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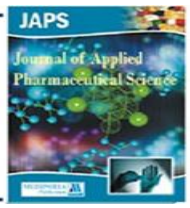

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Development of a Topical Formulation Containing *S. Lutea* Extract: Stability, *In Vitro* Studies and Cutaneous Permeation

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ABSTRACT

Flavonoids have been widely incorporated into cosmetic and dermatological formulations, affording benefits such as antioxidant action, improved skin tone and fewer lines and wrinkles. Brazil has a huge biodiversity, with one of the richest flora in the world, and existing studies justify the quest for greater research efforts in this area. The cajazeira (*Spondias lutea* L.), a plant of the Anacardiaceae family from tropical America, is widely disseminated in Brazil. This plant was chosen because of the presence of flavonoids that exhibit antioxidant activity. The purpose of this research was to develop a stable topical formulation containing *Spondias lutea* extract with the aim of preventing skin diseases caused by UV radiation. Hydro ethanolic extract of *Spondias lutea* fruit was prepared and assayed for its the flavonoids content. The antioxidant activity was estimated by DPPH and superoxide assay. The physicochemical stability and skin permeation of the cream containing 8% (w/w) of extract were assessed. The results showed that the *S. lutea* extract possessed antioxidant activity, and that it is possible to obtain a stable cosmetic containing the extract, which is able to penetrate the skin. Thus, it is possible to use this extract to produce an anti-aging cosmetic.

Keywords: antioxidant, emulsion, cutaneous permeation, quercetin, rutin, *Spondias lutea*.

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INTRODUCTION

Cosmetology has made great progress, both in the development of products of higher quality and efficiency, and in the discovery of new raw materials. Moreover, consumers have become interested in using natural products that are less aggressive than synthetic ones. Thus, the cosmetic industry is seeking alternative products of natural origin and avoiding the use of synthetic ingredients (Packer and Da Luz, 2007). In this context, it is important to search for new active substances with potential cosmetic value. Currently, there is a strong trend in the use of plant raw materials, enabling the development of phytocosmetics. Phytocosmetics are cosmetic products made with natural plant products or raw materials of natural origin (Isaac *et al.*, 2008). The huge Brazilian biodiversity furnishes a wide range of plant secondary metabolites that possess activities sought by the cosmetic industry. The flavonoids are among the secondary metabolites extracted from plants with great cosmetic potential because they have antioxidant, anti-inflammatory, antibacterial and other activities (Packer and Da Luz, 2007; Perruchon, 2002).

In view of the cosmetic potential found in flavonoids and the significant presence of flavonoids in the cajá-manga fruit, the latter was chosen for the development of a new cosmetic. Cajazeira (*Spondias lutea* L. sometimes also referred to as *Spondias mombin* L.) is a tropical American tree that belongs to the family Anacardiaceae. It is widespread in Brazil, especially in the Northeast (Bora *et al.*, 1991; Gomes, 2007; Schultz, 1991). The cajá-manga, fruit of the cajazeira, shows excellent sensorial quality, thus, in the Brazilian Northeast region its pulp is widely used and commercialized (Pinto *et al.*, 2003). The aim of this study was to assess the properties of the *S. lutea* fruit extract and to develop a physically stable cream formulation containing this extract to be applied topically, to prevent the skin damage caused by UV radiation. The skin permeation of flavonoids through cellulose, silicone and skin membranes was also studied.

MATERIALS AND METHODS

Spondias lutea extract

The extract was prepared in 70 ºGL ethanol by percolation of the fresh fruit cut in small pieces, using 200 g of the fruit and 1000 mL of the solvent, which was evaporated after the extraction to leave a dark syrup.

