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Article

In vitro evaluation of copaiba oil as a kojic acid skin enhancer

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The capacity of copaíba oil to act as a skin penetration enhancer for the depigmenting agent kojic acid was evaluated using an *in vitro* diffusion system with static flux and shed rattlesnake skin membrane, *Crotalus durissus terrificus,* in saline solution at 34±2 °C as the fluid receptor. The quantities of kojic acid liberated into the fluid receptor were determined by spectrophotometry at 268 nm with intervals of one and a half hours. The membranes, pretreated with copaíba oil at 25% and 50% v/v, gave flux values of 8.0 and 12.7 µg/cm^2/h, permeability values of 2.0 and 3.3 cm×10^{-4}/h, and promotion factors of 4.1 and 3.7, respectively. These results indicate that copaíba oil, at the two concentrations studied, has the capacity to promote penetration of kojic acid.

Uniterms: Copaíba oil. Kojic acid. Skin penetration/ *in vitro* studies .UV Spectrophotometry.

A propriedade do óleo de copaíba como agente promotor de penetração cutânea do despigmentante ácido kójico foi avaliada utilizando-se sistema de difusão *in vitro* com fluxo estático, membrana de pele da serpente cascavel - *Crotalus durissus terrificus* e solução salina a 34±2 °C como fluido receptor. As quantidades liberadas do ácido kójico no fluido receptor foram determinadas por espectrofotometria em 268 nm em intervalos de 1:30 h. As membranas pré-tratadas com óleo de copaíba a 25 e 50% v/v apresentaram valores de fluxo de 8,0 e 12,7 µg/cm^2/h, permeabilidade de 2,0 e 3,3 cm×10^{-4}/h, e fatores de promoção de 4,1 e 3,7, respectivamente. Os resultados obtidos indicaram que o óleo de copaíba, nas duas concentrações estudadas, apresentou capacidade de promoção da penetração do ácido kójico.

Unitermos: Óleo de copaíba. Ácido kójico. Penetração cutânea/ *in vitro*. Espectrofotometria UV.

INTRODUCTION

Hyper pigmentation of the skin can be caused by a range of factors such as aging, pregnancy, endocrine disturbances, hormonal treatment and sun exposure to varying degrees. Several substances are commonly employed as depigmentation agents in the manufacture of cosmetics used for the reduction of hyper pigmentation, one of these being kojic acid (Cabanes, Garcia-Carmona, 1994; Su, 1999; Nicoletti *et al.*, 2002). Kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pirone) is a depigmenting agent obtained from rice fermentation (Burdock *et al.*, 2001) as a fungus metabolite from the genera *Aspergillus* and *Penicillym,* and acts by inhibiting tyrosinase activity (Cabanes, Garcia-Carmona 1994).

In order to be effective, depigmenting agents incorporated in topical formulas must cross the stratum corneum to act on the more inner layers situated towards the basal lamina of the epidermis. To this end, the addition of other compounds with a greater capacity for skin penetration, also known as absorption promoters or "enhancers", can result in an increase in diffusion of substances by disorganizing the lamellas of the stratum corneum (Williams, Barry, 2004). The incorporation of these substances in formulations allows for the development of topical products with a lower concentration of active ingredients, thereby increasing both the efficacy and safety of the product (Yourik, Bronaugh, 1999).

Various species of Brazilian flora such as copaiba are being investigated for their industrial potential in the formulation of cosmetics and medications (Veiga Junior *et al*., 2007; Stupp *et al.*, 2008). Copaibas are frequently cited

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as a source of ingredients possessing anti inflammatory, anti infectious, anti tumoral and wound healing properties, among others (Veiga Junior, Pinto, 2002). Copaibas are trees that are native to the tropical regions of Latin America and Western Africa, belonging to the family Leguminosae Juss, sub-family Caesalpinoideae Kunth. These trees furnish an oily substrate consisting of 40 to 50% essential oil and 40 to 60% resin, whose composition is made up principally of esters and terpenes (Cascon, Gilbert, 2000; Pinto *et al.,* 2000; Biavati *et al*., 2006).

