Carapa guianensis Aublet oil: potentiation of anti-inflammatory effects in mice skin by emulsion formulation

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ABSTRACT: *Carapa guianensis* Aublet is an evergreen typical Amazonian tree and the oil obtained from its seeds (OCg) are widely used in folk medicine of many countries in order to treat different inflammatory conditions. Therefore, the emulsions of *Carapa guianensis* oil (EOCg) were developed and their stability determined after storage under different conditions, showing stability and the rheological measurements showed a non-Newtonian flow behavior of the pseudoplastic type and thixotropy. Analyzes of the anti-inflammatory action on the skin of mice after administration of carrageenan indicated intense leukocyte migration and degranulation of the mast cells, and the experimental groups treated whith OCg, EOCg e EDexa (emulsion of silicone oil with dexamethasone as standard drug) showed a reduction of leukocyte migration and mast cell degranulation. The annexin-A1 (AnxA1) protein was expressed in leukocytes, but without any statistical significance. The pro-inflammatory cytokines TNF-α, IL-1β and IL-6 were significantly increased in the inflammed groups and reduced in the treated groups and the anti-inflammatory cytokine IL-10 was significantly increased in the treated groups. This experimental inflammation model showed a putative anti-inflammatory activity of *Carapa guianensis* oil through the suppression of inflammatory cytokines and induction of IL-10. These results suggest that *C. guianensis* oil can be used on the skin as a potent anti-inflammatory agent.

Keywords: *Carapa guianensis* oil; emulsion; skin inflammation; annexin-A1.

RESUMO: Efeitos anti-inflamatórios da emulsão do óleo de *Carapa guianensis* Aublet na pele de camundongos. *Carapa guianensis* Aublet é uma árvore típica da Amazônia e o óleo obtido a partir de suas sementes (O*Cg*) é conhecido popularmente pela sua atividade anti-inflamatória. No presente trabalho, foram desenvolvidas emulsões do óleo de *Carapa guianensis* (EO*Cg*) e determinada a sua estabilidade após armazenamento em diferentes condições, onde verificou-se que são estáveis e as medidas reológicas apresentaram comportamento de fluxo não-Newtoniano do tipo pseudoplástico e tixotropia. As análises da ação anti-inflamatória na pele de camundongos, após administração de carragenina, indicaram intensa migração de leucócitos e desgranulação dos mastócitos. Os grupos tratados com o OCg, EOCg e EDexa (emulsão de óleo de silicone com dexametasona como padrão) mostraram uma diminuição na migração de leucócitos e desgranulação de mastócitos. A proteína anexina-A1 (AnxA1) foi expressa nos leucócitos, mas sem significância estatística. As citocinas pró-inflamatórias TNF-α, IL-1β e IL-6 foram significantemente elevadas nos grupos inflamados e reduzidas nos grupos tratados. A citocina anti-inflamatória IL-10 aumentou significantemente nos grupos tratados. O modelo experimental de inflamação mostrou possíveis atividades anti-inflamatórias do óleo de *C. guianensis* através da supressão de citocinas inflamatórias e da indução de IL-10. Estes resultados sugerem que o óleo de *C. guianensis* pode ser utilizado na pele como um potente agente anti-inflamatório.

Palavras-chave: Óleo de *Carapa guianensis*; emulsão; inflamação de pele; Anexina A1.

INTRODUCTION

Carapa guianensis Aublet is an evergreen or deciduous tree that belongs to the Meliacea family. It is popularly known in Brazil as “Andiroba” and may grow up to 60 m. Concerning the use by traditional communities in the Amazon, its oil shows insect repellency and larvicidal effect (Miot et al., 2004; Silva et al., 2004; Mendonça et al., 2005; Silva et al.,

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2006). Furthermore, it can be used as febrifuge, anti-bacterial, anti-parasitic and anti-inflammatory remedy (Penido et al., 2006a, BRASIL, 2015). Due to its large use, therapeutic interest and the reproductive safety of this oil (Costa-Silva et al., 2006, 2007, 2008). it has been recommended by the Developmental Program of United Nations, and it is also listed by the National Relation of Medicinal Plants of Interest from the Brazilian National Health System (Sistema Único de Saúde/SUS-Brazil) (Hammer & Johns, 1993; BRASIL, 2009). The andiroba oil (CgO) is a source of fatty acids, such as oleic, palmitic, stearic and linoleic acids. The non-saponifiable part of the oil, between 2 and 5%, basically consists of a rich fraction containing limonoids. The compounds methyl angolensate, 7-deacetoxy-7-oxogedunin, deacetylgedunin, 6α-acetoxygedunin, gedunin, andirobin, 17β-hydroxyazadiradione, 1,2-dihydro-3β-hydroxy-7-deacetoxy-7-oxogedunin and xyloccensin were previously isolated from *C. guianensis* by various chromatographic techniques (Ambrozin et al., 2006; Silva, et al., 2009).

Several ways have been used to improve the CgO properties, like soap, macerate, cream, oil capsules and gel (Costa-Silva et al., 2008). The oil from *C. guianensis* has been widely used for pharmaceutical purpose (Ferraz et al., 2002; Ferrari et al., 2007). However, due to the water-insoluble nature of the oil, the emulsion should be of interest to cosmetic applications (Andrade et al., 2007; Lima et al., 2008). Emulsions are heterogeneous mixtures that consist of droplets of a liquid dispersed in a second continuous immiscible liquid phase. The liquid/liquid immiscibility creates an interfacial tension between the two liquids that assign thermodynamic instability to such systems (Westesen, 1999; Rahate & Nagarkar 2007).

