTAS%20MEDICINAIS%20COMERCIALIZADAS%20POR%20RAIZEIROS%20DE%20UMA%20FEIRA%20LIVRE%20DE%20MUNIC%C3%88PIO%20PATOS%20%20ESTABILIDADE%20DE%20PRODUTOS%20%20.pdf

[11] Neves MCA, Neves PCA, Zanini JC, et al. Analgesic and anti-inflammatory activities of the crude hydroalcoholic extract from the bark of Hymenaea martiana. Phytother Res. 1993;7(5):356–362.

[12] Anselmo AF, Silva CG, Marinho MGV, et al. Levantamento etnobotânico de plantas medicinais comercializadas por raizeiros em uma feira livre no município de Patos – PB. Biofar Especial. 2012;2012;39–48. http://sites.uemp.br/biofar/download/v-especial-2012/LEVANTAMENTO%20ETNOBOTANICO%20DE%20PLANTAS%20MEDICINAIS%20COMERCIALIZADAS%20POR%20RAIZEIROS%20DE%20UMA%20FEIRA%20LIVRE%20DE%20MUNIC%C3%88PIO%20PATOS%20%20ESTABILIDADE%20DE%20PRODUTOS%20%20.pdf

[13] Gazzaneo LRS, Lucena RFP, Albuquerque UP. Knowledge and use of medicinal plants by local specialists in a region of Atlantic Forest in the state of Pernambuco (Northeastern Brazil). J Ethnobiology Ethnomedicine. 2005;1(1):9. https://doi.org/10.1186/1746-4269-1-9

[14] Carneiro E, Calixto JB, Delle Monache F, et al. Isolation and chemical identification and pharmacological evaluation of eucryphin, astilbin and engelitin obtained from the bark of Hymenaea martiana. Int J Pharmacoc. 1993;31(1):38–46.

[15] Calixto JB, Yunes RA, Medeiros YS. Differential antagonistic effect of hydroalcoholic extract from Hymenaea martiana Hayne arzeik on kinin and other agonist-induced contractions of the isolated rat uterus and guinea-pig ileum. Phytother Res. 1992;6(6):322–326.

[16] Calixto JB, Yunes RA, Medeiros YS. Vascular action of the crude hydroalcoholic extract from Hymenaea martiana on the isolated rat and rabbit aorta. Phytother Res. 1992;6(6):327–331.

[17] Closa D, Torres M, Hotter G, et al. Roselló-Catafau, Prostanoids and free radicals in C14-activated hepatoxicity in rats: effect of astilbin. Prostaglandins Leukot Essent Fatty Acids. 1997;56(4):331–334.

[18] Bobin MF, Raymond M, Martini MC. UVA/UVB absorption properties of natural products. Cosmet Toiletries. 1995;109:63–78.

[19] Almeida JRGS, Oliveira MR, Guimarães AL, et al. Quintans-Júnior, phenolic quantification and antioxidiant activity of Anaxagorea dolichocarpa and Dugetia chrysocarpa (Annonaceae). Int J Pharm Biol Sci. 2011;2:367–374. https://ijpbs.net/subcription_renewals.php?articleid=MTA4OA==

[20] Marques GS, Monteiro RPM, Leão WF, et al. Avaliação de procedimentos para quantificação espectrofotométrica de flavonoides totais em folhas de Bauhinia forficata Link. Quim Nova. 2012;35(3):517–522. http://dx.doi.org/10.1590/S0100-40422012000300014

[21] Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem. 1999;269(2):337–341.

[22] Re R, Pellegrini N, Protegente A, et al. Antioxidant activity applying an improved ABTS radical. Free Radic Biol Med. 1999;26(9-10):1231–1237.

[23] Brand-Williams W, Cuvelier M, Berset C. Use of free radical method to evaluate antioxidant activity. Lebensm Wiss Technol. 1995;28(1):25–30.

[24] Mansur JS, Breder MNR, Mansur MCD, et al. Determinação do fator de proteção solar por espectrofotometria. An Bras Dermatol. 1986;61:121–124. http://www.anaisdedermatologia.org.br/detalhe-artigo/421/Determinacao-do-fator-de-protecao-solar-por-espectrofotometria

[25] Sayre RM, Agin P, LeVee GJ, et al. A comparison of in vivo and in vitro testing of sunscreens formulas. Photochem Photobiol. 1979;29(3):559–566.

[26] Oliveira GG, Carnevale-Neto F, Demarque DP, et al. Dereplication of flavonoid glycoconjugates from Adenocalymma imperatoris-maximilianii by untargeted tandem mass spectrometry-based molecular networking. Planta Med. 2017;83(7):636–646.

