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ORIGINAL ARTICLE

Synergistic enhancement SPF from annatto seed oil and alkaline extracts in association with organic UV filters

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Abstract

Objective: This study investigated the antioxidant activity of oil and alkaline aqueous extracts obtained from *Bixa orellana* seeds and evaluated their potential to modulate the in vitro sun protection factor (SPF) of a sunscreen formulation containing avobenzone (5% w/w) and ethylhexyl methoxycinnamate (10% w/w).

Methods: Alkaline aqueous extracts from whole (AAE1) and ground seeds (AAE2) were prepared, freeze-dried and chemically characterised. Extracted oil (EO) and commercial oil (CO) were also evaluated. Phytochemical screening, chromatographic analyses (GC–MS and LC–MS), carotenoid quantification and in vitro antioxidant assays (DPPH, ABTS + and CUPRAC) were performed. Sunscreen formulations containing 5% (w/w) of each sample were developed, and in vitro SPF was determined before and after UV irradiation.

Results: AAE1, AAE2 and EO exhibited relevant antioxidant activity ($IC_{50} < 50\,\mu g/mL$), whereas CO showed no detectable activity under the tested conditions. Formulations containing AAE1 and AAE2 demonstrated a marked increase in in vitro SPF prior to irradiation (86.7 and 86.3, respectively) compared to the base formulation (23.3). However, after irradiation, SPF values decreased substantially for AAE1 and AAE2 (11.51 and 21.36, respectively), indicating limited photostability. LC–MS analysis confirmed the presence of bixin and norbixin in aqueous extracts, while GC–MS revealed a more complex profile in EO. Notably, AAE2 exhibited greater SPF retention after irradiation, which may be associated with the presence of triterpenes.

Conclusion: Although annatto-derived extracts enhanced in vitro SPF prior to irradiation, their limited photostability restricts their direct application as

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standalone photoprotective agents. These findings suggested that such extracts may be better positioned as auxiliary antioxidant ingredients in sunscreen formulations, particularly when combined with stabilisation strategies to mitigate photodegradation.

KEYWORDS
antioxidant activity, *Bixa orellana* L., chemical analysis, emulsions, formulation, SPF in vitro

Résumé

Objectif: cette étude avait pour objectif d'examiner l'activité antioxydante d'extraits d'huile et d'extraits aqueux alcalins obtenus à partir de graines de *Bixa orellana*, et a permis d'évaluer leur capacité à moduler l'indice de protection solaire (SPF) *in vitro* d'une formulation de crème solaire contenant de l'avobenzone (5 % p/p) et du méthoxycinnamate d'éthylhexyle (10 % p/p).

Méthodes: des extraits aqueux alcalins de graines entières (AAE1) et de graines broyées (AAE2) ont été préparés, lyophilisés et caractérisés chimiquement. L'huile extraite (HE) et l'huile commerciale (HC) ont également été évaluées. Un criblage phytochimique, des analyses chromatographiques (chromatographie en phase gazeuse couplée à la spectrométrie de masse [GC–MS] et chromatographie en phase liquide couplée à la spectrométrie de masse [LC–MS]), une quantification des caroténoïdes et des tests antioxydants *in vitro* (DPPH•, ABTS•+ et CUPRAC) ont été réalisés. Des formulations de crèmes solaires contenant 5 % (m/m) de chaque échantillon ont été élaborées, et le SPF *in vitro* a été déterminé avant et après exposition aux rayons UV.

Résultats: les AAE1, les AAE2 et l'HE ont présenté une activité antioxydante significative (IC_{50} < 50 µg/ml), alors que l'HC n'a montré aucune activité détectable dans les conditions testées. Les formulations contenant des AAE1 et des AAE2 ont présenté une augmentation marquée du SPF *in vitro* avant l'exposition aux rayons (86,7 et 86,3, respectivement) par rapport à la formulation de base (23,3). Cependant, après l'exposition aux rayons, les valeurs du SPF ont considérablement diminué pour les AAE1 et les AAE2 (11,51 et 21,36, respectivement), ce qui indique une photostabilité limitée. L'analyse LC–MS a confirmé la présence de bixine et de norbixine dans les extraits aqueux, tandis que la GC–MS a révélé un profil plus complexe dans l'HE. Il convient de noter que les AAE2 ont présenté une meilleure conservation du SPF après l'exposition aux rayons, ce qui pourrait être lié à la présence de triterpènes.

Conclusion: bien que les extraits dérivés du rocou aient amélioré le SPF *in vitro* avant l'exposition aux rayons, leur photostabilité limitée restreint leur utilisation directe en tant qu'agents photoprotecteurs autonomes. Ces résultats suggèrent que ces extraits pourraient trouver une meilleure application en tant qu'ingrédients antioxydants auxiliaires dans les formulations de crèmes solaires, en particulier lorsqu'ils sont associés à des stratégies de stabilisation visant à atténuer la photodégradation.

INTRODUCTION

Skin cancers are the most common groups of cancers diagnosed worldwide, with more than 1.5 million new cases estimated in 2022. In 2022, an estimated 330 000 new cases of melanoma were diagnosed worldwide and almost 60 000 people died from the disease [1]. These statistics underscore the critical need for preventive measures against skin cancer, particularly through sunscreens.

Bixa orellana L., commonly known as annatto, is a plant native to tropical regions of the Americas, including Brazil [2]. The plant is of small stature, reaching a height of less than 6 meters. Its fruits have spiny husks that harden as they mature, and when ripe, the husks open to release seeds [3]. These seeds are rich in chemical compounds, especially carotenoids such as bixin and norbixin, which are concentrated in the aril (seed covering) [2] (Figure 1).

Carotenoids can neutralise free radicals, byproducts of normal metabolic processes that can damage cells and impair bodily functions, by donating hydrogen or electrons, thus stabilising these radicals [4]. To assess antioxidant capacity in vitro, common assays include DPPH (2,2-diphenyl-1-picrylhydrazyl) radical inhibition, ABTS+ (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) radical inhibition and CUPRAC (cupric ion reducing antioxidant capacity) [5, 6].