However, the anti-inflammatory effect of *Carapa guianensis* emulsion oil (CgOE) has not been reported yet. Thus, the aim of this study was to formulate O/W emulsions containing *Carapa guianensis* oil and to analyze its effect on the leucocytes during an inflammatory process.

MATERIAL AND METHODS

Emulsion preparation

The emulsions were prepared following raw materials: *Carapa guianensis* seed oil (10%), caprylic/capric triglyceride (3%), oleyl alcohol (2%) (Croda do Brasil Ltda, São Paulo, Brazil), butilhydroxitoluene (0.05%), ethylenediaminetetraacetic acid disodium (0.1%), triethanolamine (0.6%) (Henrifarma Ltda, Campinas, Brazil), methyldibromo glutaronitrile (and) phenoxyethanol (0.2%) (Galena Química e Farmacêutica Ltda, Campinas, Brazil), acrylate/C10-30 alkyl acrylate crosspolymer (0.3%), polymers of carboxyvinilic acid (0.2%) (BF Goodrich, USA) and distilled water.

The emulsions were obtained by the phase inversion temperature method (Santos et al., 2005). Both phases were heated separately to 70 ± 5ºC and then interdispersed. Stirring constant was added to the Acrylates/C10-30 Alkyl Acrylate Crosspolymer in the oil phase lasting for 1 h until total dispersion, then poured in the aqueous phase, being kept stirring at 1200 rpm for 25 min. After this time, it was added Methyldibromo glutaronitrile (and) phenoxyethanol (Ferrari et al., 2008; Pemulen, 2007). Three differents batches of *Carapa guianensis* emulsion oil (CgOE) were obtained and stored at 25 ± 2°C.

Performed after 24 h of sample preparation and during all evaluations, the prepared emulsions were examined by filter paper as well as by test tube methods in order to identify the type of emulsion (water-in-oil or oil-in-water) (Prista, Alves, Morgado, 2002).

Characterization of the Emulsions

Macroscopic Analysis. Each emulsion was evaluated to detect visible modifications or instabilities such as color, creaming, coalescence, and/or separation of phases. This analysis was performed for both storage conditions.

Stability Studies

Preliminary tests of emulsion stability. The CgOE was preliminarily evaluated by the centrifugal test, thermal stress, freeze thaw cycles, using the following parameters of evaluation: microscopic analysis, organoleptic characteristics, formulation homogeneity. The pH measurements of the emulsions were performed using a pre-calibrated pHmeter (mod. LS300-01, Alpax LOGEN, Brazil), inserting the electrode directly into the aqueous dilution 1:10 (w/w), and the electrical conductivity was measured using a conductivimeter (mod. ACA – 150, Alpax, Brazil), inserting the electrode directly in the sample, and the centrifugation test (mod. Excelsa Baby II, Fanem, Brazil) was performed on CgOE, freshly prepared and after 24 h, at 1000, 2500 and 3500 rpm for 15 min in each rotation. Samples were inspected for eventual phase separation after 15 and 30 min of centrifugation. All tests were made at 25°C and in triplicate (Davis, 1977; Latreille & Paquin, 1990; Nakhare & Vyas, 1996; Vasiljevic, Vuleta, Primorac, 2005).

Thermal stress. Emulsions of CgOE were submitted to a heated thermostatic bath (mod. 500/2D, Nova Ética Ltda, Brazil) set for the temperature range of 40 to 80 °C, with the temperature increase at intervals of 5 °C, and holding at each temperature for 30 min. The organoleptic

characteristics, pH value determination, electrical conductivity measures, and homogeneity, were obtained to evaluate the formulations before and at the end at 80 °C, after the natural cooling of the samples at room temperature (25 ± 2 °C) (Lima et al., 2010; Daher et al., 2014).

Freeze/thaw cycles. Samples CgOE were subjected to 4 ± 2 °C/24 hours (CONSUL, CFC 28A, Brazil), and then 45 ± 2 °C/24 hours (Fanem, mod. 502, Brazil), thus completing a cycle. This cycling was repeated 6 times. The same parameters were used to evaluate the thermal stress test and recorded after each cycle. As a control, emulsions kept at 25°C were also analyzed for 12 days (Lima et al., 2010).

Accelerated stability test. The samples considered stable by preliminary tests were stored under different conditions: 4 ± 2 °C (CONSUL, CFC 28A, Brazil); 25 ± 2 °C (room temperature), 37 ± 2 °C, 45±2 °C and 75 ± 5% relative humidity (RH) (climatic chamber Nova Ética, mod. 420 – CLD 300, Brazil), 45 ± 2 °C and 75 ± 5% RH (Nova Etica, mod. 520-CLDTS 150, Brazil). The samples were maintained under these conditions for 90 days. The macroscopic analyses (appearance, homogeneity and organoleptic characteristics), pH value determinations and rheological behavior were evaluated at different time intervals (24 h after preparation of formulations and on the 30th, 60th and 90th days) (Ferrari & Rocha-Filho, 2011; ANVISA, 2004). All samples were analyzed in triplicate.

Physical stability was assessed through rheological determinations that were obtained using a model DV-III Brookfield rotational rheometer (Stoughton, MA, USA) with a cone-plate configuration and a Brookfield software program (Rheocalc version v3.2). Rheological parameters were determined at 25±2°C using a CP 52 spindle (d=12 mm, θ=3.0) and 0.5 g of each sample. The consistency index, flow index and hysteresis area were calculated. Three batches were prepared and measurements repeated three times for each sample.

