Development and Stability Testing of Emulsions with Myrciaria Cauliflora (Jaboticaba) Peel Extracts for Cosmetic Application

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Abstract

Interest in healthy lifestyles has renewed the importance given to nature and treatments with natural products. Among such products, the use of jaboticaba (Myrciaria cauliflora), a native fruit from Brazil, can be highlighted for its high phenolic content and good antioxidant activity, especially when using extracts made from its peel. The aim of this study was to analyze the phytochemical profile of jaboticaba peel extracts in order to incorporate them in emulsions, targeting the production of cosmetic formulations. A hydroglycolic extract (HGE) and a hydroethanolic extract (HEE) were prepared by maceration followed by percolation. Physicochemical analysis, thermogravimetric analysis, determination of total phenolic content, and antioxidant activity by DPPH and FRAP assays were performed. The content of metals with antioxidant properties in peels in natura and peels after extraction was also determined. Non-ionic emulsions were formulated to receive the addition of the extracts, which were then analyzed for their organoleptic and physicochemical characteristics, subjected to viscosity and accelerated stability tests, and analyzed for antioxidant activity by DPPH assay. The HGE was shown to be more thermodynamically stable, had a higher total phenolics content and a greater antioxidant capacity by FRAP assay. The HGE also presented a higher concentration of Fe and Mn, considering that lower levels of these metals were detected in the analysis of the peels after extraction. Emulsions showed a pseudoplastic behavior. When different concentrations of HGE were tested by DPPH assay, it was observed that the higher the concentration of extract in the formulation, the better the antioxidant activity. Although both extracts achieved good results, the hydroglycolic jaboticaba extract is more suitable for the type of formulation targeted by the present study.

Keywords: Jaboticaba; Peels; Extratc; Emulsion; Cosmetic



Research Article

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Introduction

The interest in natural products and their benefits for a healthier lifestyle is associated with the global tendency seen in cosmetic industries, which aim to innovate with raw materials of botanical origin, such as fruit and vegetable extracts, as a means to reduce consumer exposure to synthetic substances [1].

Natural constituents have diverse biological actions, including antioxidant, anti-inflammatory, and photo protective properties. These biological effects can be explained by the presence of secondary metabolites in their composition, exemplified by phenolic compounds such as flavonoids and tannins. Although substances of natural origin have promising applications, further scientific studies are necessary to confirm their beneficial effects when employed in different cosmetic formulations, in different concentrations, thus aiming to ensure their safety and efficacy [2].

Jaboticaba (Myrciaria cauliflora) is a native fruit from Brazil that deserves special emphasis for its application as a raw material of botanical origin. Its fruits are also largely used in the preparation of syrups, jams, fermented beverages, homemade liqueur, vinegar, and ice cream [3-6].

In industrial processes, peels and seeds are generally discarded, representing a loss of approximately 50% of the fruit. This residue is partially destined for animal feed, but the major part is disposed of or directed to composting [7-9]. These subproducts can be employed as alternative sources of micronutrients in pharmaceutical and cosmetic industries, which not only can contribute to reducing waste but also to decreasing environmental impacts and adding value to fruits such as jaboticaba [9-11].

Jaboticaba is one of the richest tropical fruits in relation to phenolic compounds, showing a pronounced antioxidant activity when compared to similar fruits, especially when using extracts made from its peels [3,12]. However, few studies on its chemical constituents can be found in the literature, above all studying the cosmetic properties of the jaboticaba sub-products [6,7,13].

The incorporation of plant extracts into basic cosmetic formulations is a widespread practice, being of fundamental importance the choice of the vehicle to which the active compounds will be added, thus ensuring the expected stability and biological actions. In the industrial context, extracts can be incorporated into various pharmaceutical forms; emulsions are particularly important as they have good consumer acceptance for their pleasant sensorial characteristics, in addition to providing high cutaneous permeation of active compounds [1].

Stability testing of finished cosmetic products involves the analysis of varied factors, such as phase separation, pH variation, and organoleptic and rheological properties. Evaluation of rheological properties, i.e. parameters related to formulation flux properties, is important for the comprehension of a vehicle's physicochemical nature and the quality control of raw materials and finished products [14]. Based on products stability profile, it is possible to analyze its performance, safety, and efficacy, as well as its acceptance by the consumer [15].