Antioxidants can protect skin cells from free radical damage and enhance the photoprotective effects of sunscreens. Photoprotective products work by shielding the skin from ultraviolet (UV) light across various wavelengths, as prolonged UV exposure can cause conditions ranging from dermatitis to skin cancer [7].

Growing demand for safer, more efficient and sustainable sunscreens has intensified the search for botanically derived actives that can act synergistically with established organic filters. Evidence remains scarce on whether annatto-seed carotenoids can synergistically boost conventional organic filters. By comparing oil and alkaline aqueous extracts of *Bixa orellana*, both rich in bixin and norbixin, this study assesses their antioxidant potential and tests whether adding 5% (w/w) of each extract to a sunscreen containing 5% (w/w) avobenzone and 10% (w/w) ethylhexyl methoxycinnamate measurably enhances in-vitro SPF. Notably, no previous studies have evaluated the association of annatto aqueous extracts with chemical UV filters, highlighting the novelty and relevance of the present work.

MATERIALS AND METHODS

Collection and handling

The *Bixa orellana* seeds were collected from two trees located in Itarana-ES (19°56′43.9″S 40°49′45.2″ W). Access to genetic resources was registered with the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the number AF36277. The cleaned seeds were then weighed and stored in a dark environment.

Oil extraction

Fixed oil extraction from *Bixa orellana* seeds followed the methodology described in the Brazilian Pharmacopoeia for determining fixed oils (5.4.1.7) [8]. After extraction, the solvent was evaporated using a rotary evaporator, and the oil yield was determined by weighing the oil on an analytical balance. For comparison, commercial annatto oil (CO) was purchased from Mundo dos Óleos® and obtained through cold pressing.

Alkaline aqueous extracts

The alkaline aqueous extracts were prepared following an adapted method [9]. The extraction was performed with

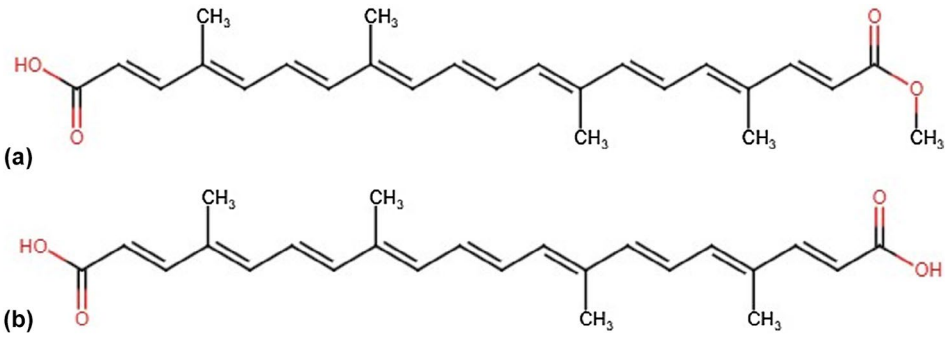


FIGURE 1 Chemical structure of (a) Bixin and (b) Norbixin.

both whole seeds (AAE1) and ground seeds (AAE2). The extracts were frozen at −20°C, transferred to a −80°C freezer for 72 h, and then lyophilised for 48 h.

Characterisation of carotenoids in oils and alkaline aqueous extracts from *Bixa orellana* seeds

Phytochemical screening

Preliminary phytochemical tests were adapted to assess the presence of flavonoids, coumarins, tannins, alkaloids, saponins, triterpenes and steroids [10, 11]. For the tests, the extracted oil (EO) and commercial oil (CO) samples were diluted in ethanol P.A. while the alkaline aqueous extracts from whole seeds (AAE1) and ground seeds (AAE2) were diluted in a 70:30 ethanol-water solution due to their low solubility in organic solvents.

Gas chromatography

The two oils, extracted and commercial were analysed using a GC–MS QP2010 Ultra (Shimadzu) equipped with a quadrupole analyser and a DB-5MS capillary column (5% phenyl-methylpolysiloxane, 30 m × 250 µm i.d. × 0.25 µm film thickness). For the analysis, a 1.0 µL aliquot was injected in split mode (20:1), with an injector temperature of 290°C and an interface temperature of 280°C. The carrier gas used was helium (99.999%) at a constant flow rate of 1.30 mL/min. The oven temperature was initially set at 40°C, held for 3.0 min, followed by a heating rate of 5°C/min up to 110°C, where it was held for 1.0 min. Then, the temperature increased at 12°C/min up to 290°C and maintained for 15 min. The mass spectrometer, equipped with a quadrupole analyser (qMS) and operating under electron ionisation (EI, 70 eV), was run in scan mode with a mass range of 50–600 Da and a solvent cut time of 4.0 min. Each analysis had a total run time of 48 min [12].

Liquid chromatography coupled to mass spectrometry

The characterisation of liquid chromatography coupled to mass spectrometry (Nexera X2 Liquid Chromatography System, Shimadzu; and Maxis Impact, ESI-Q-TOF configuration, Bruker), the column used was C18 (150 × 2.1 mm, 3 µm particle size) (ODS Hypersil, Thermo Fisher Scientific), with negative mode. The mobile phase used was a 2% (v/v) acetic acid solution: methanol (35:65) with a flow rate of 1 mL/min and an injection volume of 20 microliters [13]. The AAE1 and AAE2 samples were solubilised in a 1:1 (v/v) methanol: water solution to a final concentration of 10 mg/mL and then diluted 10 times in a 7:3 (v/v) acetonitrile: water solution. The EO sample was diluted 900 times in a 7:3 (v/v) acetonitrile: water solution [13].

Quantification of carotenoids (β-carotene) by spectrophotometric Method

Total carotenoids were extracted using petroleum ether and quantified according to a standard procedure [14]. The absorbance of the extracts was measured at 450 nm using a spectrophotometer. The molar absorptivity coefficient 2592 was used to calculate the total β-carotene concentration (Equation 1). Total carotenoid values, in mg/100 g, were calculated based on the absorbance of the pigment solution, correlating the concentration of total carotenoids (expressed as β-carotene) (Equation 2).