[27] PUBCHEM. PubChem Open Chemistry Database [accessed 2017 Nov 13]. Available from: https://pubchem.ncbi.nlm.nih.gov/

[28] Isaac VLB, Cefali LC, Chiari BG, et al. Protocolo para ensaios físico-químicos de estabilidade de fitocosméticos. J Basic Appl Pharm Sci. 2008;29(3):763–796. https://www.repositorio.unesp.br/handle/11449/70617

[29] Khan BA, Akhtar N, Khan H, et al. Development, characterization and antioxidant activity of polysorbate based O/W emulsion containing polyphenols derived from Hippophae rhamnoides and Cassia fistula. Braz J Pharm Sci. 2013;49(4):763–773. http://dx.doi.org/10.1590/S0100-42622013000400016

[30] ANVISA Agência Nacional de Vigilância Sanitária. Guia de Estabilidade de Produtos Cosméticos, ANVISA (Eds.). Brasília, 2004; 52 p. Available from: http://e-protecao-solar-por-espectrofotometria. An Bras Dermatol. 1986;61:121–124. http://www.anaisdedermatologia.org.br/detalhe-artigo/421/Determinacao-do-fator-de-protecao-solar-por-espectrofotometria

[31] Khan BA, Akhtar N, Khan H, et al. Development, characteristic and antioxidant activity of polysorbate based O/W emulsion containing polyphenols derived from Hippophae rhamnoides and Cassia fistula. Braz J Pharm Sci. 2013;49(4):763–773. http://dx.doi.org/10.1590/S0100-42622013000400016

[32] ANVISA Agência Nacional de Vigilância Sanitária. Guia de Estabilidade de Produtos Cosméticos, ANVISA (Eds.). Brasília, 2004; 52 p. Available from: http://portal.anvisa.gov.br/documents/106351/107910/Guia-de-estabilidade-de-produtos-cosmicos.pdf

[33] Cechinel-Filho V, Vaz ZR, Zunino L, et al. Antinociceptive and anti-oedematogenic properties of astilbin, taxifolin and some related compounds. Arzneimittelforschung. 2000;50(3):281–E285. Balasundram N, Sundram K, Samman S. Phenolic compounds in plants and agri-industrial by-products: an assessment of their antioxidant activity, occurrence, and potential uses. Food Chem. 2006;99(1):191–203.
[34] Pacheco AGM, Branco A, Câmara CA, et al. Identification of flavonoids in *Hymenaea martiana* Hayne (Fabaceae) by HPLC-DAD-MS¹ analysis. Nat Prod Res. 2019;1:9–16. doi:10.1080/14786419.2019.1672062. Online ahead of print.

[35] Monsalve-Bustamante YA, Puertas-Mejia MA, Mejia-Giraldo JC. Comparison of the photoprotective effect between hydrolyzed and aglycones flavonoids as sunscreen: a systematic review. J Appl Pharm Sci. 2020;10:116–123.

[36] Baldisserotto A, Buso P, Radice M, et al. *Moringa oleifera* leaf extracts as multifunctional ingredients for “natural and organic” sunscreens and photoprotective preparations. Molecules. 2018;23(3):664.

[37] Chinh NT, Anh NTL, Thao PT, et al. Photoprotective properties of natural antioxidant flavonoids: a DFT and TD-DFT study on acridone derivatives. VJCH. 2020;58(2):157–161.

[38] Bianchini Silva LS, Perasoli FB, Carvalho KV, et al. *Melaleuca leucadendron* (L.) L. flower extract exhibits antioxidant and photoprotective activities in human keratinocytes exposed to ultraviolet B radiation. Free Radic Biol Med. 2020;159:54–65.

[39] Lefahal M, Makhloufi E, Trifa W, et al. Phytochemical screening, total phenolic content and antiradical properties of *Hordeum vulgare* leaf extracts as multifunctional ingredients for “natural and organic” sunscreens and photoprotective preparations. Molecules. 2018;23(3):664.

[40] Battia CM, Alves AVF, Queiroz LA, et al. The photoprotective and anti-inflammatory activity of red propolis extract in rats. J Photochem Photobiol B. 2018;180:198–207.

[41] Kostyuš V, Potapovich A, Albuhyadar AR, et al. Natural substances for prevention of skin photoaging: screening systems in the development of sunscreen and rejuvenation cosmetics. Rejuvenation Res. 2018;21(2):91–101.

[42] ANVISA, Agência Nacional de Vigilância Sanitária. Resolução RDC n° 30 de 1 de junho de 2012 [accessed 2018 Sep 18]. Available from: http://www.cosmetic-ra.org/10.5897/JMPR2015.5979

[43] FDA. Sunscreen: how to help protect your skin from the sun, food and drug, 2017 [accessed 2018 Sep 18]. Available from: https://www.fda.gov/Drugs/ResourcesForYou/Consumers/BuyingUsingMedicineSafely/UnderstandingOver-the-CounterMedicines/ucm239463.htm

[44] Badea N, Giurinca M, Meghea A. Complex effects of sunscreen agents and flavonoid antioxidant devoted to enhance photoprotection of dermal tissues. Mol Cryst Liq Cryst. 2008;48:183–192.