In this context, the present study analyzes the phytochemical profile of hydroglycolic and hydroethanolic extracts of jaboticaba fruit peels and their incorporation in non-ionic oil-in-water (O/W) simple emulsions, targeting the production of cosmetic formulations. Therefore, the jaboticaba extract with the best potential to be used as an active raw material will be outlined.

Material and Methods

Extracts

Harvesting of Botanical Material: The jaboticaba fruits were harvested from the jaboticaba orchard of the Fazenda e Vinícola Jabuticabal, located in the district of Nova Fátima, Hidrolândia, Goiás, Brazil, from 10 thousand trees randomly selected, in October 2015.

Preparation of Jaboticaba Peel Extracts: In order to obtain the extracts, this study used the method proposed by Borella, et al. with adaptations. The peelings were submitted to maceration followed by percolation [16,17]. Two types of extracts were obtained through this technique, using different extraction solutions: water and ethanol in the proportion of 32:68, respectively, for the hydroethanolic extract (HEE) and water and propylene glycol in the proportion of 1:9, respectively, for the hydroglycolic extract (HGE) [18]. In both cases, a proportion of 1:5 (20% m/v) peel to extract solution was employed. After 96 hours of maceration, the percolation step was carried out with a drip speed of approximately 1 mL min⁻¹.

Thermal Analysis: The obtained extracts were submitted to a thermogravimetric analysis (TGA), which measures

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mass alteration and is one of the most used thermoanalytical techniques. By employing a thermoscale, it is possible to measure gain or loss of sample mass as a function of controlled temperature variation [19,20]. Weighting (between 12 and 20 mg) was carried out with the pure extracts directly onto the thermoscale of the thermogravimetric analyzer, with the samples being submitted to an initial temperature of 25 °C to observe their rate of evaporation.

Determination of Antioxidant Metal Concentration in Jaboticaba Peels: Atomic absorption spectrometric (AAS) methods were applied to measure in the jaboticaba fruit peels in natura and in the peels after extraction the content of the following metals with antioxidant properties: Cu, Fe, Mn, Se and Zn. Briefly, 250 mg of fruit peels were mineralized in the presence of concentrated ultrapure nitric acid in a micro-wave digester oven (CEM, Mars 6, Matthews, NC, USA). Upon process completion (approximately 40 minutes), the mineralized sample was volumetrically transferred to polypropylene graduated centrifuge tube (St. Louis, MO, USA) and volume made up to 10 mL with ultrapure water (Merck-Millipore®, Billerica, MA, USA). Analyses were performed in duplicate. Cu, Fe, and Zn determinations were performed by flame atomic absorption spectrometry (FAAS), while the determination of Mn and Se was carried out using a graphite furnace atomic absorption spectrometer equipped with a Zeeman corrector. Certified reference materials: Oyster tissue NIST 1566b and Spinach leaf NIST 1570a (National Institute of Standards and Technology, Gaithersburg, MD, USA) were employed for quality assurance for all determinations along with reagent blanks.

Determination of Total Phenolic Content: The total phenolic (TP) content was determined in triplicate according to the Folin-Ciocalteu traditional spectrophotometric method developed by Singleton and Rossi [21]. For TP determination, extracts were diluted in their respective extraction solution in the proportion of 1:10 to attend the requisites of the Beer-Lambert law, with absorbance values between 0.200 and 0.800. After, 200 µL of the diluted extracts were mixed with 2.5 mL of 10% Folin-Ciocalteu reagent solution and 2.0 mL of 7.5% (m/v) Na2CO3 solution. In total, six samples were prepared. Two control samples were prepared by mixing 2.5 mL of 10% Folin-Ciocalteu reagent and 2.0 mL of 7.5% (m/v) N2CO3 solution to 200 µL of the hydroglycolic solution or the hydroethanolic solution. After 2 h of incubation in the dark at room temperature, sample absorbance was measured at λ = 760 nm. Results were

Determination of Antioxidant Activity of Jaboticaba Peel Hydroglycolic and Hydroethanolic Extracts

