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Anti-inflammatory effects and acute oral toxicity of *Copaifera* spp. essential oil-loaded nanoemulsion

[Efectos antiinflamatorios y toxicidad oral aguda de nanoemulsión cargada de aceite esencial de *Copaifera* spp.]

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Abstract: *Copaifera* spp. essential oil (EOC) was extracted by hydrodistillation of *Copaifera oleoresin* (COR). The EOC was characterized by GC/MS and a novel EOC-loaded nanoemulsion was developed to enhance the EOC solubility and to evaluate its utility as antinflammatory. EOC contain 14 volatile compounds (including β-caryophyllene: 51.52%) having a required HLB of 11. The Surfactant: EOC: Water ratio of 13:15:75 (%, w:w:w) produced the optimal formulation (particle size: 94.47 nm). The EOC-loaded nanoemulsion presented a pseudoplastic/thixotropic behavior with excellent shelf stability for 6 months. The anti-inflammatory effect of the nanoemulsion was more potent than that of the EOC, and statistically equal to diclofenac (50 mg/kg). The EOC-loaded nanoemulsion showed no oral acute toxicity (in mice) at 2000 mg/kg; hence, it is considered a nontoxic product. The development of the EOC-loaded nanoemulsion added value to both the COR and the EOC by providing a suitable formulation that could be used as an anti-inflammatory product.

Keywords: *Copaifera*; Essential oil; Oleoresin; Nanoemulsion; Anti-inflammatory.

Resumen: El aceite esencial (EOC) fue extraído por hidrodestilación de oleoresina de *Copaifera* spp. El EOC fue caracterizado químicamente por GC/MS. Se formuló una nanoemulsión con EOC para mejorar la solubilidad del EOC y evaluar su utilidad como antiinflamatorio. El EOC contiene 14 compuestos volátiles (incluido el β-cariofileno: 51,52%) con un HLB requerido de 11. La relación Tensioactivo: EOC:Agua de 13:15:75 (%, p:p:p) produjo la formulación óptima (tamaño de partícula: 94,47 nm).. La nanoemulsión cargada con EOC presentó un comportamiento pseudoplástico/tixotrópico con una excelente estabilidad en almacenamiento durante 6 meses. El efecto antiinflamatorio de la nanoemulsión fue más potente que el del EOC y estadísticamente igual al diclofenaco (50 mg/kg). La nanoemulsión cargada con COE no mostró toxicidad aguda oral (en ratones) a 2000 mg/kg; por lo tanto, se considera un producto no tóxico. El desarrollo de la nanoemulsión cargada con EOC agregó valor tanto al COR como al EOC al proporcionar una formulación adecuada que podría usarse como un producto antiinflamatorio.

Palabras clave: *Copaifera*; Aceite esencial; Oleorresina; Nanoemulsión; Antiinflamatorio

INTRODUCTION

Medicinal plants are recognized for their great therapeutic value, and generally induce fewer adverse effects than synthetic drugs (Melro *et al*., 2019). Therapies using medicinal plants should be developed using a scientific approach to reduce the frequency of adverse events and the quantity of materials to be used, thereby increasing patient compliance and crop sustainability (Ansari *et al*., 2012, Melro *et al*., 2019). In contrast, botanical extracts usually possess an unpleasant flavor and aroma, poor water solubility, susceptibility to damage from light, and other factors that affect stability, which hinder their manipulation, management, and utilization. In this context, encapsulating medicinal plant derivatives in nanoparticle systems to enhance bioavailability, stability, and potency, is an appropriate methodology for the use and preservation of the nation's genetic patrimony.

Species of the *Copaifera* genus (Fabaceae) are spread throughout the Amazonian forest, including Brazilian states and other countries, such as Colombia, Venezuela, and Perú (Cascón & Gilbert, 2000; Costa *et al*., 2002; Kar, 2003; Barreto *et al*., 2005). The most abundant species of Copaifera in the Amazon are *Copaifera cearensis* Huber ex Ducke, *Copaifera reticulata* Ducke, and *Copaifera multijuga* Hayne (Veiga *et al*., 2007). Species of this genus produce a crude oleoresin (COR) that has been used for several centuries as an insect repellent and for the treatment of stomach pains, arthritis, and skin disorders. The COR is informally commercialized in small markets of all states of Brazil; however, its utilization as a drug is still restricted owing to the large variability in the chemical composition of the COR produced by distinct species, which affects the efficacy and the anti-inflammatory potency (Cascon & Gilbert, 2000; Veiga *et al*., 2007).