Copaiba oil, given its terpine compounds such as sesquiterpenes, oxygenated sesquiterpenes, hydrocarbon diterpenes and oxygenated diterpenes (Pinto *et al.,* 2000) is a good choice as a permeability promoter for other compounds. Terpenic compounds are well known for their ability to increase cutaneous penetration (El-Kattan *et al*., 2001; Narishetty, Panchagnula, 2004).

In vivo and *in vitro* (Franz *et al.*, 1993*)* diffusion methods are used to study the efficacy of skin penetrating agents. For *in vitro* methods, diffusion cells or the Franz method are used where, *i*n the literature, many studies have used diffusion cells with membranes from the skin of rats, mice, guinea pigs, shed snake skin, human or synthetic skin to assess the penetration promoting powers of different substances (Franz *et al.*., 1993; Bronaugh, Collier, 1993). Different experimental conditions involving modifications of temperature, agitation, different pretreatment temperatures, collection of samples and trial duration have been described (Akimoto, Nagase, 2003; Andega *et al*., 2001; Babu, Pandit, 2004; Cotte *et al*., 2004; El-Kattan *et al.*, 2001).

The use of shed snake skin as a membrane in *in vitro* systems is justified due to the similarities between shed snake skin and the stratum corneum of human skin, in terms of structure, lipid composition, water permeability, and other substances with different functional groups. (Itoh *et al*., 1990). Additionally, shed snake skin has advantages in terms of low cost, ease of use, absence of hair, and lack of need to sacrifice animals. (Lin *et al.,* 1992; Haigh *et al.*,1998; Chang, Zheng, 2003).

For the above reasons and due to the need to investigate properties of vegetable compounds, the aim of this study was to evaluate *in vitro,* copaiba oil as a penetrating agent for kojic acid in a shed snake skin model through diffusion studies in an effort to develop more effective topical depigmenting agents.

MATERIAL AND METHODS

Material

Substances used were AP grade, kojic acid was 90% purity and the solutions were prepared using freshly distilled water. Copaiba oil was of pharmaceutical purity and was kindly donated by Ionquímica. The membranes used as diffusion cells were obtained from the shed skin of the rattle snake *Crotalus durissus terrificus*.

Methods

Preparation of membranes

Square or rectangular sections measuring about 4 cm per side were cut from the ventral part of the shed snake skin, containing four sections of rigid scales and three flexible sections. These were placed in distilled water at room temperature for 48 hours to hydrate. The membranes were then dried on absorbent paper and placed in a receptacle with an external diameter of about 3.7 cm, and an area available for diffusion of 3.14 cm^2.

Quantification of kojic acid in receptor fluid

Quantification of the kojic acid was done by spectrophotometry (Beckman-Coulter® DU-640) at 268 nm (Oliveira *et al*., 2007) using a method adapted from Majmudar and collaborators (1998). This method gave a quantification limit of 0.17 µg/mL and a detection of 0.057 µg/mL according to Oliveira and collaborators (2007).

Readings were taken from 0.5 mL aliquots of the fluid receptor after dilution up to10 mL with saline solution (sodium chloride 0.9% p/v) and filtration through a 0.45 µm pore membrane.

Diffusion Assays

The (Logan® SDFC-6 VTC-200) *in vitro* cell diffusion system was used, composed of three vertical diffusion cells with donation compartments of 2 cm diameter, receptor compartments with a capacity of 15.0, 15.1 and 15.2 mL, a support stand, and a magnetic agitation system equipped with a pump system and water heater.

The circulating water temperature was adjusted to 34±2 °C and the fluid receptor (saline solution) was maintained under agitation at 100 rpm.

Diffusion studies were carried out by applying saturated solutions of kojic acid with pretreatment of the membranes using oil solutions, and then quantifying the amount of kojic acid in the samples, extracted at different time intervals. (Colipa, 2000). Installed membranes were treated with 0.8 mL of saline solution initially and after 30 minutes were removed with the help of cotton. In the first cell, another 0.8 mL of saline solution was added and in the second cell, 0.8 mL of copaiba oil at 25% dilution in isopropyl – propylene glycol 95:5 v/v alcohol, and in the third, 0.8 mL of copaiba oil diluted in 50% isopropyl – pro-