Equation 1 – Carotenoid Concentration Equation (source: Rodriguez-Amaya, 2001).

$$x\,(\mu g) = \frac{A \cdot y\,(mL) \cdot 10^6}{A^{1\%}_{1cm} \cdot 100} \quad (1)$$

A, absorbance; A1cm1% %, extinction coefficient; x(µg), carotenoid mass; y, solvent volume.

Equation 2 – Carotenoid Concentration in Sample (µg/g) (Source: Rodriguez-Amaya, 2001).

$$x\left(\frac{\mu g}{g}\right) = \frac{x\,(\mu g)}{massa\ da\ amostra\ (g)} \quad (2)$$

Antioxidant activity

DPPH assay

The DPPH antioxidant assay was conducted using spectrophotometric analysis, and the IC50 values, representing the concentration of antioxidants required to reduce the initial radical concentration by 50%, were calculated and statistically analysed. The antioxidant capacity was assessed using a DPPH solution (6.34 mM in ethanol), following the modified methodology of Ak and Gülçin [5]. After 30 min, the absorbance was recorded at 517 nm using the EPOCH 2 microplate reader with Gen 5 software.

ABTS assay

The $ABTS^+$ radical scavenging activity was evaluated using a modified method of Re et al. [6]. After 30 min, the

absorbance was recorded at 734 nm using the EPOCH 2 microplate reader with Gen 5 software.

Cupric ion reducing antioxidant capacity (cuprac) assay

The Cupric Ion Reducing Antioxidant Capacity (CUPRAC) assay, developed in the early 2000s, assesses total antioxidant capacity by measuring the reduction of cupric ions (Cu^{2+}) to cuprous ions (Cu^{+}). Neocuproine (Nc; 2,9-dimethyl-1,10-phenanthroline) is commonly used as a ligand in this method, which is advantageous for evaluating both lipophilic and hydrophilic antioxidants [6]. The CUPRAC assay was performed following the method [6] and the results were expressed in μg/mL of Trolox Equivalents (TE), calculated from a Trolox calibration curve (6.25 to 37.5 μg/mL; r2 = 0.9952) [6].

Sunscreen formulations

The formulation was developed as an oil-in-water (O/W) emulsion containing 5% (w/w) of the oils and alkaline aqueous extracts from annatto seeds, combined with organic sunscreen filters: 10% (w/w) methoxycinnamate and 5% (w/w) avobenzone, in Table 1. This formulation aimed to assess the impact of annatto seed oil and extracts on in vitro photoprotection. The concentrations of chemical filters adhered to RDC N° 600/2022 standards of ANVISA [15] and the Scientific Committee on Consumer Safety [16]. The emulsion was prepared by heating and mixing the aqueous and oily phases, followed by stirring at room temperature [8].

In vitro SPF

The Sun Protection Factor (SPF) of the formulations was determined using a Labsphere® UV-2000S ultraviolet transmittance analyser [17]. Spectrophotometric transmittance values were recorded from 290 to 400 nm. Each formulation (1.3 mg/cm^{-2}) was applied to polymethylmethacrylate (PMMA) plates ($n = 3$) (Helioplate HD 6, Helioscreen) and left to dry for 30 min [18]. The in vitro SPF was calculated using the UV-2000 software, with five readings taken at different points of each plate, allowing a maximum coefficient of variation of 40% between points and 20% between plates.

Statistical analysis

Data was statistically analysed using Minitab Statistical Software (Version 21.1.0). The Ryan-Joiner test will be applied to assess data normality. One-way ANOVA was used, with Dunett's method for post-hoc analysis and $p < 0.05$.

TABLE 1 Qualitative and quantitative composition (% w/w) of the photoprotective formulations evaluated for in vitro sun protection factor.

Components				Concentration (% w/w)				
INCI name	Commercial name	Function in formulation	Phase	BaseF	AAE1F	AAE2F	EOF	COF
Phenoxyethanol (and) caprylyl glycol	Optiphen®	Preservative	W	1,0	1,0	1,0	1,0	1,0
Cetearyl alcohol (and) dicetyl phosphate (and) ceteth–10 phosphate	Crodafos™ CES	Non-ionic Self-emulsifying Wax	O	6,0	6,0	6,0	6,0	6,0
Butyl Methoxydibenzoymethane	Avobenzone	UVA Filter	O	5,0	5,0	5,0	5,0	5,0
Ethylhexyl Methoxycinnamate	Methoxysinamate	UVB Filter	O	10,0	10,0	10,0	10,0	10,0
Freeze-dried aqueous extract AAE1	–	Active	–	–	5,0	–	–	–
Freeze-dried aqueous extract AAE2	–	Active	–	–	–	5,0	–	–
Annatto Seed Oil (EO)	–	Active	–	–	–	–	5,0	–
Commercial Annatto Oil (CO)	–	Active	–	–	–	–	–	5,0
Purified Water q.s.p.	–	Solvent	W	100,0	100,0	100,0	100,0	100,0

Abbreviations: F, final phase; O, oily phase; q.s.p, quantity sufficient for; W, aqueous phase.

RESULTS

Phytochemical screening

The first test conducted was phytochemical screening to identify the compounds present in the samples. This is a standard method used for the identification of phytochemical constituents in the samples. The results of the test for the alkaline aqueous extracts and oils are presented in Table 2.

The triterpenes present in the AAE2 and EO are secondary metabolites consisting of 30 carbon atoms formed by the combination of six isoprene units and as the result of the cyclisation and oxidation of two C15 units, squalene, or related acyclic 30-carbon C30 precursors, different triterpenes can be formed. Based on structure, the triterpenes are divided into two main groups: tetracyclic and pentacyclic triterpenes. These biological activities are related to their chemical structure. In preclinical studies, it was shown that triterpenes have a wide range of pharmacological effects including anticancer, antioxidant, anti-inflammatory, antiatherosclerotic, antiviral, hepatoprotective and immunomodulatory activities [19].

The triterpenes and carotenoids are potent antioxidants that can neutralise free radicals-byproducts of normal metabolic processes that, in excess, can damage cells and impair bodily functions by donating hydrogen or electrons, thus stabilising these radicals and with this mechanism may contribute to the stabilisation of UV filters [4].