Experimental model of cutaneous inflammation-

Animals. Balb/c mice (*Mus musculus*) (male, 20-25g body weight) were used. They were housed in polypropylene cages with water and food (Purina®) *at libitum*, and kept at ± 25°C with controlled 12h light/dark. All procedures were made under the approval of the Ethical Committee in Animal Research (CEPA-UFMT, Brazil, 23108.002115/09-9).

Carrageenan-induced cutaneous inflammation. Mice were anesthetized with ketamine chloridrate (ketamine Syntec®, Brazil, 80 mg/kg, i.m.) and xylazine chloridrate (xylazine Syntec®, Brasil 10 mg/kg, i.m.). Then, they were tricotomized in the dorsal region (electric shaver, mod. LuxShav-722, China). Animals were submitted to the inflammatory process by topical administration of 75 µg/cm^2 carrageenan (type λ; Sigma Chemical Co. Poole, Dorset, UK), diluted in 50 µL liquid crystal emulsion (Uniox® C, Chemyunion®, Brazil), dispersed in 1 cm^2 of dorsal skin. This concentration is equivalent to 3 mg/Kg. After 1 h of the inflammatory induction, a group of mice was anesthetized again, and 4.7 µg/cm^2 of CgO or CgOE was administered topically (56.25 µL in a concentration equivalent to 0.187 g/kg). As the standard drug, we used 0.25 mg/cm^2 emulsion of silicone oil with dexamethasone (DexaE, volume of 56.25 µL in a concentration equivalent to 10 mg/kg). As negative controls, different groups of mice were treated with: PBS vehicle (PBS group), silicon emulsion oil (SEO), liquid crystal emulsion (only LCE) or CgO only. The volume and the concentrations were equivalent to that used in other experiments.

Histological analysis. After experimental procedures, animals were euthanized by deep anesthetization. Then, skin samples were obtained and fragments were histologically processed as described by Damazo et al. (2006). Tissue was fixed in 4% paraformaldehyde and 0.5% glutaraldehyde, 0.1 M sodium cacodylate buffer, pH 7.4 for 18 h at 4 °C, dehydrated in ethanol, clarified in xylene and embedded in histosec® (Merck, Darmstadt, Germany). Sections (3 µm thick) were stained with 0.25 % toluidine blue in 0.25 % borax solution. The samples were analyzed in the light microscope AxioScope A1 with the software AxioVision Release 4.8.1 (Carl Zeiss, Germany), by counting cells per area. The degree of leukocytes and mast cells (intact and activated) were determined. Data are reported as mean of cells/mm^2 ± SEM.

Immunohistochemical analysis for annexin-A1. The detection of AnxA1 protein by light microscopy was performed as described by Damazo et al. (2006) in the leukocytes and mast cells skin. A polyclonal rabbit anti-AnxA1 antibody (Invitrogen, USA) was added (1/200 in 1% PBSA), and slides incubated overnight at 4°C. As a control for the reaction, some sections were incubated with nonimmune rabbit serum (1/200 working dilution; Sigma-Aldrich) instead of the primary Ab. The goat anti-rabbit IgG nanogold® (Fab′ fragment) (1/100; Invitrogen, USA) was used as secondary antibody. At the end of the reaction, sections were counterstained with hematoxylin. Skin leukocytes and mast cells immunostained to AnxA1 were analyzed with the software AxioVision.

TNF-α, IL-1β, IL-6 and IL-10 detection. Fragments of skin were weight, frozen in liquid nitrogen, homogenized at 4°C in 50 mM/l Tris buffer rich in protease inhibitors (1 mM/l phenylmethyl

sulfonyl fluoride, 1.5 mM/l pepstatin A, and 0.2 mM/l leupeptin, pH 7.4) and centrifuged (4,000 g, 5 min, 4°C). Supernatants were used to determined cytokine levels. Then, concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and IL-10 were measured using specific enzyme-linked immunosorbent assay (ELISA) kits purchased from eBioscience (San Diego, USA).

Data handling and statistical analysis. The cell counting, cytokine release and preliminary stability data were reported as mean ± SEM of ten mice per group. Statistical differences between groups were determined by ANOVA followed, if significant, by the Bonferroni´s test using the software GraphPad Prism 5.0® (GraphPad Software Inc., San Diego, CA, EUA). Accelerated stability data were reported as mean ± SEM. Statistical analysis were performed using the univariate analysis of variance, and the post test was the Tamhane's test ($p<0.05$). In all cases, a probability value < 0.05 was taken as significant.

RESULTS

Preliminary stability analysis of the *C. guianensis* oil emulsion

The preliminary stability studies are used to delineate the initial phase of product development. The emulsions did not show any changes in its macroscopic characteristics. Also, the microscopic analysis showed the homogeneity of the samples. The dilution test showed that the external phase was water, which indicates this emulsion as one oil/water (O/W) and the hydrophilic character of the polymeric emulsifier used. The centrifugation test was performed for the macroscopically stable emulsions. No phase separation or organoleptic characteristic changes were observed after centrifugation for any of the samples. After the thermal stress test, the pH value was still at 5.3. However, a small variation in the electrical conductivity was observed at the emulsion (Table 1).

Accelerated stability analysis

Accelerated stability tests employ less extreme conditions compared to the previous test. After the accelerated stability test, all of the formulations maintained their organoleptic characteristics. The samples showed a variation of pH values (5.6 to 6.05) compared to the data obtained in the day 1 (Table 2).

The CgOE physical stability was assessed through rheological determinations. Three batches were prepared, and the results were obtained with three separated evaluations. The CgOE initial consistency value was 3660.85 ± 44.89 cP (Table 2). The other time points did not present any differences. Since no changes were observed between the selected time points, rheogram curves were constructed with ascendant and descendant segments at days 1 and 90 (Figure 1). The CgOE initial flow index was calculated at 0.23 ± 0.01. After the described time and temperature, a variation among 0.08 to 0.23 was observed (Table 2). The analysis of hysteresis area and the rheogram observations characterized the thixotropic system of the CgOE, demonstrating that the formulation has a difference in capacity and time to return to its initial structure after the application of a force (Table 2 and Figure 1).