pylene glycol 95:5 v/v. alcohol. After one hour, the solutions were removed with cotton and the membranes washed with distilled water. The first cell of the receptor compartment containing 15.1 mL, received 0.8 mL of saline solution (white) while the second (15.0 mL) and third (15.2 mL) both received 0.8 mL of kojic acid 3.9% p/v equivalent to 31.2 mg of kojic acid. The experiment was run for 22 h 30 min, with removal of aliquots and replacement of the volumes of the receptor compartments at times points 0; 1:30; 3:00; 4:30; 6:00; 7:30; 9:00; 10:30; 12:00; 13:30 and 22:30 hours. Concentrations for kojic acid were determined spectrophotometrically and these values used to calculate the diffusion parameters. Four replicates of each experiment were done; however, the first determinations of kojic acid gave results lower than the quantification limits due to the heterogeneity of the shed snake skin membrane, and therefore these first results were omitted for the calculation of the mean values.

The mean quantity of kojic acid by area was obtained using the mean circular membrane values and the kojic acid concentration. Concentration of kojic acid was obtained from the mean values of absorbency. Kojic acid liberation curves by membrane area (cm^2) against hours of treatment were constructed (Asbill, Michniak, 2000).

Flux ($\mu g/cm^2/h$) was considered equal to the inclination coefficient of those lines with a linear correlation coefficient that yielded values greater than 0.9.

Permeability, which was equivalent to the depth of the membrane the substance managed to penetrate (cm) against time (h), was calculated by dividing the flux values ($\mu g/cm^2/h$) by the kojic acid solution concentration (39 mg/cm^3) applied to the membrane (Asbill, Michniak, 2000).

The latency time (hours), indicating the moment the membrane allowed Constant liberation of kojic acid, was extrapolated from the point where the regression lines crossed the axis of the abscissa (Barry, 1987).

To compare the effect on the oil solutions and their controls, a "promotion factor" calculated based on the mean flux ($\mu g/cm^2/h$) of kojic acid in the sample divided by the mean flux of the control (without pretreatment with oil) (Bronaugh, Collier, 1993).

Statistical Analysis

Fisher's and Student's t tests were used to compare the data, means and standard deviations (Bussab, Morettin, 1985; Botter *et al.*, 1996).

RESULTS AND DISCUSSION

The quantification of kojic acid began only from the three hour time interval in membranes treated with both 25 and 50% solutions of copaiba oil giving mean liberation values per area of 20.6 and 27.4 $\mu g/cm^2$, respectively (see Table I). Prior to this three hour time point, the kojic acid concentration did not reach the quantification limit for the method employed (Oliveira *et al*., 2007). The solution of kojic acid 3.9% p/v used in these penetration studies, with a pH around 4.0, favored the non ionized form of kojic acid, and allowing its penetration into the membranes.

Compared with the controls, membranes pretreated with oil solutions of 25 and 50% allowed better diffusion of kojic acid from the seven and one half hour time point, and after 12 hours the amounts of kojic acid liberated were 80.2 $\mu g/cm^2$ and 135.2$\mu g/cm^2$, respectively (Table I). The lines associated to these oil solutions present greater inclination indicating a larger angular coefficient, and thus a higher flux (Figures 1 and 2). Figures 1 and 2 also show variable coefficients of correlation which can be explained by the irregular thickness and tortuosity inherent to the snake membrane (Ostrenga *et al.,* 1974). The characteristics of animal membranes also influence the values of the variation coefficients. Lopes (1999), in his study of diffusion with sodium diclophenate on treatment with 0.2% papain solution in human membranes, found a coefficient of variation of up to 73% in the liberated amounts. Sato and colleagues (2007) also observed high standard deviations in their studies assessing kojic acid formula penetration in porcine skin membrane.

Based on the regression lines in the diffusion curves (Figures 1 and 2), the flux parameters, permeability parameters and latency time were extrapolated, as shown in Table II. The table demonstrates that latency times for the constant liberation of kojic acid were reduced with increased copaiba oil concentration. This represents a beneficial characteristic for depigmentation agents. The promotion factors for liberation of kojic acid obtained from treatment with 25% and 50% copaiba solutions v/v were equivalent to 4.0 and 3.7, values greater than those obtained from control solutions.