Quantification of carotenoids (β-carotene)

Subsequently, a spectrophotometric assay was conducted to quantify the β-carotene present in the samples and oils; hence, the β-carotene is one of the carotenoids in the seeds of *Bixa orellana* and has antioxidant activity.

TABLE 2 Results of the preliminary phytochemical screening.

	Samples			
Metabolites	**AAE1**	**AAE2**	**CO**	**EO**
Flavonoids	+	+	–	+
Coumarins	+	+	+	+
Steroids	+	+	–	+
Triterpenes	–	+	–	+
Alkaloids	–	–	–	–
Tannins	–	–	–	–
Saponins	–	–	–	–

Abbreviations: AAE1, Alkaline Aqueous Extract 1; AAE2, Alkaline Aqueous Extract 2; CO, commercial oil; EO, extracted oil.

The results obtained from the calculations for β-carotene quantification are presented in Table 3.

Gas chromatography

Gas chromatography–mass spectrometry (GC–MS) analysis of the extracted and commercial *Bixa orellana* oils revealed relevant qualitative differences in their chemical profiles, with the extracted oil presenting a higher diversity of bioactive constituents (Table 4). This compositional richness is particularly significant for cosmetic formulations containing UV filters, in which antioxidants and anti-inflammatory components contribute to the mitigation of UV-induced oxidative stress [20, 21].

Liquid chromatography coupled to mass spectrometry

After the characterisation by gas chromatography, 3 of 4 samples (Figures 2–4), were sent for characterisation by liquid chromatography coupled to mass spectrometry. The commercial oil was not sent because in the phytochemical analysis and in the gas chromatography, it did not show compounds of interest.

Antioxidant activity

DPPH assay

Table 5 displays the mean IC_{50} values for each sample and their statistical groupings. It is evident that EO, AAE1 and the Trolox control are grouped under letter A, indicating no statistically significant differences between them.

ABTS assay

The $ABTS^{+}$ assay is commonly used to assess antioxidant activity in both hydrophilic and lipophilic compounds. The $ABTS^{+}$ radical can be generated through chemical, electrochemical, or enzymatic reactions, providing a versatile methodology for evaluating antioxidant capacity [22].

Cupric ion reducing antioxidant capacity (CUPRAC) assay

In the CUPRAC assay (Cu^{2+} – neocuproine complex is the chromogenic oxidising agent) the reduction of Cu^{2+}

TABLE 3 Results of *β*-carotene quantification by UV–Visible spectrophotometry in aqueous extracts, extracted oil, commercial oil and *β*-carotene standard.

Samples	Concentrations*	ABS (absorbance)	β-Carotene concentration (μg/mL)	β-Carotene concentration (μg/g) in sample masses
AAE 1	C1	0.787	30.36	76673.73
	C2	0.368	14.19	14341.41
	C3	0.174	6.71	6780.80
AAE 2	C1	0.745	28.74	69092.31
	C2	0.331	12.77	12278.85
	C3	0.159	6.13	5898.08
CO	C1	0.020	0.62	1455.66
	C2	0.013	0.50	473.58
	C3	0.006	0.23	217.92
EO	C1	1.087	41.94	98907.55
	C2	0.551	21.25	20054.72
	C3	0.278	10.72	10117.92

Abbreviations: AAE1, Alkaline Aqueous Extract 1; AAE2, Alkaline Aqueous Extract 2; CO, commercial oil; EO, extracted oil.
*C1 = 0.4 mg / mL; C2 = 0.2 mg / mL; C3 = 0.1 mg / mL.

TABLE 4 GC–MS results of fixed and commercial oils of *Bixa orellana*.

Suggested compounds	M+	MS	SI	FO	CO
Hexadecanoic acid	257.43	74, 87, 129, 143, 171 185, 199, 227	94	+	+
9-Hexadecenoic acid (palmitic acid)	255.41	55, 236, 268	92	+	+
Cis-10-Heptadecenoic acid	269.43	55, 83, 110, 137, 250, 282	90	+	+
Heptadecanoic acid	271.45	74, 87, 129, 143, 253, 284	94	+	+
Octadecanoic acid (stearic acid)	285.48	54, 74, 87, 157, 213, 255	93	+	+
9,12-Octadecadienoic acid (linoleic acid)	281.45	81 164 191, 263, 294	94	+	+
6-Octadecenoic acid	283.46	55, 84, 264, 296	93	−	+
9-Octadecenoic acid (oleic acid)	283.46	55, 83, 110, 292	90	+	+
9,12,15-Octadecatrienoic acid (*α*-linolenic acid)	279.43	67, 79, 236, 261	91	+	+
11-Eicosenoic acid	311.51	55, 83, 250, 292 324	92	+	+
Tetracosanoic acid	369.64	74, 87, 382	92	+	+
Tetradecanoic acid	229.38	54, 74, 87, 242	94	+	+
Eicosanoic acid	313.53	74, 87 143, 283, 326	92	+	+
Docosanoic acid	341.59	74, 87 129 143 241, 255 354	93	+	+
α-Amorphene	205.35	55, 94, 105, 161, 204	90	+	−
(E,E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene	273.48	69, 93, 133 189, 272	94	+	−
(E,E,E)-3,7,11,15-Tetramethylhexadeca-1,3,6,10,14-pentaene	273.48	69, 81, 107, 272	95	+	−
Farnesol (E)	223.37	69, 93, 136, 236	90	+	−
Trans-Geranylgeraniol	291.48	69, 81 107, 136, 189, 290	92	+	−

Abbreviations: −, absence; +, presence; CO, commercial oil; FO, fixed oil; M+, molecular ion; MS, mass spectrometry; SI, similarity index.

in the presence of neocuproine produces a Cu^{+} complex, with maximum absorbance at 450 nm. The results, shown in Figure 5, indicate that AAE1 and AAE2 samples presented absorbance values comparable to the blank, making it difficult to assess their reducing activity. Conversely, the EO sample demonstrated significant reducing activity, whereas the CO sample showed none.