Moreover, we observed that flow index (Table 2) was below 1.0 in all formulations, indicating non-Newtonian pseudoplastic flow behavior, which is a desirable rheological property in these formulations. In this case, the viscosity decreases with the increase of the shear rate.

Experimental model of inflammation and CgOE anti-inflammatory effect

Macroscopic and microscopic analyses were used to evaluate mice skin submitted to inflammatory

TABLE 1. Results of preliminary stability tests of emulsions containing *Carapa guianensis* oil.

PARAMETERS	Bach 1	Bach 2	Bach 3	Mean ± SEM
AFTER 24 H				
Centrifugation	N	N	N	---
pH value	5.33	5.34	5.32	5.3 ± 0.01
Electrical conductivity (μS/cm³)	0.74	0.75	0.78	0,75 ± 0.01
AFTER TS				
pH value	5.34	5.35	5.32	5.3 ± 0.01
Electrical conductivity (μS/cm³)	0.70	0.68	0.72	0.71 ± 0.01
AFTER FTC				
Ph value	5.36	5.40	5.42	5.4 ± 0.03
Electrical conductivity (μS/cm³)	0.78	0.76	0.77	0.77 ± 0.01

FTC = Freeze/thaw cycles; TS = Thermal Stress; N= Normal. Data were expressed as mean ± Standard Error of mean (SEM).

TABLE 2 – Values of pH, minimal apparent viscosity, flow index and hysteresis area values of O/W *Carapa guianensis* oil emulsion during accelerated stability studies at different temperatures.

Time (days)	Temperature (°C)	pH value	Minimal apparent viscosity (cP)	Flow index	Hysteresis area (d/cm².s)
1	25	6.01 ± 0.01	3660.85 ± 44.89	0.23 ± 0.01	23062.89 ± 415.65
	4	6.05 ± 0.01	3976.89 ± 89.11	0.20 ± 0.01	25864.11 ± 647.92
90	25	6.00 ± 0.01	3633.29 ± 50.55	0.19 ± 0.01	23878.89 ± 523.61
	37	5.72 ± 0.01***	3325.71 ± 76.77	0.10 ± 0.01	21725.67 ± 533.73
	45	5.60 ± 0.01***	3402.88 ± 51.13	0.08 ± 0.01	22509.18 ± 341.10

***p<0.001 *vs* day 1. Data were expressed as mean ± Standard Error of mean (SEM). (n=9).

process induced by carrageenan. These data demonstrated a high hyperemia, vasodilatation, and edema observed in the skin dermis and hypodermis. Also, high leukocyte migration (611.30 ± 53.05/mm²) and mast cell degranulation (~49%) were observed. In negative control mice (LC, SOE, CgO, CgOE, DexaE), only resident leukocytes (149.30 ± 21.44) and a few degranulated mast cells (~9%) were observed (Figure 2A-B and Table 3).

CgO, CgOE and DexaE treatment after carrageenan administration reduced the leukocyte migration (356.30 ± 24.06, 222.00 ± 40.43 and 180.00 ± 37.55, respectively) and mast cell granules release (~16%, 18% and 13%, respectively) (Figure 2C-D and Table 3).

Annexin-A1 immunohistochemical analysis

The AnxA1 expression was analyzed in leukocytes and mast cells skin. After carrageenan administration, a strong AnxA1 expression was observed in the leukocytes (213.30 ± 25.71) and mast cells (210.5 ± 30.15), when compared to the NC groups (154.85 ± 2.96 and 145.50 ± 2.05) (Figure 3 and Table 4).

Mice treated with CgO, CgOE and DexaE also showed a high expression of AnxA1 in the leukocytes (211.04 ± 1.13, 217.10 ± 1.00 and 209.50 ± 1.00, respectively) and mast cells (209.10 ± 2.05, 211.10 ± 2.00 and 211.80 ± 3.00, respectively) (Figure 3 and Table 4).

Analysis of TNF-α, IL-1β, IL-6 and IL-10 levels

To evaluate the molecular mechanism of the inflammatory process inhibition by the CgO, CgOE and DexaE treatments, the cytokine levels assay was performed. The topical administration of carrageenan induced higher levels of TNF-α

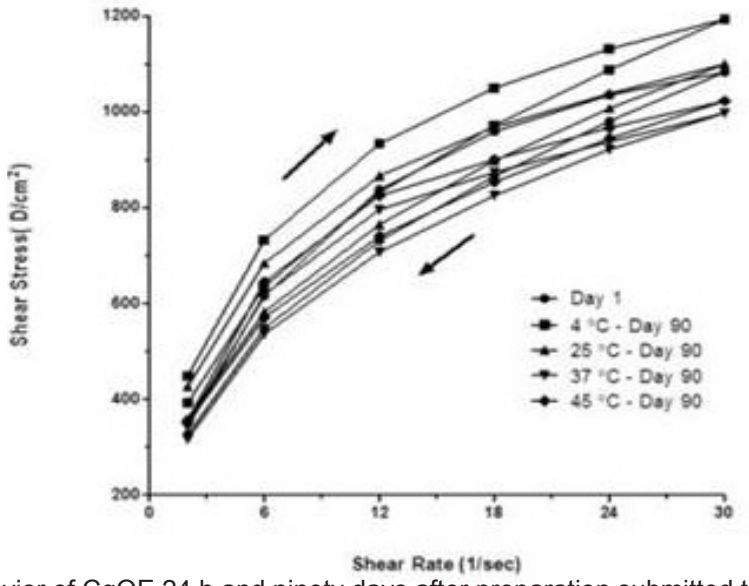

FIGURE 1. Flow behavior of CgOE 24 h and ninety days after preparation submitted to different temperatures. Values of each point correspond to mean ± SEM of 3 readings of each batch (n=3).