[45] Lins-Neto JR, Uchôa ADA, Moura PA, et al. Phytochemical screening, total phenolic content and antioxidant activity of some plants from Brazilian flora. J Med Plants Res. 2016;10:409–416. https://doi.org/10.5897/JMPR2015.5979

[46] Wolfender JL, Waridel P, Ndjoko K, et al. Evaluation of Q-ToF–MS/MS and multiple stage IT-MSn for the dereplication of flavonoids and related compounds in crude plant extracts. Analusis. 2000;28(10):895–906.

[47] Lambert M, Staerk D, Hansen SH, et al. Rapid extract dereplication using HPLC-SPE-NMR: analysis of isoflavonoids from *Smirnowia iranica*. J Nat Prod. 2005;68(10):1500–1509.

[48] Diao H, Kang ZK, Han F, et al. *Astragalus mongholicus* Bge. protects diabetic rat heart against ischemia-reperfusion injury via blockade of HMGB1-dependent NF-kB signaling pathway. Food Chem Toxicol. 2014;63:104–110.

[49] Bakota EL, Winkler-Moser JK, Berhow MA, et al. Antioxidant activity of hybrid grape pomace extracts derived from midwestern grapes in bulk oil and oil-in-water emulsions. J Am Oil Chem Soc. 2015;92(9):1333–1348.

[50] Gardi C, Bauerova K, Stringa B, et al. Quercetin reduced inflammation and increased antioxidant defense in rat adjuvant arthritis. Arch Biochem Biophys. 2015;583:150–157.

[51] Chobot V, Hadacek F, Bachmann G, et al. Pro- and antioxidant activity of three selected flavon type flavonoids: catechin, eriodictyol and taxifolin. Int J Mol Sci. 2016;17(12):1986. https://doi.org/10.3390/jims17121986

[52] Jin Y, Lu Y, Han G, et al. Comparative study on in vitro anti-free radical activities of quercetin, isoquercetin, and rutin. Chin Trad Patent Med. 2007;38:408–412. http://en.cnki.com.cn/Article_en/CJFDTOTAL-ZCYQ200703032.htm

[53] Liu H, Mou Y, Zhao J, et al. Flavonoids from *Halostachys caspica* and their antimicrobial and antioxidant activities. Molecules. 2010;15(11):7933–7945.

[54] Chua MT, Tung YT, Chang ST. Antioxidant activities of ethanolic extracts from the twigs of *Cinnamomum osmophloeum*. Bioresearch Technol. 2008;99(6):1918–1925.

[55] Zhu L, Chen J, Tan J, et al. Flavonoids from *Agrimonia pilosa* Ledeb: free radical scavenging and DNA oxidative damage protection activities and analysis of bioactivity-structure relationship based on molecular and electronic structures. Molecules. 2017;22(3):195–111.

[56] Yu D, Duan Y, Bao Y, et al. Isoflavonoids from *Astragalus mongholicus* protect PC12 cells from toxicity induced by L-glutamate. J Ethnopharmacol. 2005;98(1-2):89–94.

[57] Genovese MI, Hassimotto NMA, Lajolo FM. Isoflavone profile and antioxidant activity of Brazilian soybean varieties. Food Sci Technol Int. 2005;11(3):205–211. https://doi.org/10.1177/1082013205054499

[58] Abdelhady MIS, Kamal AM, Othman SM, et al. Total polyphenolic content, antioxidant, cytotoxic, antidiabetic activities, and polyphenolic compounds of *Sophora japonica* grown in Egypt. Med Chem Res. 2015;24(2):482–495.

[59] Tarallo V, Lepore L, Marcellini M, et al. The biflavonoid amentoflavone inhibits neovascularization preventing the activity of proangiogenic vascular endothelial growth factors. J Biol Chem. 2011;286(22):19641–19651.

[60] He M, Min JW, Kong WL, et al. A review on the pharmacological effects of vitexin and isovitexin. Fitoterapia. 2016;115:74–85.

[61] Ferreres F, Sousa C, Valentão P, et al. New C-deoxyhexosyl flavonies and antioxidant properties...
of *Passiflora edulis* leaf extract. J Agric Food Chem. 2007;55(25):10187–10193.

[62] Chen XM, Tait AR, Kitts DD. Flavonoid composition of orange peel and its association with antioxidant and anti-inflammatory activities. Food Chem. 2017;218:15–21.

[63] Oliveira FGS. Influência do método extrativo sobre a produção de compostos fenólicos em *Hymenaea maritima* (Fabaceae) e controle de qualidade da droga vegetal. Dissertation presented for the Post-graduation program in Natural Resources of the Semiarid). Federal University of the São Francisco Valley, Brazil, 2015 [accessed 2019 Mar 26]. Available from: http://www.cpgrnsa.univasf.edu.br/uploads/7/8/9/0/7890742/disserta%C3%A7%C3%A3o_fernanda_granja_reunida.pdf

[64] Ribeiro C. Fotoproteção e fotoprotetores. Cosmetologia aplicada à Dermoestética, Pharmabooks, São Paulo, 2006. 270 p. Available from: https://www.pharmabooks.com.br/livros/details.aspx/z-cosmetologia-aplicada-a-dermoestetica-z/?isbn=858973109X