The main difference in the chemical composition is the content of β-caryophyllene, the major constituent of the COR from all species. Terpenoids, such as α-humulene, α-copaene, α-bergamotene, and δ-cadinene, are present in the oleoresin at different concentrations (Veiga *et al*., 2007; Leandro *et al*., 2012). Variability in the chemical composition of the oleoresin makes it difficult to standardize this plant-derived product as a raw material for use in pharmaceutical and cosmetic preparations (Rigamonte *et al*., 2004; Biavatti *et al*., 2006; Herrero *et al*., 2011).

In contrast, the essential oil (EOC) obtained by steam distillation of COR has a homogeneous composition, regardless of the species (Veiga *et al*., 2007; Sousa *et al*., 2011). In minor grade, the EOC is used by Amazonian populations for treating joint pains and inflammation. Independent of the species, the EOC content of β-caryophyllene is 45% or more. The use of EOC instead of the COR can be a method for using this extractivist product from the Amazon region as an ingredient in pharmaceuticals and cosmetic products.

β-Caryophyllene is a potent anti-inflammatory, antimicrobial, and antioxidant compound (Veiga *et al*., 2007; Liu *et al*., 2013). The high content of β-caryophyllene is a justification for the ethnobotanical use of the EOC as an anti-inflammatory agent. However, EOC has poor water solubility, which hinders its utility in pharmaceutical preparations. Encapsulating the EOC in a nanoemulsion using surfactants may be a good strategy for use in pharmaceutical preparations, because nanoemulsions can improve the water solubility, stability, and the anti-inflammatory potency of EOC (Tadros *et al*., 2011; Ansari *et al*., 2012). This study aimed to develop and characterize an EOC-loaded nanoemulsion, and to evaluate the anti-inflammatory effect in mice.

MATERIAL AND METHODS

Vegetal material and chemicals

As farmers collect COR from any *Copaifera* species and mix them for commercializing purpose, the sample used in this work was a pool (3 kg) made with COR collected from plants of the species *Copaifera cearensis* Huber ex Ducke (1 kg), *Copaifera reticulata* Ducke (1kg), and *Copaifera multijuga* Hayne (1 kg). The collect was made in Tupé, community of Rio Negro (3°03'10.3" S, 60°18'23.4" W), Amazonas, Brazil. The oleoresin was collected between days 10 and 14 of April 2017 in the morning hours. The collect was performed by the manual traditional method, drilling the trunk at a height of 1.5 m from the ground, using a drill of 1.8 cm on diameter. This method allows to preserve the integrity of the collected trees, for future harvests. The pool of COR was submitted to hydrodistillation using a Clevenger apparatus for obtain the volatile fraction (EOC). EOC was kept in an amber flask at room temperature until use.

All chemicals and the β-caryophyllene standard (Sigma, USA) used in this work were purity for analysis.

Physicochemical characterization of Copaifera spp. essential oil

To characterize the EOC, the aspect, color, and aroma were qualitatively evaluated. The relative density (at 25°C), refractive index, viscosity, and pH were also evaluated using the methodologies proposed in the Brazilian Pharmacopoeia (Brazil, 2007).

GC/MS analysis

The quantitation of the EOC constituents was performed by GC-MS with FID detection (QP2010 Plus, Shimadzu, Japan), using a Rtx-5 MS capillary column (30 m × 0.25 mm × 0.25 mm) with helium as the carrier gas at a flow rate of 1.0 mL/min. Aliquots of EOC (10 mg) were dissolved in 1 mL of dichloromethane, and 10 µL of this solution was injected with a split ratio of 1:20. The column temperature was programmed from 60°C to 280°C, increasing at 3°C/min, and the injector and detector were kept at 220°C and 260°C, respectively. The retention indexes were obtained using a homologous series of linear hydrocarbons (C_6–C_{30}, Sigma, USA). The identification was made comparing the acquired spectra with those stored in the Wiley 8th edition library and by comparison of its retention indices with literature data (Adams, 2017).

β-Caryophyllene quantitation

β-Caryophyllene was quantified by gas chromatography as described by Xavier *et al*. (2017). The quantitation was conducted by the standard internal method, using a β-caryophyllene standard (Sigma, USA). The standard was dissolved in dichloromethane to prepare a solution of 1 mg/mL. Subsequently, samples of EOC were enriched to known amounts of the standard solution (5, 10, 25, 50, 100, 250, 500, and 1000 µg/mL). The calibration curve was constructed plotting the peak area *vs.* sample concentration. The least-squares linear regression was used for fitting the calibration curve. The concentration of β-caryophyllene was expressed as a percentage.