Estimation of phenolic compounds and flavonoids in the *Spondias lutea* extract

The amount of total phenolics in extracts was determined by the Folin-Ciocalteau procedure described by Singleton and Rossi (1965) and Kähkönen *et al.* (1999). A sample of 400 µL of fruit extract (3 mg/mL) was mixed with 2 mL of the Folin-Ciocalteau reagent (tenfold diluted) in a test tube. After 8 minutes, 1.6 mL of sodium carbonate (7.5%) were added to the reaction tube. The absorbance was read in a spectrophotometer (Hitachi U-2001), after 30 minutes, at 765 nm. The results were calculated from an analytical curve of gallic acid, so the total phenolic content was expressed as gram of gallic acid equivalents per 100 g of the *S. lutea* extract. Flavonoid content was analyzed by the method described by Zishen *et al.* (1999). Aliquots of 5 mL of a 6 mg/mL solution of extract were allowed to react with 0.3 mL of 5% $NaNO_2$. After five minutes, 0.6 mL of 10% $AlCl_3$was added. Six minutes later, 2 mL of 1M NaOH was added and the volume completed to 10 mL with distilled water. The absorbance was read at 510 nm in a spectrophotometer. The results were calculated from an analytical curve for quercetin and rutin, allowing the quantity of flavonoids equivalent to quercetin and rutin in the *S. lutea* extract to be estimated. The assays were repeated three times.

Assessment of the antioxidant acitivity of *Spondias lutea* extract

DPPH assay

Aliquots of the hydro-ethanolic extract were added to a DPPH ethanolic solution to a final concentration of 60 µM DPPH. A control tube, without extract but with the same volume of ethanol to substitute the sample, was used to measure the maximum absorbance of the test at λ = 531 nm, corresponding to 100% of free radical in solution. In the presence of the antioxidant test sample, there was a fall in the absorbance compared to the control, proportional to the antioxidant potential and to the concentration added. Therefore, in order to evaluate the antioxidant potential of the *S. lutea* Hydro-ethanolic extract, a range of concentrations of extract were tested. The absorbance of each reaction mixture was evaluated at 531 nm after incubation for 30 minutes in the dark. The percentage of inhibitory activity was calculated as in Cuendet *et al.* (1997). All tests were performed twice.

Superoxide radical assay

In two tubes for each concentration of *S. lutea* extract, the following components were added in this order to the reaction mixture:

1. Buffer (25 mM sodium pyrophosphate solution– sufficient to give a final volume of 1 mL,
2. 25 µL of 372 µM Phenazinemethosulfate (PMS) freshly prepared,
3. 75 µL of 600 µM nitrobluetetrazolium (NBT), freshly prepared,
4. Extract sample (in sufficient volume to each final concentration),
5. 50 µL of 1560 µM NADH.

The solution was mixed and after 90 seconds the reaction was stopped by adding 100 µL of glacial acetic acid, followed by 900 µL of distilled water. After 20 minutes, the absorbance was read in a spectrophotometer at 560 nm (Kakkar and Viswanathan, 1984). Controls were prepared with propylene glycol and without NBT.

Emulsion preparation

To prepare the O/W emulsion (raw material content shown in Table 1) the oily and aqueous phases were heated separately to 75 °C, then the oily phase was stirred into the water phase and the system mixed (Helipath®at 130 r.p.m.) with constant agitation until it had cooled to 30 °C. At this point, 8% of the hydro-ethanolic extract of *S. lutea* was added to the emulsion.

Table. 1: Percent composition of O/W emulsion.

Component	Content (wt%)
Ceteareth-20	3.0
Cetearyl Alcohol	6.0
Dimethicone	1.0
Cetearyl Palmitate	3.0
Isopropyl Myristate	4.0
BHT	0.05
Propyl paraben	0.02
Methyl paraben	0.18
Propylene Glycol	3.0
PEG-75 Lanolin 50%	1.0
Distilled water	70.75
Hydro-ethanolic extract of *S. lutea*	8.0

Assessment of the physicochemical stability of the topical formulation

For a preliminary estimatation of stability, 10 g of the formulation with extract were centrifuged at 3000 rpm for 30 minutes. Samples (three for each condition) were exposed to

thermal stresses (45 ºC and -5 ºC), as stipulated in the ANVISA Stability Guide and Isaac *et al.* (2008). Their organoleptic properties (appearance and smell) and physicochemical characteristics (pH, viscosity, density and active substance contents) were tested on day 0, 1, 7, 15, 30, 60 and 90.

The pH was measured with Micronal BGH 874 digital pH-meter in an aqueous dispersion of 10% of the emulsion (Davis, 1977).

Viscosity was measured in a Visco Star L viscometer, with spindle L4. Samples were subjected to increases and decreases in rotation speedy to identify the appropriate speed for analysis.

Density (d) was measured in a pyknometer (5 mL), the difference between the initial (empty and dry pyknometer) and final (pyknometer with cream) weights being the mass of cream that occupies 5 mL. Thus:

$$d = \frac{W_{final} - W_{pyknometer}}{5}$$

A spectrophotometer (Hitachi U-2001) was used to assay the flavonoid concentration (relative to the extract content) in the O/W emulsion. The analysis was performed at room temperature. Test conditions were: cream solution (5 mg/mL) in isopropanol/chloroform (1:1).