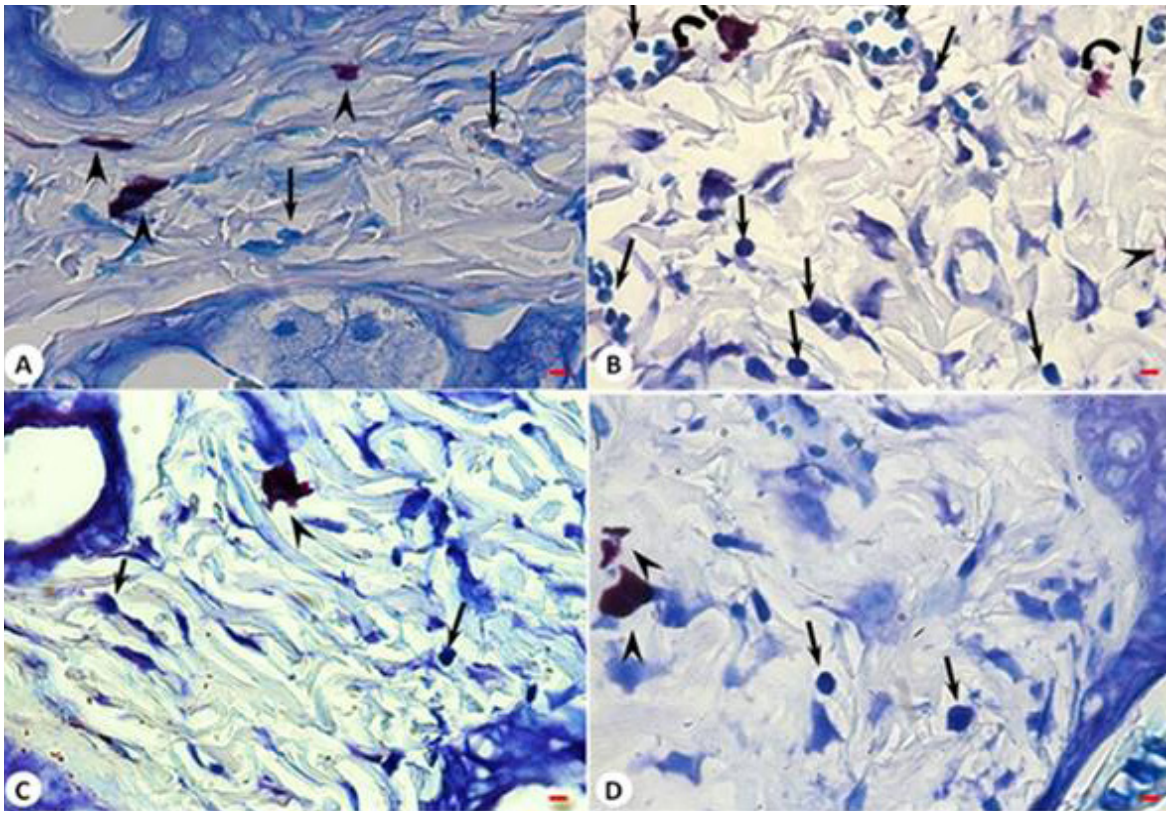

FIGURE 2. Histological analysis of leukocytes and mast cells in skin mice. (A) Negative control mice showing intact mast cells (arrowheads) and resident leukocytes (arrows). (B) Mice treated with carrageenan presented an intense leukocyte migration and mast cell degranulation. (C) Carrag+CgOE and (D) Carrag+DexaE groups showed a reduction in leukocyte migration and mast cell degranulation. Stain: toluidine blue. Barr: 5 µm.

TABLE 3. Quantitative analysis of leukocytes and mast cells in the mice skin.

Groups	Leukocyte	Mast cells	
		Total	% Degranulation
NC	149.30 ± 21.44	140.00 ± 12.41	9.00 ± 2.30
Carrag	611.30 ± 53.05***	278.70 ± 50.04	48.87 ± 2.45***
Carrag + SOE	700.00 ± 48.17***	193.30 ± 27.24	41.98 ± 10.40***
Carrag + CgO	222.00 ± 40.43###	175.00 ± 21.84	16.39 ± 4.54###
Carrag + CgOE	356.30 ± 24.06###	109.30 ± 16.55	18.24 ± 5.18#
Carrag + DexaE	180.00 ± 37.55###	182.20 ± 5.90	13.34 ± 3.02##

Data represents mean of cells ± SEM per mm^2 in the skin (n=10 per group). *** $p < 0.001$ *vs* negative control (NC); # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ *vs* Carragenann group (one way ANOVA followed by Bonferroni´s test).

(74.60%), IL-6 (76.50%) and IL-1β (80.90%) release but not IL-10, when compared to the negative control groups (Figure 4).

However, the induction of the inflammatory process followed by the CgOE and DexaE treatments reduced the TNF-α (34.30% and 43.60%, respectively) and IL-6 (36.23% and 43.35%, respectively) levels (Figure 4). The levels of IL-1β were also reduced in all treatments: CgO (46.66%), CgOE (76.49%) and DexaE (45.40%). Also, the analysis of IL-10 levels showed an increase of this cytokine after CgOE (46.70%) and DexaE (51.15%) treatment (Figure 4).