The extracts antioxidant potential was FRAP Assav: determined in triplicate using the ferric reducing antioxidant power (FRAP) assay, as proposed by Benzie and Strain, with small modifications [22]. The FRAP reagent was prepared by mixing 25 mL of acetate buffer (300 mM, pH 3.6), 2.5 mL of TPTZ solution (10 mM in 40 mM HCl), and 2.5 mL of FeCl₃ (20 mM), in the ratio of 10:1:1 (v/v/v). Subsequently, 3.0 mL of the freshly prepared FRAP reagent was added to 10 µL of 10% extract solutions. Samples were homogenized and incubated in a water bath at 37°C for 30 min, and the absorbance was recorded at $\lambda = 593$ nm. The results were calculated according to a standard ferrous sulfate curve (R2 = 0.992; y = 0.0006x + 0.0266) and expressed in μ M $FeSO_4/g$ of extract.

DPPH Assay: This method is based on the capture of the (2,2-diphenyl-1-picryl-hydrazyl) radical DPPH by antioxidant substances [23]. A 0.004% (w/v) ethanolic solution of DPPH was prepared on the day of analysis. Antioxidant activity determinations were performed in triplicate, and the reaction medium consisted of 3.750 mL of the ethanolic DPPH solution added to 250 µL of the extract solutions. The reaction control was prepared using ethanol and ethanolic DPPH solution. The first spectrophotometric reading at 517 nm was performed with the control only, considered time 0. Samples were left to stand protected from light, and after 30 minutes, the second absorbance reading was taken at 517 nm. The percentage of DPPH free radical scavenging (%FRS) and the EC50, the minimum concentration required by an antioxidant substance to reduce the initial concentration of DPPH by 50%, were calculated.

Emulsions

Development of Emulsions: Three O/W emulsions (Table 1) were formulated by the phase inversion method, according to the ANVISA Brazilian Pharmacopoeia National Form [24]. The aqueous and oily phases were heated separately at 75° C ± 2°C, and the aqueous phase was slowly poured onto the oily phase under constant stirring at 430 rpm until the emulsion cooled. After reaching 40°C, 2.5% of the extracts were incorporated into the emulsions. One emulsion was kept without extract addition to be used as control.

Raw material (%) INCI name	F1	F2	F3
GLYCERIN	5	5	5
METHYLPARABEN	0.1	0.1	0.1
AQUA	qs 100	qs 100	qs 100
CETYLSTEARYL ALCOHOL (AND) POLYSORBATE-60	12	12	12
DECYL OLEATE	2	2	2
MINERAL OIL	6	6	6
PROPYLPARABEN	0.1	0.1	0.1
ВНТ	0.05	0.05	0.05
HE	-	2.5	-
HEE	-	-	2.5

Table 1: Composition of experimental non-ionic formulations developed to test the effects of jaboticaba peel extracts.

Emulsion Physicochemical Stability Evaluation

After preparation, the developed formulations were submitted to stability tests. This evaluation involves several steps, such as centrifugation, accelerated stability tests, and pH determination [15]. In the first step, approximately 5 g of each formulation was centrifuged for 40 min at 1000 rpm and, then, for 1 min at 3000 rpm. After the centrifugation test, samples were subjected to accelerated stability tests under the following conditions: vortex-induced stress vibration for 10 minutes; alternated freezer cooling (approximately -5°C) and heating (ambient temperature of approximately 25°C) cycles of 24h each for 12 days; and exposure to light radiation for 7 days, using sunlight as the source of illumination.

Each sample had its pH determination performed in triplicate. Color, phase separation, and homogeneity parameters were analyzed in the organoleptic evaluation.

Rheological Evaluation by Viscosity

The viscosity of formulations that remained stable after being submitted to the accelerated stability tests was determined using a Brookfield Viscometer. A cylindrical LV spindle no. 4 was used with a radius of 0.1588 mm in diameter, a real height of 3.101 mm, and a corrected height of 3.396 mm, at 20°C.

DPPH Assay for the Analysis of Antioxidant Activity of the Emulsions

As in the analysis of the extracts, a 0.004% (m/v) ethanolic solution of DPPH was prepared. All emulsions

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were diluted in absolute ethanol in the ratio of 1:10 (w/v). Determinations were performed in triplicate. The reaction medium consisted of 3.0 mL of the ethanolic DPPH solution added to 1000 μ L of the 10% emulsions. The control was prepared using ethanol and the ethanolic DPPH solution. The first spectrophotometric reading at 517 nm was performed with the control only, considered time 0. Samples were left to rest in the dark, and, after 30 min, the second absorbance readings were taken at 517 nm. The percentage of DPPH free radical scavenging and the EC50 were calculated.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 5.0 software using non-parametric tests, with a two-way ANOVA and a Bonferroni post-test at a significance level of 5% (p < 0.05) for comparison of means of dimensional components.