The absorbance at 247 nm was used to determine the concentration of extract in the sample, using the equation for an analytical curve of absorbance *versus* concentration of *S. lutea* extract.

In vitro release and permeation studies

In vitro release and permeation studies were performed in Franz cells of static flow with 3 types of membrane: cellulose and silicone (Sil-Tec 500-2, 0002") membranes, obtained from Technical Products Inc. (Georgia, USA), used to test the ability of the cosmetic to release the extract, and a biological membrane (human skin), used to study the permeation of the extract through human epidermis. This biological tissue came from surgical intervention to reduce the abdomen of a Caucasian woman of 54 years, after ethical approval and informed consent. After excision, the tissue was kept in saline solution at 2-8 °C and then frozen at -20 °C until used. The stratum corneum and viable epidermis were obtained by a warming process (OECD, 2004) in which the tissue was immersed for 45 seconds in a solution of isotonic phosphate-buffered saline (PBS) at pH 7.4 and 60 ± 2 ºC. After this procedure, the epidermis was removed.

In the release and permeation study, the area of the membrane in contact with the donor and receptor phases was approximately 1.0 cm^2. The receptor compartment had a capacity of approximately 5.0 mL. The receptor phase was a mixture of ethanol and propylene glycol (1:1). The system was maintained at 32 °C for about 30 minutes before starting the experiment. Samples of 200 µL were collected at predefined times. After sampling, the same volume was replaced with fresh receptor phase maintained at the same temperature. The tests were performed with 6 cells per formulation. The amount of cream applied to the membrane was about 200 mg (equivalent to 625.6 µg of quercetin and 225.6 µg of rutin). The amount of extract permeated was determined by UV absorption, as described above.

The release and skin permeability data were subjected to kinetic analysis, to establish the extract-release mechanism. The data were fitted to zero-order, first-order and Higuchi model equations.

Statistical analysis

The statistical and graph package Origin 7.0 was used to analyze the data and plot the graphs.

RESULTS AND DISCUSSION

Contents of phenolic compounds and flavonoids in the *S. lutea* extract

Quantities of phenolics and flavonoids in the samples were calculated from analytical curves for gallic acid, quercetin and rutin. The results are shown in Table 2. This flavonoids content expressed in rutin equivalents was similar to results obtained by Dong *et al.* (2009), who studied the flavonoids content of wild *Lycium barbarum* L. Meda *et al*. (2005)studied the phenolics and flavonoids contents in honey, expressing the results in gallic acid and quercetin equivalents respectively, as in this study. Among the twenty seven samples of honey used in that study, the one with the highest phenolics content had 114.75 ± 1.30 mg GAE/ 100 g, or approximately 13 times less than the content found in the *Spondias lutea* extract. Similarly, the best flavonoids content was 468 times less than in the *Spondias lutea* extract. Plants or natural compounds that contain flavonoids may be good sources of anti-radiation food products or anti-UV cosmetics (Dong *et al*., 2009). Another interesting result is that we found a larger number of quercetin equivalents of flavonoids than of gallic acid equivalents of total phenolics. This is a surprising result, since the flavonoids content should be less than the total phenolics content, given that the latter includes the former as flavonoids are a class of phenolic compounds. This anomaly should be caused by the calibration curves which are probably unsuitable to quantify either the phenolic compounds or the quercetin equivalents of flavonoids. To verify this hypothesis, it would be necessary to make detailed evaluations, using High Performance Liquid Chromatography (HPLC) to identify the phenolic compounds, including the flavonoids that are present in *Spondias lutea*.

Table. 2: Amount of phenolic compounds and flavonoids in the *S. lutea* extract (n=3 ± SDev).

	Equivalent to	Amount (g/100 g of extract)
Phenolic compounds	Gallic acid	1.55 ±0.083
Flavonoids	Quercetin	3.91 ±0.017
	Rutin	1.41 ±0.004

Antioxidant activity in the *Spondias lutea* extract

DPPH assay

The extract showed a great capacity to scavenge the DPPH radical, as shown in Figure 1, exhibiting an IC_{50} of 21.30 mg/mL. This property may indicate that the *S. lutea* extract affords protection against *in vivo* damage mediated by free radicals (Chen

et al., 2005). The IC_{50} value of the *S. lutea* extract is similar to results obtained for the honey samples, the best of which had and IC_{50} of 29.13 ± 1.50 mg/mL (Meda*et al.*, 2005).