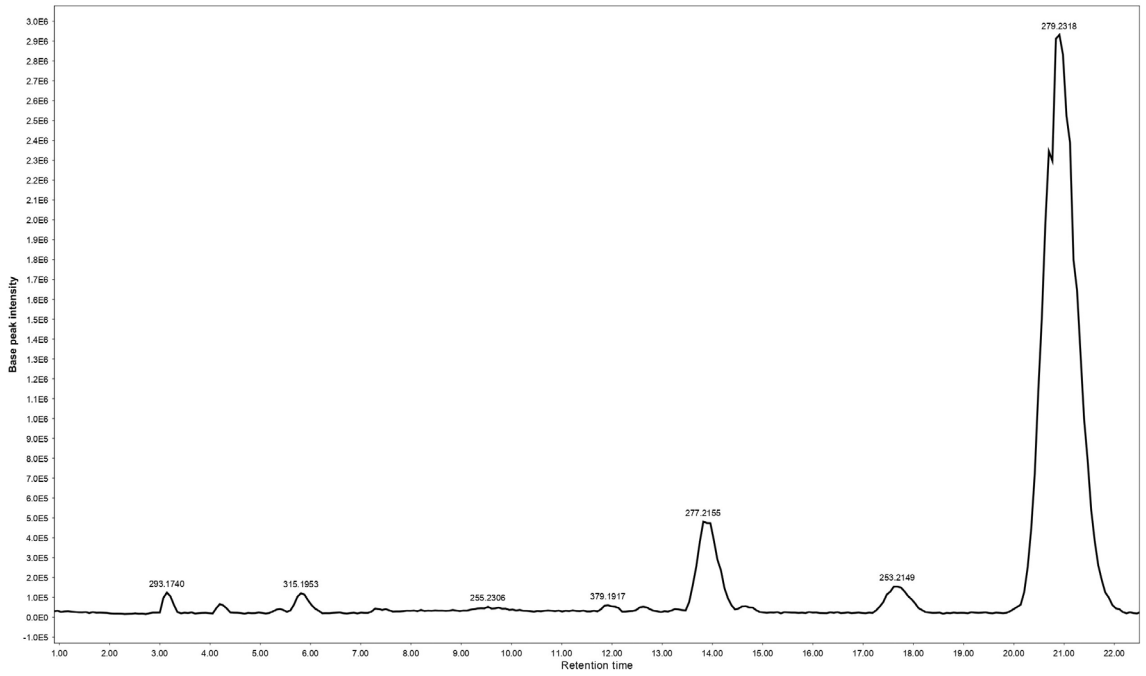


FIGURE 2 Composition of constituents (LC–MS in negative mode) found in *Bixa orellana* L. extracted oil (EO).

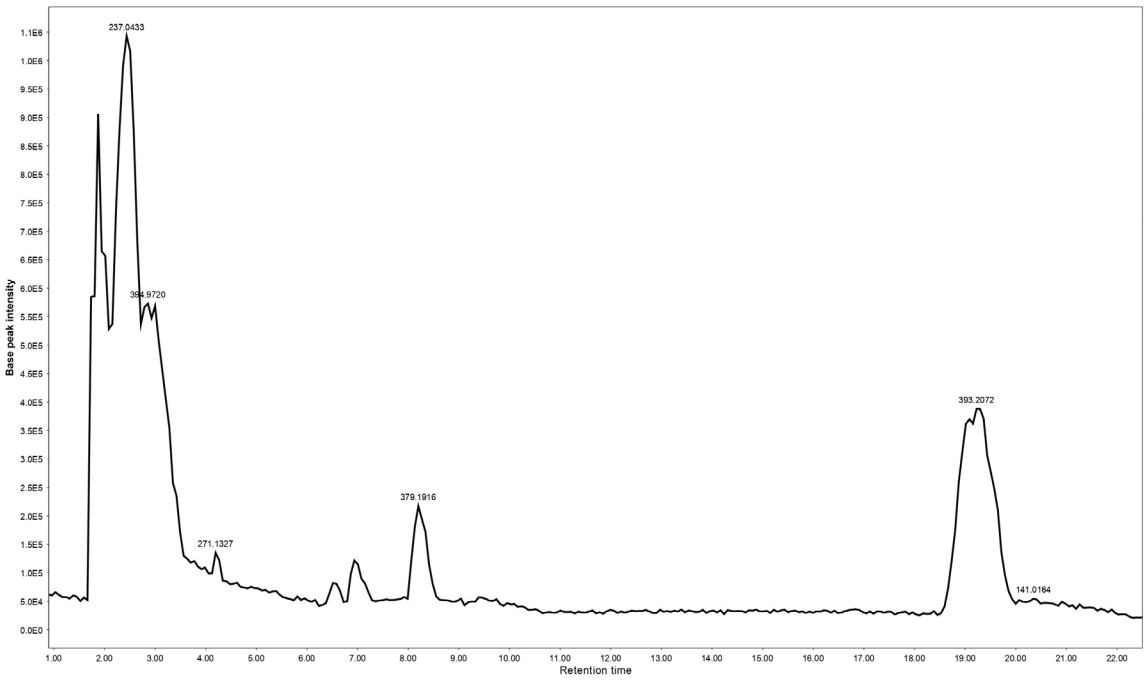


FIGURE 3 Composition of constituents (LC–MS in negative mode) found in *Bixa orellana* L. alkaline aqueous extract with whole seeds (AAE1). Bixin at 393 m/z and Norbixin at 379 m/z.

Sunscreen formulations

Subsequently, the active ingredients were incorporated into the base cream, forming the final formulation. The preparation of the base cream occurred several days prior to conducting the in vitro SPF test to ensure no issues arose with the incorporation of actives.