DISCUSSION

Natural products synthesized by plants are knowledge by its pharmacological diversified activities and are wildly used therapeutically. The CgO has been indicated as a relevant natural product

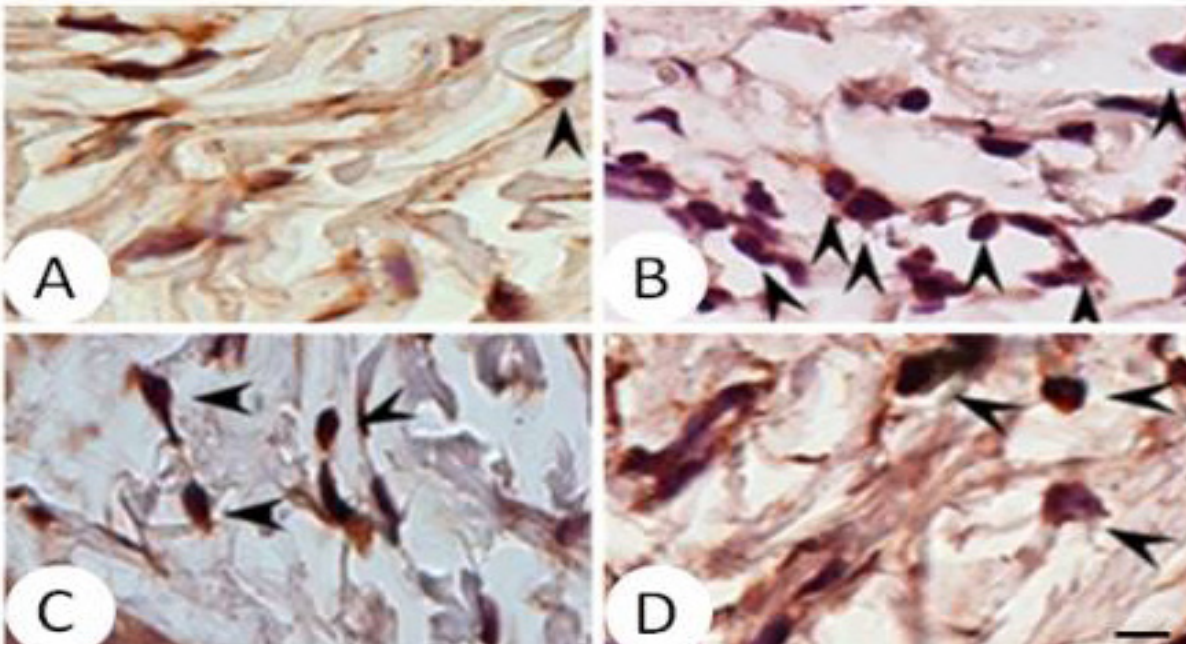

FIGURE 3. AnxA1 immunostain in leukocytes and mast cells. (A) Group negative control showed immunostain for AnxA1 in leukocytes (arrowhead). (B) The leukocytes from the groups Carrag, Carrag+CgOE and Carrag+DexaE showed an intense immunostain for AnxA1. Counterstain: hematoxilin. Barr: 5 µm.

TABLE 4 - Densitometric analysis of AnxA1 expression in the leukocytes and mast cells.

Groups	MOD (a.u.)	
	Leukocyte	Mast cell
NC	154,85 ± 2,96	145.50 ± 2.05
Carrag	213,30 ± 2,52***	210.5 ± 30.15***
Carrag + SOE	202,50 ± 1,32***	211.3 ± 25.10***
Carrag + CgO	211,00 ± 1,30	209.10 ± 2.05
Carrag + CgOE	217,10 ± 1,00	211.10 ± 2.00
Carrag + DexaE	209,50 ± 1,00	211.80 ± 3.00

Data represents mean of cells ± SEM (n=10 per group). MOD = Mean Optic Density. a.u. = arbitraries units.*** $p < 0.001$ *vs* negative control (NC) (one way ANOVA followed by Bonferroni´s test).

from the Brazilian biodiversity. Several studies have indicated its anti-inflammatory and anti-allergic activity in experimental models (Penido et al., 2006a, 2006b; Costa-Silva et al., 2007; Henriques & Penido, 2014). The medicinal properties of *C. guianensis* have been attributed to the presence of limonoids, which are tetranortriterpenoids. We have previously demonstrated that the oil obtained from C. guianensis seeds contains different tetranortriterpenoids, including 6α-acetoxygedunin, 7-deacetoxy-7-oxogedunin, andirobin, gedunin and methyl-angolensate (Henriques & Penido, 2014).

Also, a few works have evaluated the OCg application in the pharmaceutical technology through emulsification process (Ferreira et al., 2010). This is a relevant system because the emulsions have lipophilic and hydrophilic characteristics and are relevant vehicles to the drug vehiculation/ transport, which facilitates the application on the skin (Vasiljevic et al., 2006). In the present study, we have demonstrated that the *C. guianensis* oil extracted from the seeds of the tree has a considerable capacity of emulsification, using polymeric tensoactives, and presents a high stability even when submitted to temperature variations.