Nanoemulsion preparation

The EOC-loaded nanoemulsion was prepared as described by Ostertag *et al*. (2012), with modifications. The organic phase was composed of 5% of EOC and 5% of surfactants (Tween 80-Span 80) in different proportions, to obtain hydrophilic-lipophilic balance (HLB) values between 6 and 15. The organic phase was stirred for 10 min at 400 rpm using a magnetic stirrer (Ika, Switzerland). The aqueous phase, Milli-Q water ($\Omega < 5$ µΩ), consist of the remainder of the emulsion. The aqueous phase was added to the organic phase at a rate of 1 mL/min, with stirring at 400 rpm for 5 min. Subsequently, the mixture was homogenized for 10 min using an UltraTurrax homogenizer (IKA, Switzerland) at 5000 rpm. The final volume of nanoemulsion was made up to 50 g using Milli-Q water. The nanoemulsion was stored in an amber flask at 25°C until the characterization. It was selected as the HLB required by EOC for nanoemulsion development (HLBr), the EHL value that produced the nanoemulsion with the small particle size and more homogeneous particle size distribution (Griffin, 1949).

Selecting the surfactant system

To select the surfactant system yielding the more stable nanoemulsion with minor particle size and more homogeneous size distribution, three different systems of non-ionic surfactants were used (Tween 20: Span 20, Tween 60: Span 60; and Tween 80: Span 80), in proportions that achieve HLB=11 (HLBr of the EOC). For this assay was used a ratio of 1:1 (Surfactant: EOC).

Effect of the Surfactant: EOC: Water (SOW) ratio

The effects of changes in the relative amounts of the components on the properties of EOC nanoemulsion were evaluated. The surfactant amount varied from 5% to 15%, the EOC varied between 5% and 15%, and the amount of water used to complete the volume to 100%. Up to 15% surfactant was evaluated because this is the limit for this type of surfactant for use in oral formulations (Rowe, 2009). Forty-one nanoemulsions were prepared to construct the ternary phase diagram. The starting point was the ratio 5:5:90 (% SOW; w:w:w), which was the ratio used for the formulation tests. The best formulations were selected in terms of the smallest and most homogeneous particle size and the highest modular value of the zeta potential. Finally, the better formulations were evaluated every 24 h, for 3 days, to select the optimal nanoemulsion for the stability and pharmaco-toxicological evaluation.

Particle size evaluation

The particle size and the polydispersity index were measured by dynamic light scattering (DLS), using a Zetasizer Nano-ZS (Malvern, UK). The nanoemulsion was appropriately diluted with Milli-Q water (1:25, v:v). Measurements were performed in

triplicate and the mean ± standard deviation was reported (Rodriguez *et al*., 2020).

Zeta potential

Zeta potential was determined, indirectly, using electrophoretic mobility measurements using a Zetasizer Nano-ZS (Malvern, UK). The nanoemulsion was appropriately diluted with Milli-Q water (1:25, v:v). Measurements were performed in triplicate and reported as the mean ± standard deviation (Rodriguez *et al*., 2020).

Rheology

Rheology of the nanoemulsion was assessed in a rotational rheometer (Brookfield R/S Plus, UK) with a cone-plate system (C50-1). The assay was performed at 25 ± 1°C using a Julabo water bath (GmbH, Germany). The flow and viscosity curves were constructed over a range of shear rates from 0 to 600 s^{-1} by measuring the produced shear stress (τ) and the viscosity (η). Different mathematical models were evaluated to select the model that better fix the rheological dataset. Finally, the consistency index (k) was calculated according to the Ostwald-de-Waele equation (selected model):

$$\tau = k \cdot (\Upsilon)^n \qquad (1)$$

where $\boldsymbol{\tau}$ was the shear stress, $\boldsymbol{\Upsilon}$ was the shear rate, and $\boldsymbol{n}$ was the flow index.

Effect of temperature and pH

The nanoemulsion (1 mL) was previously diluted with Milli-Q water (1:24, v:v) to a final volume of 25 mL. The sample was heated at a constant rate of 5°C/min from 5 to 75°C. The measurements were made every 10°C. Each measurement was made in triplicate and the result was graphically represented.