In vitro SPF determination

Initially, the active ingredients were incorporated into the base cream (BC), and then in vitro SPF was determined. The mixture was homogenised and applied to PMMA plates. After 30 min of application, the plates were analysed. The in vitro SPF measurements for the

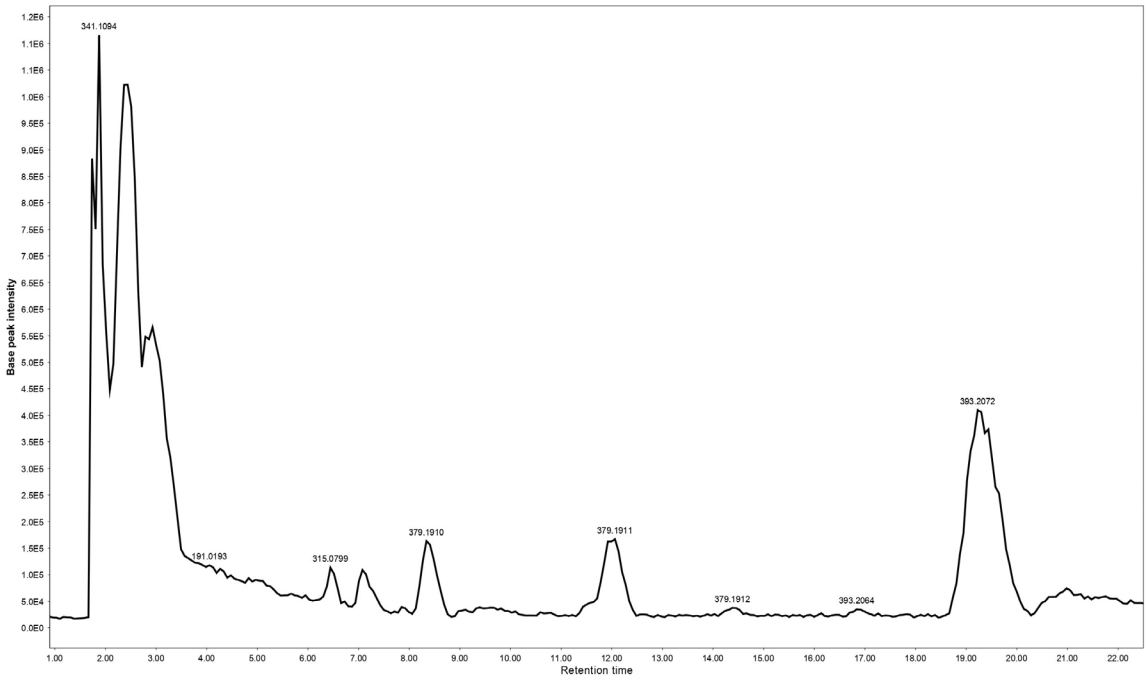


FIGURE 4 Composition of constituents (LC–MS in negative mode) found in *Bixa orellana* L. alkaline aqueous extract with crushed seeds (AAE 2). Bixin at 393 m/z and Norbixin at 379 m/z.

TABLE 5 Statistical results of the DPPH and $ABTS^{+}$ antioxidant assays.

		DPPH results		ABTS results	
Factor	***N***	**Mean**	**Grouping**	**Mean**	**Grouping**
Trolox (control)	2	3.978	A	2.340	A
AAE 2	3	59.6	–	43.123	–
AAE 1	3	33.30	A	39.000	–
EO	3	22.88	A	37.700	–

Note: Means not labelled with the letter A are significantly different from the control mean level.

Abbreviations: AAE1, Alkaline Aqueous Extract 1; AAE2, Alkaline Aqueous Extract 2; EO, extracted oil; $p < 0.05$.

concentrated BC without active ingredients resulted in a value of 23.33, and it is shown in Table 6 together with the other in vitro SPF values.

DISCUSSION

The primary objective of the preliminary phytochemical analysis, presented in Table 2, was to identify the metabolites present in the samples and compare them with those reported in the literature. According to Silva, Bizerra and Fernandes [23], ethanolic extracts of *Bixa orellana* seeds contain flavonoids, flavones, flavonols, xanthones, saponins, anthraquinones, alkaloids, triterpenoids and steroids. Silva et al. [24] also reported that aqueous extracts of *Bixa orellana* seeds contain alkaloids, steroids, phenols, flavonoids, flavones, flavonols, quinones, saponins and xanthones. Most metabolites were identified in agreement with literature, like flavonoids, steroids, triterpenes and flavonoids are one of the compounds that are responsible for antioxidant activity. However, there were discrepancies in two metabolites across all samples: alkaloids and saponins. Two factors may explain these discrepancies: the alkaline medium hampers alkaloid extraction, and saponins might be below the colorimetric detection limit in the alkaline aqueous extraction. Alkaloids are typically extracted under acidic conditions, and since the medium used was alkaline, this could explain their absence. Furthermore, no references to tests for coumarins were found in the literature. The discrepancy observed in the oil samples could be related to the cold-press extraction method, and the absence of triterpenes in AAE1 might be due to the use of whole annatto seeds during the extraction process.

TABLE 6 In Vitro sun protection factor (SPF) values, statistical results of Dunnett's method, $p < 0,05$.

Samples	N	SPF in vitro ± standard deviation pre-irradiation	SPF in vitro ± standard deviation post-irradiation	Grouping
BC	3	23.33 ± 5.86	9.73 ± 0.99	A
AAE1	3	86.7 ± 18.4	11.51 ± 1.94	–
AAE2	3	86.33 ± 14.36	21.36 ± 3.30	–
EO	3	26.00 ± 3.61	23.60 ± 4.56	A
CO	3	14.67 ± 2.52	14.34 ± 1.49	A

Note: Samples not labelled with the letter A are significantly different from the control mean level.

Abbreviations: AAE1, Alkaline Aqueous Extract 1; AAE2, Alkaline Aqueous Extract 2; BC, base cream; CO, commercial oil; EO, extracted oil.