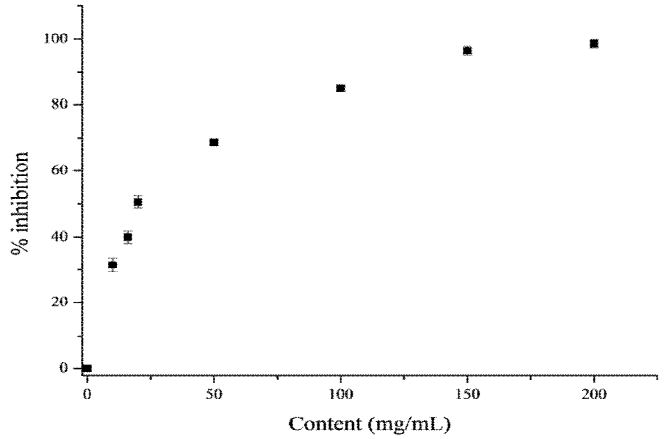

Fig. 1: Inhibition profile of DPPH radical by *S. lutea* hydro ethanolic extract.

Superoxide radical assay

The antioxidant activity *in vitro*, assayed with the superoxide radical, although less intense than that observed with DPPH, was also significant, reaching 45% inhibition of the free radical at the highest tested concentration (Figure 2).

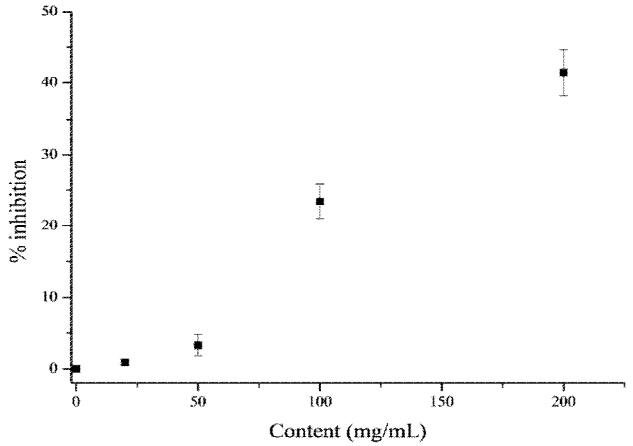

Fig. 2: Inhibition profile of superoxide radical by *S. lutea* hydro ethanolic extract.

However, these two analyses methods, taken together, show with greater certainty the effective activity of the *S. lutea* extract (Cervellati *et al.*, 2002; Ghiselli *et al.*, 1995; Proteggente *et al.*, 2002; Schlesier *et al.*, 2002).

Many secondary metabolites produced by plants can be used for different applications in nutrition, health and cosmetology (Curtis *et al.*, 2009; González-Molina *et al.*, 2010; Guimarães *et al.*, 2010). Thus, the presence of these metabolites in the *S. lutea* extract indicates the cosmetic potential of this fruit.The topical application of antioxidant plant extracts (green and black tea, carotenoids, coffee and many flavonoids from fruits and vegetables) can protect skin from UV-induced erythema, early aging and irradiation-induced cancer (Reuter *et al.*, 2010).

Physicochemical stability of the topical formulation

Analytical curve

The analytical curve constructed showed perfect correlation between concentration and absorbance, according to the Lambert-Beer Law. This curve is described by the equation: y=0.0673x + 0.0304 (r=0.9999).

Centrifugation test

The emulsion containing *S. lutea* extract maintained its homogeneity, with no phase separation, even after centrifugation at 3000 rpm for 30 minutes. This indicates stability of this product.

Accelerated stability

All tests were performed in triplicate. In Table 3 are the mean results measured. The pH data showed that this emulsion was stable and suitable for skin application (pH 3.79-3.94). The density did not vary significantly.

Viscosity was also stable over the study period. A slight increase in viscosity was observed in the samples subjected to low temperature (-5 ºC). Macroscopic organoleptic characteristics were stable under the experimental conditions.