Results and Discussion

Jaboticaba Peel Extracts

Table 2 presents the yield and thermal analysis results of jaboticaba peel extracts. In the extraction process, HGE had an initial volume of 337.5 mL and HEE an initial volume of 500 mL. As can be observed in Table 2, HEE had a loss of 14% in relation to its initial volume by the end of the extraction process. On the other hand, there was no volume loss for HGE, which achieved a 100% yield and a better performance than HEE.

Analysis	Hydrogycolic extract	Hydroethanolic extract
Yield (mL)	337.5	430
Temperature range of maximum rate of degradation (°C)	175-185	95-105

Table 2: Yield and thermal analysis of jaboticaba peel extracts.

Fig. 1 shows the extracts TGA graphs, with the mass degradation as a function of temperature. The initial degradation temperature was 25°C. HGE presented a significantly higher temperature range of maximum rate of degradation than HEE (Table 2), implying that HGE is more thermodynamically stable and, therefore, more indicated to be employed as a raw material in cosmetic formulations.



Concentration of Antioxidant Metals in Jaboticaba Peels

The contents of the five antioxidant metals analyzed in jaboticaba peels in natura and peels after extraction can be seen in Table 3. Analyses were performed in triplicate. According to ANVISA Resolution RDC No. 79, the maximum level of heavy metals permitted in cosmetic products is 100 ppm, with the exception of lead, of which the maximum concentration allowed is 20 ppm [25]. All metals evaluated in the studied samples were within the limits permitted by Brazilian legislation, being the highest concentration 13 ppm for Fe in peels in natura. Comparing the results of peels in natura with peels after extraction, a significant reduction in Fe, Zn, Cu, and Mn contents was observed, concluding that these metals passed from the peels to the solutions during the extraction process. No significant detection of Se was observed in the analyses. Peels after hydroglycolic extraction presented a lower content of Fe and Mn when compared to peels after hydroethanolic extraction, with a significant statistical difference. Therefore, it is possible to infer that a greater amount of these metals was extracted from the peels and are present in the HGE solution. Zn and Cu levels were not statistically different for both peels after extraction.

Samples	Fe (µg.g ⁻¹)**	Zn (µg.g-1)**	Cu (µg.g ⁻¹)**	Mn (μg.g ⁻¹)**	Se (µg.g ⁻¹)**
Peels in natura	13.00 ± 0.41^{a}	6.00 ±0.21 ^a	3.00 ±0.09 ^a	11.00 ± 0.57^{a}	< 0.1
Peels -HEE extraction	10.00 ± 0.20^{b}	5.00 ± 0.07^{b}	2.00 ± 0.13^{b}	8.00 ± 0.55^{b}	< 0.1
Peels -HGE	8.00 ±0.22 ^c	5.00 ± 0.40^{b}	2.00 ± 0.17^{b}	6.00 ±0.96 ^c	< 0.1

*Mean of triplicates ± standard deviation.

**Means in the same column followed by different letters (a to c) are significantly different (p < 0.05) based on Bonferroni post-hoc test.

Table 3: Antioxidant metal contents in jaboticaba peels*.

The studied metals are known to play an important role as non-enzymatic antioxidants. While Cu, Zn, and Mn participate in the antioxidant process in association with the SOD enzyme, Fe is one of the most important catalase cofactors [26,27]. Differently, Se acts as a cofactor to glutathione peroxidase, an enzyme capable of reducing

not only H_2O_2 but also other organic hydroperoxides. The presence of these metals may contribute to the antioxidant performance of the extracts.

Determination of Total Phenolic Content

The TP contents of the jaboticaba peel HGE and HEE are shown in Table 4. The results (Table 4) show that HGE had a higher TP content than HEE. Extract characterization by TP determination is an important step in the development of cosmetic products. The results may indicate the characteristics of ingredients and predict their potential action on the finished product's effectiveness. In addition to their antioxidant activities, phenolic compounds have been attributed relevant properties for topical application, such as protective action against UV damage, inhibition of dermal proteinases, and antimicrobial and anti-carcinogenic activities [28].