The CgOE formulations were standardized containing 10% of CgO. This concentration was chosen in accordance to toxicological studies, which showed that this oil was safe for oral administrations (Costa-Silva et al., 2007, 2008). Besides, this oil concentration was similar to the one used by Lima et al. (2010) with *babassu* oil and Pianovski

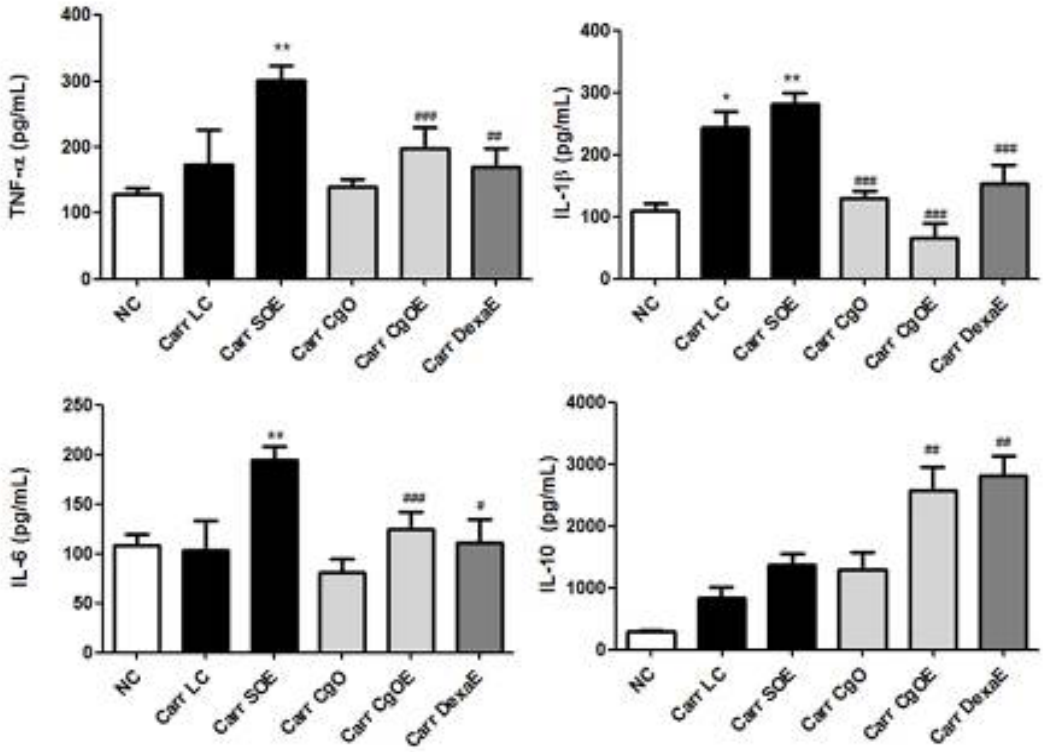

FIGURE 4 – Detection of cytokine levels in mice skin. (A) TNF-α, (B) IL-6, (C) IL-1β and (D) IL-10. ** $p < 0.01$; *** $p < 0.001$ *vs* negative control group; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ *vs* carrageenan group. N=10 per group (One way ANOVA, followed by Bonferroni test).

et al. (2008) with *pequi* oil, both using polymeric tensoactives. This compound was used because the emulsions are thermodynamically unstable and they provide the required stability. At this study, we have used the emulsifier Acrylate/ C10-30 Alkyl Acrylate Crosspolymer (Pemulen® TR1). This polymeric tensoactive has significant advantages, because it provides a low concentration of use, it is easily dispersed and can be used in the oleic or the aqueous phase (Pemulen, 2007). We have also used the polymer carboxyvinilic acid (Carbopol® ultrez) at the aqueous phase to provide a better stabilization into the emulsion. This polymer increases the emulsion viscosity and reduces the transit of the globules, avoiding its coalescence (Tados et al., 2004).

According to ANVISA (National Agency of Sanitary Vigilance - Brazil), the study of preliminary stability must be focused on the product formulation and there is no need to determine its expiration date (ANVISA, 2004). Similar to previous investigations (Lima et al., 2010; Pianovski et al., 2008) after 24 h the CgOE showed cream characteristics, with a milky and uniform aspect, macroscopically stable, maintaining the CgO odor, absence of creaming or phase separation, which indicates stability. Then, we used the centrifugation test, which can induce the sedimentation, creaming or coalescence of droplets in unstable emulsions (Latreille & Paquin, 1990; Tadros et al., 2004). The CgOE did not present the phase separation, indicating the emulsion stability as obtained in *pequi* (Pianovski et al., 2008), *babassu* (Lima et al., 2010) and *Carapa guianensis* emulsions (Ferreira et al., 2010).

The primary stability of the emulsion was also evaluated by the thermal stress test and freezing-thawing cycle. These data indicate that there were no changes in pH and electrical conductivity of the formulations. The measurement of pH in emulsions is a parameter that measures the formation of fatty acids during storage after partial hydrolysis of triglyceride. Also, conductimetric analysis is often used to determine the nature of an emulsion and control its stability over time (Ferreira et al., 2010; Masmoudi et al., 2005). In fact, this method is sensitive to small changes in the physical structure of emulsions, which may show creaming, sedimentation or phase inversion (Ferreira et al., 2010).

After establishing the preliminary stability, we evaluated the accelerated stability up to 90 days. It was identified a decrease in pH. These results could be explained by the fact that vegetable oils are susceptible to oxidation with the formation of oxidized chains (hydroperoxides) or hydrolysis of triglycerides, which is manifested by the formation

of free fatty acids (Ferreira et al., 2010; Masmoudi et al., 2005). However, this change was considered acceptable because it is still dermatologically compatible (Hathout et al., 2010). The decrease in pH values after 90 days might indicate evidences of instability. Decreases in pH values are expected due to the complex nature of the CgO (Ferreira et al., 2010). These changes did not affect the organoleptic and macroscopic characteristics, indicating that the emulsion systems have a high ability to trap the oil components and protecting them from oxidative degradation.