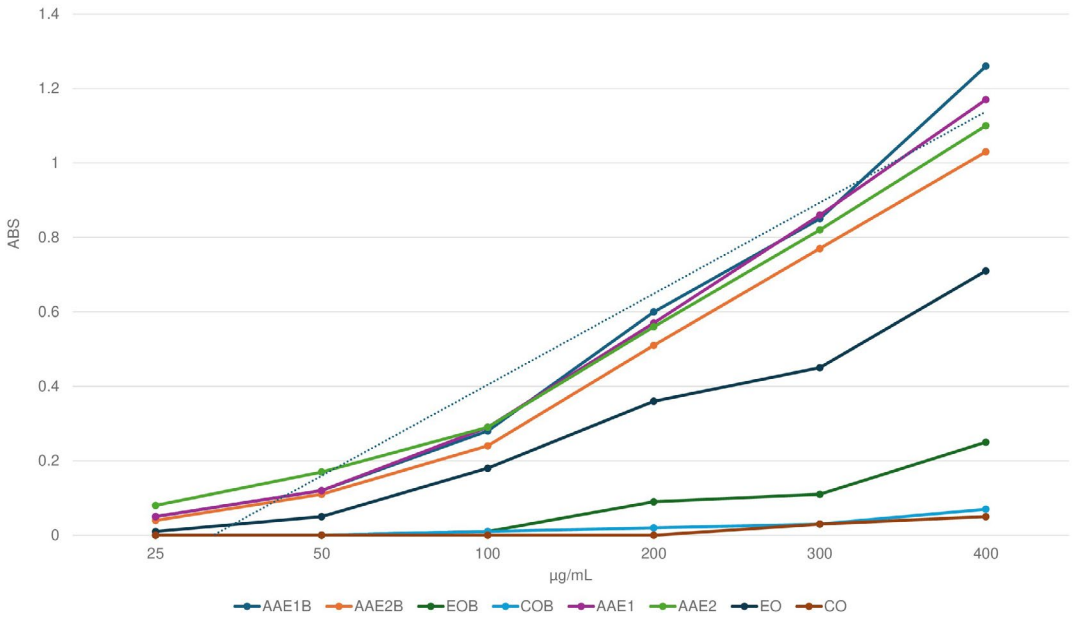


FIGURE 5 Reducing activity of the cupric ion (CUPRAC) for all samples. AAE1 – Alkaline Aqueous Extract 1; AAE2 – Alkaline Aqueous Extract 2; EO – Extracted Oil; CO – Commercial Oil. AAE1B – Blank of Alkaline Aqueous Extract 1; AAE2B – Blank of Alkaline Aqueous Extract 2; EOB – Blank of Extracted Oil; COB – Blank of Commercial Oil. Source: Author's Collection.

According to the results obtained for the *β*-Carotene analysis, a higher quantity of this carotenoid was observed in the extracted oil (EO), followed by the non-crushed seed extract (AAE 1) and the crushed seed extract (AAE 2). This result is attributed to the presence of this carotenoid in the outer part of the seed (aril). The extracted oil contains a higher amount of *β*-Carotene compared to the commercial oil. This difference may be due to the distinct extraction methods employed (organic solvent and cold pressing, respectively). Furthermore, the lower concentration of carotenoids in the commercial oil, as compared to the aqueous extracts, can also be explained by the extraction method used.

In Table 4, both oils contain saturated and unsaturated fatty acids, including palmitic, stearic, oleic, linoleic and *α*-linolenic acids. Polyunsaturated fatty acids such as linoleic and α-linolenic acids are associated with indirect antioxidant activity, as they function as precursors of lipid mediators involved in the regulation of oxidative stress and inflammation [25, 26]. Oleic acid has also been reported to enhance membrane resistance to lipid peroxidation, contributing to improved oxidative stability in biological systems and cosmetic formulations [27].

Notably, the extracted oil exhibited several compounds absent from the commercial oil, including sesquiterpenes and diterpenoid-related compounds such as α-amorphene, (E)-farnesol, and trans-geranylgeraniol, as well as highly unsaturated isoprenoid hydrocarbons. The exclusive presence of these compounds suggests that the extraction process favoured the preservation or recovery of minor lipophilic constituents with relevant biological activity.

Sesquiterpenes such as α-amorphene have been associated with antioxidant and anti-inflammatory effects through modulation of reactive oxygen species and inflammatory

pathways [22]. (E)-Farnesol is a well-documented multifunctional compound exhibiting antioxidant, anti-inflammatory and skin-conditioning properties, with established relevance in cosmetic and dermatological applications [28].

In addition, the highly unsaturated isoprenoid hydrocarbons identified exclusively in the extracted oil contain multiple conjugated double bonds, a structural feature associated with radical stabilisation via electron delocalisation. Although less explored in cosmetic science, structurally related isoprenoids have been reported to contribute to antioxidant activity, either directly or through synergistic interactions with fatty acids and terpenoids [23, 29].

Overall, the GC–MS results demonstrate that, while both oils contain fatty acids associated with antioxidant effects, the extracted *Bixa orellana* oil displays a more complex chemical profile enriched with terpenoid and highly unsaturated compounds. This profile supports the premise that the extracted oil may exhibit enhanced antioxidant and anti-inflammatory potential compared to the commercial oil, particularly in cosmetic formulations designed to counteract UV-induced oxidative stress. This hypothesis will be further substantiated by in vitro antioxidant assays.

In the analysis of the sample of extracted oil (EO) (Figure 2), the compound found in the greatest quantity was linoleic acid (LA) with a value of 279.2318 m/z. This substance is a polyunsaturated fatty acid (PUFA) that has many benefits, including cardiovascular health; skin health; brain health and others, but the most important is antioxidant activity [30, 31]. This analysis showed that oil contains various compounds, however, only linoleic acid exhibits antioxidant activity compounds like bixin and norbixin were not found despite being the focus of the research.

The samples AAE 1 and AAE2 (Figures 3 and 4) had the presence of substances called bixin and norbixin (carotenoids), with values within 393 and 379 m/z, different from the extracted oil. These carotenoids have various biological activities, such as antioxidants, anti-tumour, anti-diabetic, anti-ageing and anti-inflammatory activities [32], and those substances promote health benefits, such as reduction of the risk of developing cancer, cardiovascular disorders, age-related macular degeneration and cataract formation [33].

In Table 5, AAE2 displayed a less favourable antioxidant capacity, with a mean value further from the control. The CO sample was excluded from statistical analysis due to the absence of detectable antioxidant activity at the highest concentration evaluated (200 μg/mL); higher concentrations are necessary for further evaluation.

The DPPH assay and $ABTS^{+}$ assay were performed, and IC_{50} values were calculated and statistically analysed. The results, summarised in Table 5, show that the Trolox control group is the only one grouped under letter A, as none of the samples achieved mean values comparable to Trolox, but it has to be considered that Trolox is a pure substance, which underwent treatments to be used as a control and the samples contain several mixed compounds. Nonetheless, AAE1, AAE2 and EO exhibited promising antioxidant activity, because their mean was below 50, and that is a good result for mixed substances present in the extracts and oil. As with the DPPH assay, the CO sample did not show antioxidant activity at the maximum concentration evaluated (100 μg/mL), indicating that higher concentrations are needed for further evaluation.