Analysis of the mean flux values shows that a 50% distilled water solution may provide improved shed snake skin membrane hydration allowing for greater penetration of kojic acid. Copaiba oil was diluted in isopropyl alcohol associated with propylene glycol (95:5) v/v, in a bid to ascertain the most powerful solvent, combined with preservation of integrity and humidification of the membrane and thus less interference in diffusion results owing to liberation of contaminants. Propylene glycol usually acts as a penetration promoter for substances and was therefore included in the controls (Larrucea *et al*., 2001). However, this same effect was not confirmed in the present study using snake skin membrane. Comparisons of kojic acid

TABLE I - Kojic acid liberation in diffusion studies using shed snake skin membrane treated with 25 or 50% v/v copaiba oil solutions (COS) or 25 or 50% distilled water (DW) in isopropyl-propylene glycol (95:5 v/v) alcohol after application of kojic acid solution 3.9% p/v

Time (hours)	Pre-treatment	Quantity liberated by area (μg/cm²)					
		Copaíba 25%			Copaíba 50%		
		n	Mean	SD	N	Mean	SD
3:00	DW	-	---	---	-	---	---
	COS	1	20.6	---	2	27.4	3.1
4:30	DW	-	---	---	-	---	---
	COS	1	28.3	---	3	44.2	18.8
6:00	DW	-	---	---	2	18.4	0.8
	COS	1	41.3	---	4	55.7	32.7
7:30	DW	-	---	---	2	22.4	1.0
	COS	3	31.2	21.2	4	71.9	38.1
9:00	DW	3	19.0	2.0	3	26.2	5.9
	COS	4	39.9	24.6	4	87.8	39.1
10:30	DW	3	22.9	3.6	4	28.3	7.2
	COS	4	63.8	43.7	4	118.9	58.4
12:00	DW	3	27.2	1.7	4	34.8	9.9
	COS	4	80.2	53.8	4	135.2	61.6
13:30	DW	-	---	---	4	45.9	12.3
	COS	-	---	---	4	161.9	74.5
22:30	DW	4	46.2	13.4	-	---	---
	COS	4	172.6	27.6	-	---	---

COS - Copaiba Oil solution; DW - Distilled Water solution as control; SD - standard deviation ; ---not calculated ; n – number of replicates

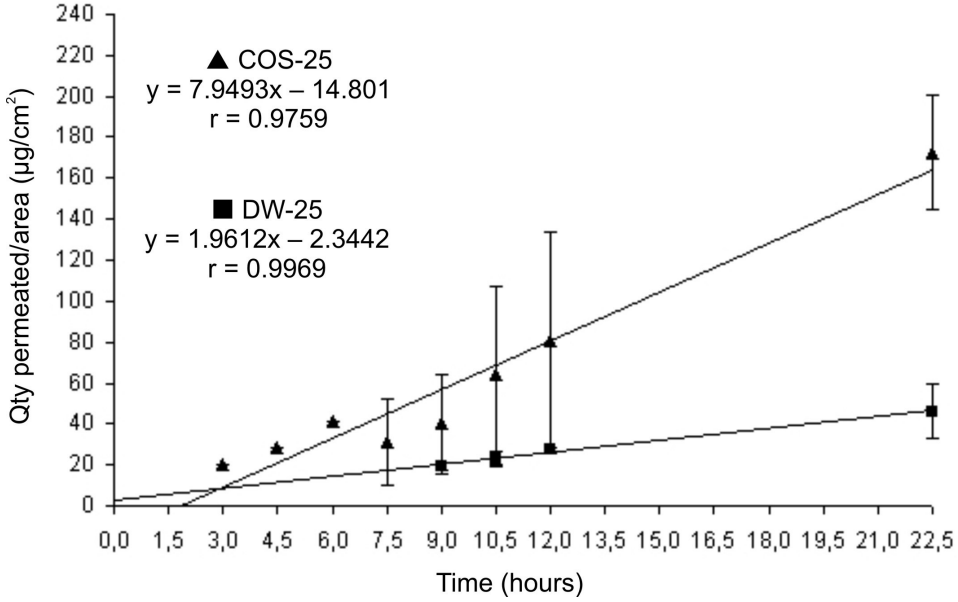

FIGURE 1 - Cutaneous diffusion profile *in vitro* of kojic acid in shed snake skin membrane treated with 25% copaiba oil in isopropyl-propylene glycol (95:5 v/v) alcohol solution (COS-25) or 25% distilled water in isopropyl-propylene glycol (95:5 v/v) alcohol solution (DW-25) and treated with kojic acid solution 3.9% p/v.