Samples	Total phenolic content (mg GAE/100 g of sample)*
HGE	4922.2 ±186
HEE	4750.0 ±125

*Mean of triplicates ± standard deviation.

Table 4: Total phenolic content of jaboticaba peel extracts.

Antioxidant Activity by FRAP and DPPH Assays

The FRAP results are shown in table 5, whereas DPPH results can be seen in table 6. In relation to the FRAP assay, the HGE had a slightly better performance than the HEE, while in the DPPH assay, results were very similar with a slightly superior performance of the HGE. However, it should be noted that different antioxidant activities can be observed between isolated extracts and extracts added to a formulation, since the interaction between formulation components may affect the final antioxidant activity [14]. In addition, it is important to consider that applying in vitro data to infer in vivo biological activities requires the understanding of the

bioavailability and metabolic processes of antioxidant compounds, as well as information on skin interaction and penetration [14,29]. Therefore, the tested extracts are potential candidates as bioactive compounds in cosmetic formulations with antioxidant properties, as demonstrated by previously discussed results.

Samples	*µM FeSO4/g of extract
HGE	27.16 ± 0.77
HEE	22.24 ± 0.29

*Mean of triplicates ± standard deviation.

Table 5: Antioxidant capacity by the FRAP assay of jaboticaba peel extracts.

Samples	*EC ₅₀ (mg/mL)	%FRS*
HGE	7.32 ± 0.033	85.30 ± 0.033
HEE	7.24 ± 0.002	86.31 ± 0.002

*Mean of triplicates ± standard deviation.

Table 6: EC_{50} values and %FRS of the DPPH assay of jaboticaba peel extracts.

Relationship between Antioxidant Metals, Total Phenolics, and Extract Antioxidant Potential

The numerical comparative relationships between antioxidant metal levels, TP, and extract antioxidant activity are shown in Table 7. Metal concentration results corroborate with TP and FRAP assay antioxidant activity. Except in the DPPH assay, HGE presented better performance when compared to HEE. In this context, it is possible to infer that HGE has the best performance for cosmetic application, besides already being commonly employed as a humectant agent in cosmetic formulations [30]. However, considering that several factors may influence the composition of botanical extracts, such as harvesting periods, extraction methods, and type of storage, it is important to review TP contents as well as the antioxidant profile of each new product in the cosmetic industry. This strategy should contribute to product safety and efficacy results [14].

Test	*HGE	*HEE	*Peels in natura	*Peels after hydroglycolic extraction	*Peels after hydroethanolic extraction
** Fe AAS (ppm)	-	-	13.00 ± 0.41^{a}	8.00 ± 0.22^{b}	$10 \pm 0.20^{\circ}$
** Mn AAS (ppm)	-	-	11.00 ± 0.57^{a}	6.00 ± 0.96^{b}	8.00 ± 0.55 ^c
TP	4922.2 ±186	4750 ±125	-	-	-
FRAP	31.36 ± 0.77	22.24 ± 0.29	-	-	-
DPPH	7.32 ± 0.033	7.24 ± 0.002	-	-	-

AAS = atomic absorption spectroscopy; TP = total phenolics (mg GAE/100 g of extract); FRAP (µM FeSO4/g of extract); DPPH (mg/mL). *Mean of triplicates ± standard deviation.

**Means in the same line followed by different letters (a to c) are significantly different (p < 0.05) based on Bonferroni post-hoc test. Table 7: Relationship between antioxidant metals, total phenolics, and extract antioxidant activity.

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Finished products should be submitted to clinical studies. Nonetheless, Brazilian laws do not provide guidelines on the frequency of such studies. Usually, they are conducted just prior to sale marketing [32]. Thus, non-recurrent clinical studies and non-periodic characterization of active substances, e.g. the extracts used, can influence the efficacy and safety of marketed products. In addition, the number of controlled clinical studies is small, even though these studies are important to the understanding of in vivo biological activity [28].