The flavonoid content decreased significantly during the test period in the samples stored at -5 ºC (from 0.060 to 0.044 mg/mL) and at 45 ºC (from 0.060 to 0.036 mg/mL). The results in Table 3 show the degradation profile of this active component incorporated into the cream and provide important data for the establishment of the shelf-life of this product (ANVISA).

The gradual fall in flavonoid concentration was less rapid in cream subjected to the low temperature. This shows the different performance of the product kept in different thermal conditions, confirming that the degradation is lower when the cream is stored at lower temperatures. This decrease in the active concentration could be counteracted by a high initial concentration of the active principle in the cream, thus ensuring that even with a marked reduction in this concentration during storage, the consumer will still have a sufficiently active concentration for product efficacy.

Table. 3: Values of the properties tested during the accelerated stability test.

Time (days)	pH		Viscosity (mPa.s)		Flavonoid contents (mg/mL)		Density (g/mL)		Macroscopic organoleptic characteristics	
	-5 ºC	45 ºC	-5 ºC	45 ºC	-5 ºC	45 ºC	-5 ºC	45 ºC	-5 ºC	45 ºC
0	3.82	3.82	22880	22880	0.060	0.060	1.71	1.71	Normal	
1	3.82	3.83	24680	22850	0.061	0.060	1.69	1.71	Normal	
7	3.94	3.78	33270	21360	0.061	0.058	1.71	1.71	Normal	
15	3.98	3.79	42820	19250	0.056	0.051	1.71	1.71	Normal	
30	3.99	3.79	35220	20370	0.049	0.040	1.71	1.71	Normal	
60	3.87	3.47	26866	28123	0.049	0.039	1.71	1.71	Normal	
90	3.79	3.47	28770	22960	0.044	0.036	1.71	1.71	Normal	

In vitro release and permeation studies

The results showed that the formulation used was able to release the extract, while the *S. lutea* extract permeated all membranes, though the permeation profiles differed among the different membranes used (Figures 3, 4 and 5).

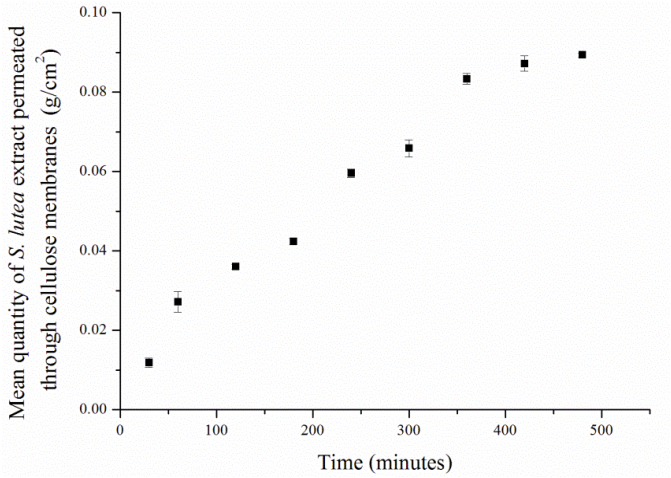

Fig. 3: Permeation of *S. lutea* extract through cellulose membranes (n=6).

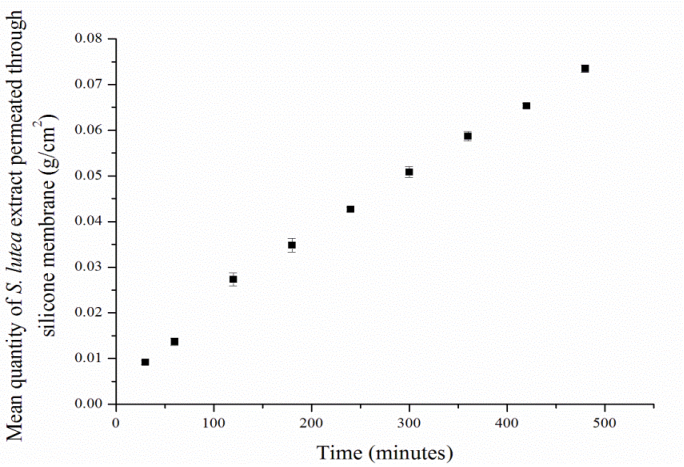

Fig. 4: Permeation of *S. lutea* extract through silicone membranes (n=6).

As expected the cellulose membrane allowed the highest rate of permeation by the flavonoids. In this membrane, the permeation tends to reach a steady state after 400 min. In silicone membranes, this state is not reached after 480 min.