The rheological evaluations are useful tools since they evaluate the emulsion physical stability, which might influence in the skin permeation of medicinal plant active principles (Derkach, 2009). Changes in the emulsion rheological behavior could be an evidence of instabilities such as flocculation, creaming and sedimentation (Derkach, 2009; Mostefa et al., 2006). Our results showed no significant variations, indicating the formulation stability.

The CgOE developed showed a non-Newtonian behavior, i.e, there is no proportionality between the tension and shear rate speed. After an increase in the shear rate, a decrease in viscosity could be observed in the emulsion. Confirmation of this profile was obtained by the flow index value which was below 1.0, indicating pseudoplastic flow behavior. This condition is desirable in formulations containing polymers and gums, such as the Pemulen® TR1 (Derkach, 2009).

Our formulation showed a decrease in viscosity shear indicating thixotropy. This fact was indicated by the continuous decrease in viscosity under shear stress followed by gradual recovery when the stress is removed (Lee, Moture, Lee, 2009). The thixotropic product is also a characteristic of pseudoplastic fluids that spread more easily in the applied surface. This characteristic contributes to the retention time on the skin and prevents drips (Gaspar & Maia Campos, 2003). All these data have a pivotal role in the therapeutic efficacy of pharmaceutical formulations, indicating stability of the formulation due to constant viscosity, avoiding the separation of the formulation constituents. Also, the modified release systems may influence the controlled release of drugs in topical formulations (Mewis & Wagner, 2009).

Since the formulation was stable, we decided to test its anti-inflammatory efficiency in an experimental model of topical inflammation induced by carrageenan. In this experimental model, the carrageenan was vehiculated with liquid crystal to avoid the skin permeation across the intact mice corneal stratum. This observation was confirmed by high hyperemia, vasodilatation and migration of leukocytes and mast cell degranulation into the site of application. Also, the administration of carrageenan with silicon oil emulsion increases the inflammatory reaction. This event could be explained by a prolonged skin hydration and allowed the dissolution of the lipids of the corneal stratum, which increased the skin permeation and potentiation of the inflammatory actions (Otto et al., 2009; Libster, et al., 2011). Carrageenan is widely used in the model of acute inflammation (Winter, et al., 1962). Some of the pro-inflammatory mediators induced by this acute inflammation model are bradykinin, histamine, serotonin, platelet activating factor and reactive oxygen species (Morris, 2003).

The CgO, CgOE, and DexaE administrations were able to inhibit the leukocytes migration and the mast cell degranulation significantly. These results are in agreement with other works (Penido et al., 2005; Penido et al., 2006a), which examined the response of the CgO tetranortriterpenoid influx using cell models of pleurisy induced by zymosan in mice. All these data are particularly noteworthy, indicating the potential CgO anti-inflammatory action and its maintenance in the CgOE formulation.

To understand the CgO anti-inflammatory mechanism, we evaluate the AnxA1 anti-inflammatory mediator expression in this experimental model. Initially, we assessed the protein expression in leukocytes after carrageenan administration. As shown by our previous work (Damazo et al., 2006; Gastardelo et al., 2009), the inflammatory processes induced by carrageenan or zymosan are capable of producing increased expression of AnxA1 in leukocyte infiltration, and it regulates leukocyte migration. Also, the CgO, CgOE and DexaE administration significantly increased the AnxA1 expression in leukocytes. It is well known that AnxA1 is an anti-inflammatory mediator triggered by pro-inflammatory mediators (Damazo et al., 2006). In our experiments, the CgO and CgOE treatment reduced the inflammatory process, but the AnxA1 levels are still high. This might indicate that this oil component stimulates AnxA1 production as seen in the DexaE treatment. Further studies are needed to explain these findings.

Subsequently, we evaluated the CgO and CgOE effect on the cytokines release. Studies show that, after carrageenan administration, TNF-α, IL-1β and IL-6 levels showed a significant increase in tissue and plasma (Loram et al., 2007). In our data, animals treated with carrageenan with silicon oil emulsion presented an increased release of all cytokines in the skin, whereas the carrageenan alone only presented detectable levels of IL-1β. These cytokines are produced by various cell types, including macrophages and mast cells, having different roles in the inflammatory

response, including activation of the endothelium and leukocytes chemotaxis in the acute phase of the inflammatory response (Medzhitov, 2010). The CgOE treatment inhibited the release of these pro-inflammatory cytokines in mice skin whereas the CgO treatment reduced the IL-1β levels. This might indicate that this delivery system promoted the skin permeation of drugs and increased its activity. Similarly, Penido and collaborators found that pretreatment with CgO tetranortriterpenoid inhibited TNF-α, IL-1β and CXCL8/IL8 levels induced by zymosan administration in mice arthritis (Penido et al., 2006a). Moreover, assessing the levels of anti-inflammatory cytokine IL-10, we found that there was a significant increase of this cytokine after treatment with CgOE and DexaE. The IL-10 is known as one of the most significant cytokines with anti-inflammatory and immunosuppressive activities, which has considerable influence on monocytes and almost all leukocytes (Sabat et al., 2010a). This cytokine is involved in antigen presentation, release of immune mediators and phagocytosis, acting on self and non-self immunity (Sabat, 2010b). All these data along with ours, suggest a pivotal role of the CgOE in the suppression of pro-inflammatory and up-regulation of anti-inflammatory mediators.

Furthermore, the CgOE remained stable and facilitates the permeation in the skin, increasing its anti-inflammatory action. As previously reported in the literature, *C. guianensis* oil has anti-inflammatory properties. In the present work we investigated its possible mechanisms of action and suggested that the emulsion associated to this oil may be used to protect the skin against potential inflammatory agents.

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