Development of Experimental Formulations

Evaluation of emulsion physicochemical stability: Stability testing of cosmetic products provides information on product performance, indicating its degree of stability in certain time intervals, under the environmental conditions to which it can be submitted, from the date of manufacture to the date of expiration [15]. Table 8 summarizes the results of the control emulsion and extract-added emulsions tests in relation to macroscopic (organoleptic) aspects and microscopic aspects after being submitted to tests of accelerated stability, centrifugation, vibration, pH, and stability after temperature variation. All emulsions presented satisfactory results, showing no signs of cream formation, flocculation or coalescence throughout all the studied period (90 days), even when subjected to accelerated stability tests.

Emulsio n	Macroscopic examination	Centrifugation	Vibration	Temperature variation (°C)	pH after preparation	pH after 90 days
1	N/A	N/A	N/A	N/A	6.76 ± 0.000	6.71 ± 0.021
2	N/A	N/A	N/A	N/A	5.51 ± 0.080	6.16 ± 0.017
3	N/A	N/A	N/A	N/A	5.73 ± 0.110	5.97 ± 0.341

N/A= No alteration after accelerated stability tests.

1- Control emulsion; 2- HEE-added emulsion; 3- HGE-added emulsion. Table 8: Emulsion performance in physicochemical stability tests.

5.5.2. Viscosity: As can be seen in Figure 2, the emulsions presented a decrease in viscosity when submitted to a higher rotation speed, i.e. a higher shear rate. Emulsions that show a decrease in viscosity as the shear rate is increased are classified as pseudoplastic and have a fluid behavior index (n) of less than 1 and greater than 0. Pseudoplastic behavior is suitable in cosmetic formulations for topical application, in which, after shearing, the initial resistance of the flowing emulsion decreases, reflecting in a greater ease of application [32]. The fluid behavior indexes of the emulsions (Table 9) together with Figure 2 confirm that the studied emulsion systems are pseudoplastic. Pseudoplasticity indicates an apparent thixotropy. Thixotropic products become more fluid when subjected to external pressures, spreading more easily in the area where they are applied and recovering their initial viscosity at the time of application preventing the product from dripping [32]. Therefore, a thixotropic behavior is ideal in cosmetic emulsions for topical application. Emulsion viscosity may widely vary depending on its constituents, with more fluid formulations called lotions (oral, topical use), or semisolid formulations denominated creams and ointments (topical use) [33]. Viscosity may be modified by emulsifier type and concentration [32].

Type of emulsion	Fluid behavior index (n)
Non-ionic control	0.45
Non-ionic with HGE	0.41
Non-ionic with HEE	0.57

Table 9: Emulsion consistency index.



DPPH Assay for Analysis of in Vitro Antioxidant Activity of Non-Ionic Formulations

Emulsions were analyzed through the DPPH assay. The results were expressed in percentage of free radical sequestration (%FRS) and in half maximal effective concentration (EC50) and can be seen in Table 10. The HGE-added emulsion presented higher in vitro antioxidant potential and achieved a better performance; therefore, it should be an object of deeper study, mainly through antioxidant activity tests in vivo to confirm that its profile will remain unchanged as a finished cosmetic product.

Samples	*EC50 (mg/mL)	*%FRS
Control emulsion	856.00 ± 0.00872	1.46 ± 0.00872
HGE-added emulsion	78.02 ± 0.00781	16.02 ± 0.00781
HEE-added emulsion	96.96 ± 0.00000	12.89 ± 0.00000

*Mean of triplicates ± standard deviation.

Table 10: EC_{50} values and %FRS of the DPPH assay of non-ionic emulsions.

Conclusions

Under the experimental conditions of this study, HGE denoted greater potential for cosmetic applicability, since it presented a superior performance in the thermogravimetric analysis, total phenolics, and antioxidant activity by the FRAP assay, in addition to presenting a higher concentration of the antioxidant metals Fe and Mn.

All formulations remained stable after being submitted to accelerated stability tests. According to the viscosity analysis, the formulations can be classified as pseudoplastic, that is, they have good fluidity, consequently implying in easier spreading and application by the consumer.

When tested by the DPPH assay, using different HGE concentrations, it was observed that the higher the concentrations of this extract in the formulations, the better the antioxidant activity.

Based on all the obtained results, it can be concluded that both extracts used in this research proved suitable for cosmetic application in emulsion systems, adding sustainable value to the jaboticaba fruit. However, the hydroglycolic extract would be ideal for the type of formulation developed in the present study.

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