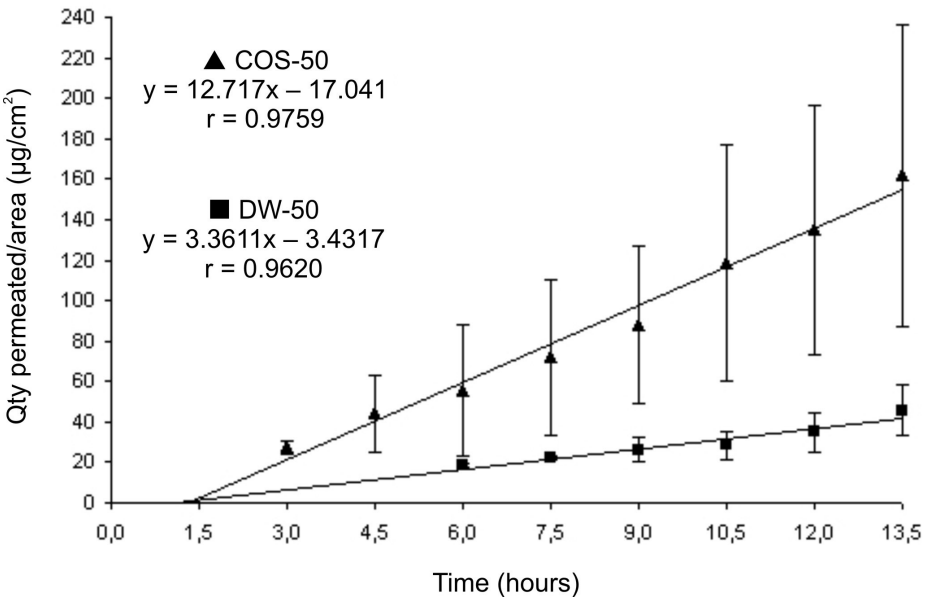

FIGURE 2 - Cutaneous diffusion profile *in vitro* of kojic acid in shed snake skin membrane treated with 50% copaiba oil solution in isopropyl-propylene glycol (95:5 v/v) alcohol solution (COS-50) or in 50% distilled water solution in isopropyl-propylene glycol (95:5 v/v) alcohol solution (DW-50) and treated with kojic acid solution 3.9% p/v.

TABLE II - Latency time (hours), flux (µg/cm^2/h) and permeability (cm/h) of kojic acid in diffusion studies of shed snake skin membrane treated with 25 or 50% copaiba oil solution in isopropyl-propylene glycol (95:5 v/v) alcohol (COS-25) or in 25 or 50% distilled water (DW) in isopropyl-propylene glycol (95:5 v/v) alcohol solution (DW-25) and treated with kojic acid solution 3.9% p/v

Treatment	Latency time (hours)	Mean Flux (µg/cm^2/h)	Mean Permeability (cm x 10^{-4}/h)
DW-**25**	-	2.0	0.5
COS-**25**	1:50	8.0	2.0
DW-**50**	1:20	3.4	0.9
COS-**50**	1:00	12.7	3.3

COS - copaiba oil solution; DW – distilled water control; SD - standard deviation.

flux values from treatment studies versus those of controls revealed a decrease in these values at higher isopropyl alcohol concentrations.

Standard deviations were calculated from the means of the flux and permeability values extrapolated on the individual graphs of each replicate experiment, and for each copaíba oil concentration (Table III). Fisher's and Student's t values were obtained from these values and are displayed in Table IV.

Student's t test revealed a significant difference in the mean flux values of kojic acid at the different copaiba oil concentrations on diffusion testing compared to the respective distilled water solutions. However, studies using 25 and 50% v/v solutions of copaiba oil showed no significant differences in the mean values of these parameters when compared with each other. These observations agree with the analysis of promotion factors which yielded similar values (3.7 and 4.0). Analysis using Fisher's test in these experiments found a significant difference in mean flux values, probably due to the shed snake skin membrane characteristic. Fisher's test also showed that the studies done with 25 and 50% distilled water in the treatment of snake skin membrane were homogeneous across all the experiments.