These results occurred, in Figure 5, probably because carotenoids absorb at 450 nm (same as Cu-NC complex) and the two samples AAE1 and AAE2 were the samples with the highest quantity of β-carotene, which could have misled the results because β-carotene has the same absorbance (450 nm) as the test reads [14]. These results suggest that other antioxidant assays, such as FRAP (Ferric Reducing Antioxidant Power) or ORAC (Oxygen Radical Absorbance Capacity), should have been used as well. When compared to Trolox, the Trolox Equivalent concentrations at 400 μg/mL were calculated as 16.34 ± 1.34 μg/mL for EO and 1.29 ± 0.12 μg/mL for CO. These results suggest that the CUPRAC assay was not effective for evaluating the antioxidant capacity of all samples.

The formulations, in Table 6, containing AAE1 and AAE2 demonstrated significantly higher in vitro SPF values than the base cream (BC) before the irradiation, whereas EO did not exhibit any noticeable difference in SPF value before and after, and CO actually reduced the SPF in vitro after the irradiation. LC–MS characterisation revealed that AAE1 and AAE2 contained bixin and norbixin, carotenoids with absorption in the UV–visible range, and the phytochemical assay also showed other structures which may account for the improved photoprotective performance before the irradiation. But the decrease of the in vitro SPF after irradiation could relate to the fact that bixin and norbixin are photosensitive and suffered degradation after exposure to sunlight, because these compounds possess a chain of conjugated double bonds (chromophore system) that absorb visible light. Exposure to excess light (especially sunlight or UV) causes these bonds to break or the molecule to isomerise, leading to loss of colour and antioxidant properties. It was proved that Z-bixin, the main component in annatto when exposed to artificial light and sunlight, could isomerise to E-bixin and after elaidinisation, it could be degraded to CO, CO_2, H_2CO and HCl through photoinduced electron transfer between bixin and chloroform to generate radical cations and chloroform-derived radicals, followed by an oxidative breakdown [34].

In contrast, EO was characterised predominantly by *β*-carotene, and CO presented the lowest concentrations of bixin, norbixin and *β*-carotene, findings that are consistent with their lower performance in the in vitro SPF assay. Subsequently, the SPF results were subjected to statistical analysis (Table 6), which showed that EO, CO and the BC did not differ significantly from one another (grouped under letter A). Conversely, AAE1 and AAE2 displayed significantly higher SPF values relative to BC control, before irradiation, but after the irradiation the sample with AAE2 had a higher SPF value, 21.36, than the AAE1, with 11.51, almost double the SPF value, and when looking at the composition of the two extracts the difference between them is the presence of triterpenes in the sample AAE2, and these compounds, as explained before, have antioxidant, antitumour, anti-microbial and anti-inflammatory activity, indicating the reason for a higher SPF value of AAE2 after irradiation [19].

The comparatively higher SPF retention observed for AAE2 after irradiation may be partially attributed to the presence of triterpenes, which were not detected in AAE1. Triterpenes are known to exhibit relevant antioxidant and anti-inflammatory properties, including the ability to scavenge reactive oxygen species and inhibit lipid peroxidation, thereby mitigating oxidative stress induced by UV radiation [19, 25]. In the context of photoprotective formulations, such properties may indirectly contribute to improved photostability by reducing the oxidative degradation of both UV filters and photoactive compounds such as carotenoids.

Additionally, triterpenes have been reported to interact with lipid matrices and biological membranes, potentially enhancing the stability of lipophilic compounds within emulsified systems [26, 27]. This effect may limit the propagation of free radical-mediated reactions initiated by UV exposure, which are known to accelerate the degradation of conjugated systems such as bixin and norbixin. Furthermore, antioxidant compounds present in complex phytochemical matrices can act synergistically, contributing to the stabilisation of UV filters through radical scavenging and energy dissipation mechanisms [7].

Although direct evidence of triterpene-mediated photostabilisation in sunscreen formulations remains limited, the present findings suggest that these compounds may play a protective role in maintaining SPF after irradiation. Therefore, the presence of triterpenes in AAE2 may represent a relevant compositional factor contributing to its comparatively improved performance, supporting the hypothesis that multifunctional plant-derived constituents can influence not only antioxidant activity but also the photostability of cosmetic formulations.

These findings are consistent with previous studies showing that antioxidant molecules can act synergistically with conventional UV filters. For example, flavonoids such as rutin and quercetin have been reported to boost the photoprotective efficacy of sunscreens by combining radical scavenging activity with UV absorption properties [17, 18]. Our results extend this concept to carotenoids from *Bixa orellana*, underscoring their dual role as antioxidants and chromophores capable of attenuating UV radiation.

CONCLUSION

The alkaline aqueous extracts of *Bixa orellana* seeds (AAE1 and AAE2) demonstrated relevant antioxidant activity and significantly increased the in vitro SPF of sunscreen formulations prior to UV irradiation. However, the pronounced reduction in SPF following irradiation highlights a critical limitation related to the photostability of these extracts, associated with the degradation of carotenoids such as bixin and norbixin.

These findings indicate that, despite their initial photoprotective enhancement, annatto-derived extracts cannot be considered effective standalone photoprotective agents under UV exposure. Instead, their role may be better defined as auxiliary antioxidant ingredients capable of contributing to multifunctional sunscreen formulations.

The comparatively improved SPF retention observed for AAE2 suggests that compositional differences, particularly the presence of triterpenes, may influence photostability and warrant further investigation. Future studies should focus on stabilisation strategies, including the combination with established photostabilisers or complementary antioxidants (e.g., vitamins C and E), as well as comprehensive photostability assessments under realistic conditions.

Overall, this study provides relevant insights into the interaction between botanical extracts and organic UV filters, supporting their potential use in the development of more complex and functionally enhanced sunscreen systems.

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CONFLICT OF INTEREST STATEMENT

All authors are employed and students at the Federal University of Espírito Santo and report no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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