The amount of *S. lutea* extract permeated through human epidermis was significantly lower than through the other membranes, showing the action of the s*tratum corneum*, which is a hydrophobic layer interfering with the permeation of the relatively hydrophilic *S. lutea* extract. A method that can be used to increase the permeation rate of this extract through skin is to use another solvent system (Levang *et al.*, 1999).

The data obtained in the *in vitro* permeation studies were used to fit mathematical models for the kinetics of the various membranes. Three models were assessed: the zero-order model, which represents a linear relation between the amount of permeated extract and time; the Higuchi model, where linearity is observed between the amount permeated and the square root of time, and the first-order model, where linearity is assumed between the log of the amount of extract permeated and time.

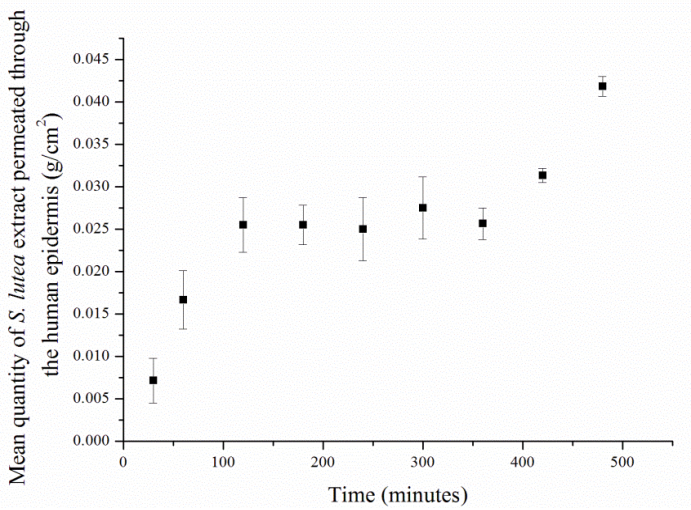

Fig. 5: Permeation of *S. lutea* extract through human epidermis (n=6).

Table 4 shows the correlation coefficient found for each kinetic model fitted to each of the membranes, and Table 5 shows the equation obtained by fitting each of these models to the data. From these equations, it is possible to determine the release rate of the system, equal to the slope of the regression line.

Table. 4: Correlation coefficient (r^2) observed for each membrane when zero-order, first-order and Higuchi models were fitted to the permeation data.

	Zero-order	Higuchi model	First-order
Cellulose membrane	0.96896	0.97671	0.84479
Silicone membrane	0.99212	0.99118	0.87691
Human epidermis	0.76752	**0.80292**	0.63951

Table. 5: Equations obtained by fitting zero-order, first-order and Higuchi models to the permeation data.

	Zero order	Higuchi model	First order
Cellulose membrane	y = 0.000174 x + 0.0135	y = 0.00486 x – 0.0154	y = 0.00167 x – 1.73
Silicone membrane	y = 0.000141 x + 0.0075	y = 0.00392 x – 0.0156	y = 0.00184 x – 1.906
Human epidermis	y = 0.0000522 x + 0.0124	y = 0.000149 x + 0.00334	y = 0.00109 x – 1.90

* For y = ax + b, a indicates J in g/cm^2 per minute; x = time (min); y = weight permeated per area (g/cm^2)

The amount of extract that permeated the membranes was a linear function of the square root of time, which indicates that the systems delivered extract according to the Higuchi model, controlled by diffusion. However, in the case of silicone membrane, showed the correlation coefficient was greatest for the linear relation between the amount of extract released and time, which describes the zero-order model.

In this study a finite dose was applied and no steady-state flux was observed.

The results obtained may be due to the lipophilic nature of the membranes used, in contrast to the continuous phase of the emulsion (aqueous phase) and to the polarity of the *Spondias lutea* extract (hydrophilic).This has been mentioned earlier (Dias *et al.*, 2007a; Dias *et al.*, 2007b).

CONCLUSION

The *S. lutea* extract contains phenolic compounds, including flavonoids. It possesses antioxidant activity, confirmed by the DPPH assay. The formulation developed as a carrier for this active principle was found to be stable. Thus, the results indicate that is possible to use the extract of *Spondias lutea* as an antioxidant for skin care products, such as an anti-aging O/W emulsified non-ionic system, which is able to permeate the skin in small concentrations. Such a cream could provide a satisfactory effect if applied to the skin frequently.

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