The effect of the pH in the particle size and the zeta potential of the nanoemulsion was evaluated by the titration technique using an MPT-2 titrator (Malvern, UK) coupled to Zetasizer (Malver, UK). It was measured the changes in particle size and zeta potential caused by pH variations. The nanoemulsion was diluted with Mili-Q water (1:9 v:v) to a final volume of 10 ml. NaOH 0.10 mol/L and HCl 0.10 mol/L were used as titrating solutions. The titrator was calibrated with buffer solutions (pH 4, 7, and 10, Alphatec, Brazil). The titration was made in two steps; firstly, from pH 1 to 5, and a fresh nanoemulsion was used for titrating from pH 6 to 10 (Rodriguez *et al*., 2020). Measurements were performed in triplicate, at 25°C, with an accuracy of 0.20 pH-units, using a voltage of 150 V (Malvern, 2015).

Shelf stability

The nanoemulsion was poured in an amber flask and kept at 25 ± 2°C for 180 days. Particle size, polydispersion index, zeta potential, and consistency index (k) were measured at 0, 15, 45, 90, and 180 days. The β-caryophyllene content of the nanoemulsion was also determined the days 0 and 180. In all cases, the measurements were performed in triplicate and reported as the mean with the standard deviation.

Zymosan-induced paw edema

The anti-inflammatory effect of EOC and the EOC loaded-nanoemulsion was evaluated in the zymosan-induced paw edema test (Pitsillides, 2003). Six to eight-week-old Swiss female mice, with a body weight of 20-25 g were used. The animals were kept for 10 days under standard laboratory conditions (25 ± 2°C; 65 ± 5% humidity), with a 12-12 h light-dark cycle, with *ad libitum* access to water and feed. All animals were fasted for 12 h before experimentation. The study was conducted according to protocol 039/2019 issued by the Ethics Committee for Experimental Animal Use, Federal University of Amazonas-Brazil.

Five experimental groups each containing five animals were created (n=5). Each animal was allocated by simple randomization into one of the five following groups:

- Group 1: blank nanoemulsion (a nanoemulsion obtained in the same conditions but without EOC)
- Group 2: received 150 µL of zymosan solution (10 mg/mL in saline solution)
- Group 3: sodium diclofenac, 50 mg/kg
- Group 4: EOC 100 mg/kg in saline solution plus 100 mg of Tween 80:Span 80 (HLB 11) used for solubilization.
- Group 5: EOC-loaded nanoemulsion, 100 mg/kg

Diclofenac was used as the standard anti-inflammatory drug (Group 2) and saline solution as the negative control (group 4) were injected into the left hind paw of each animal. The oral administration of substances was made with a gastric canula (Vigon, France) coupled to a discardable syringe, 30 minutes

before (Time = 0) the subplantar injection of zymosan (150 µg/paw) into the right hind paw of mice. The edema volume was determined by plethysmometry (NovaLab, Brazil) at time 0 and at 1, 3, and 6 h after the zymosan injection. The edema reduction was expressed as the percentage difference between the control value (paw volume before the injection) and volumes measured at each time after the treatment (Pitsillides, 2003).

Acute oral toxicity

The experiment was conducted using 4-week-old female Swiss mice, with a body weight of 20–30 g. The animals were supplied by the Central Biottery of the Federal University of Amazonas. The assay considered the rules established by the Ethics Committee of the Federal University of Amazonas, under the protocol number 039/2018. The animals were acclimated for 7 days at 25 ± 2°C and 65 ± 5% of humidity with a light/dark cycle of 12:12 h. Animals were given free access to food and water and were fasted for 12 h before the experiment.

The experiment was performed in accordance with the OECD 423 test procedure (2001). Two experimental groups of three animals each, were used. The control group (Group I) was treated with distilled water throughout the experiment; and the experimental group (Group II) received a single dose of 2000 mg/kg EOC-loaded nanoemulsion. Animals were observed for signs of toxicity, including possible changes in the posture, stool appearance, eyelid closure, piloerection, skin and coat appearance, salivation, mucosal appearance, and motor behavioral changes. Observations were made each hour for the first 4 h, and after this time, every 12 h for up to 14 days. Animals were euthanized using a ketamine injection (150 mg/kg i.p.). The internal organs (liver, heart, kidneys, lungs, and pancreas) were observed for possible signs of toxicity induced by the nanoemulsion (OECD 423, 2001).