Comparing Fisher results for each experiment versus its respective control, only the 50% p/v copaíba oil solution produced a statistically significant variation for kojic acid flux. This shows that although test solutions and their controls were submitted to the same treatment, they did not show the same pattern of variation. This is largely due to irregularities in the snake skin membrane.

Shed snake skin was used as a membrane to facilitate the penetration of the test substances compared to the use of extracts of stratum corneum isolated from the skins, as

TABLE III - Mean, standard deviation, variance and coefficient of variance for mean flux and mean permeability (μg/cm²/h and cm/h) of kojic acid from diffusion studies on shed snake skin membrane treated with 25 or 50% copaiba oil in isopropyl-propylene glycol (95:5 v/v) alcohol solution (COS-25 or COS-50) or in 25 or 50% distilled water in isopropyl-propylene glycol (95:5 v/v) alcohol solution (DW-25 or DW-50%) and treated with kojic acid solution 3.9% p/v

Experiment	Pre-treatment	*N*	Flux (μg/cm²/h)			Permeability (cm x 10⁻⁴/h)			CV%
			Mean	SD	S^2	Mean	SD	S^2	
1	DW-**25**	3	2.369	0.979	0.959	0.607	0.251	0.063	41.34
	COS-**25**	4	9.855	0.752	0.566	2.527	0.193	0.037	7.63
2	DW-**50**	4	4.665	0.953	0.909	1.196	0.244	0.060	20.43
	COS-**50**	4	13.911	5.383	28.98	3.567	1.380	1.904	38.70

CV% - coefficient of variation ; SD-standard deviation ; S^2 - variance; n- number of replicates

TABLE IV - Fisher's and Student's t test values for mean flux (μg/cm²/h) of kojic acid in diffusion membrane studies using shed snake skin treated with 25 or 50% copaiba oil in isopropyl –propylene glycol (95:5 v/v)alcohol solution (COS-25 or COS-50) or in 25 or 50% distilled water in isopropyl –propylene glycol (95:5 v/v) alcohol solution (DW-25 or DW-50%) and treated with kojic acid solution 3.9% p/v

Experiments Compared	*F* (Fisher's)			*t* (Student's)		
	DF	Tabled	Calculated	DF	Tabled	Calculated
COS-25 and DW-25	2 and 3	9.55	1.70	5	2.571	11.525*
COS-50 and DW-50	3 and 3	9.28	31.89*	6	2.447	3.383*
COS-25 and COS-50	3 and 3	9.28	51.21*	6	2.447	1.493
DW-25 and DW-50	2 and 3	9.55	0.95	5	2.571	3.119*

α = 0.05; * statistically significant difference; DF- degrees of freedom

demonstrated in the study by Lin and colleagues (1992), which found permeability values 2 to 4 times higher in shed snake skin (*Phyton molurus)* than in isolated stratum corneum for sodium diclophenate, theophyline and benzoic acid (2 mg/mL or 0.2% in aqueous solution). The use of shed snake skin in a study of copaiba oil as a promoter of skin penetration for hydrophilic substances such as kojic acid, takes into consideration the fact that these membranes have a lower permeability coefficient (3.3 to 6.1 times) for these compounds, extending the time required to carry out the experiments. With regard to lipophilic compounds, these have a permeability coefficient close those of membranes obtained from human skin (0.9 to 1.8 times) (3.3 to 6.1 times) (Ngawhirunpat *et al.*, 2006).

The percentage penetration of kojic acid for the initial amount applied was 1.63% for the 50% solution of copaiba oil after an interval of 13 and a half hours versus 1.74% for the 25 % solution of copaiba oil after a 22 and a half hour interval. These findings indicate that the 50% oil solution allowed almost the same level of kojic acid penetration but in a much shorter time frame. This indicated that a 50% concentration of copaiba oil may be optimal for developing topical formulations containing kojic acid.

CONCLUSION

Copaiba oil in both the 25 and 50% v/v solutions studied proved to be a penetration promotion factor of kojic acid in 3.9%v/v solution in the shed snake skin membranes *Crotalus durissus terrificus* compared to controls. The promotion factors were 4.0 and 3.7 for 25% and 50% copaiba oil solutions, respectively. These results allowed us to conclude that this oil has the potential to be added to topical formulations as a penetration promoter for active hydrophilic substances.

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