Statistical analysis

The means ± standard deviation was calculated and the biological effect of the EOC and the EOC-loaded nanoemulsion were analyzed as specified above. For multiple comparisons, the ANOVA test followed by HSD Tukey's test was applied using the software StatGraphic XIV.1 (StatEasy, USA). In all cases, statistically significant differences were considered at p-value of 0.05.

RESULTS

Physicochemical properties of EOC

The hydrodistillation process yielded 15.26 % (w:w) of EOC (457.8 g). EOC is an oily transparent liquid with a light-yellow color, and a pleasant woody aroma. The organoleptic and physicochemical characteristics of the essential oil used for nanoemulsion development are shown in Table No. 1. The relative density, refraction index, viscosity, and pH are also presented.

Table No. 1
Organoleptic and physicochemical properties of the essential oil

Property	Value
Aspect	Oily transparent liquid
Color	Light yellow
Odor	Pleasant woody aroma
Relative density	0.894 ± 0.005
Refraction index	1.502 ± 0.142
Viscosity (Pa.s)	10.70 ± 1.25
pH	6.00 ± 0.50
β-Caryophyllene (%)	51.52 ± 1.20

All measurements were performed at 25°C

Chemical composition

The GC chromatogram (Figure No. 1) shows the presence of 14 volatile compounds, 91.60% of the total curve area was quantitated (Table No. 2). The main constituents were β-caryophyllene (40.60%), caryophyllene oxide (13.6%), and (*E*)-*α*-bergamotene (8.02%).

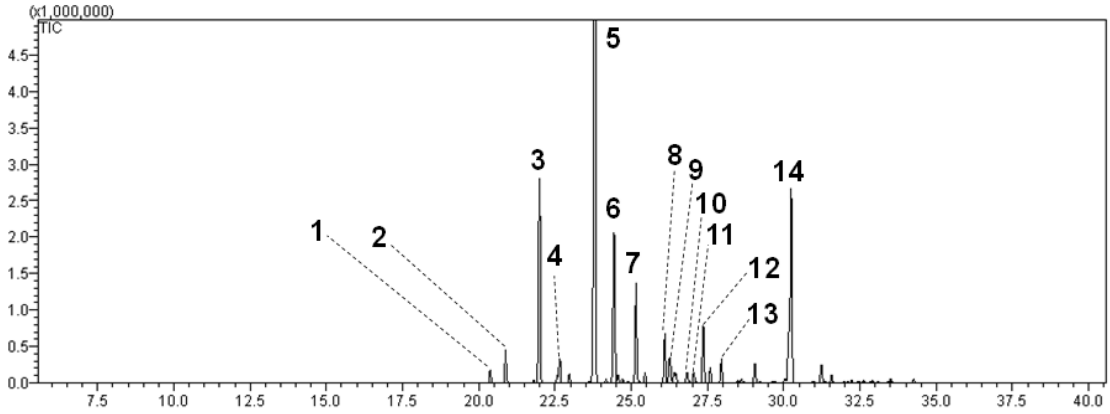

Figure No. 1
GC chromatogram of *Copaifera* spp. essential oil

Table No. 2
Chemical composition of the essential oil of copaifera *(Copaifera* spp.) oleoresin

Peak	Compound	Retention time	Retention index	Relative amount (%)
1	*δ*-Elemene	20.3	1338	0.61
2	*α*-Cubebene	20.8	1351	1.67
3	*α*-Copaene	21.9	1376	10.80
4	*β*-Elemene	22.6	1390	1.26
5	*β*-Caryophyllene	23.7	1408	47.00
6	(*E*)-*α*-Bergamotene	24.4	1412	8.02
7	*α*-Humulene	25.1	1438	5.29
8	*α*-Amorphene	26.1	1484	2.51
9	Germacrene D	26.2	1481	1.52
10	Ledene	26.8	1496	0.71
11	*δ*-Cadinene	27.0	1513	0.60
12	*β*-Bisabolene	27.3	1528	2.87
13	*α*-Cadinene	27.9	1538	1.31
14	*β*-Caryophyllene oxide	30.2	1583	13.60
	Coverage	-	-	91.50

Figure No. 2 shows the chromatogram of β-caryophyllene (Figure No. 2A) used as the internal standard and the chromatogram of EOC added with the standard (Figure No. 2B). The calibration curve coefficient of determination (R^2) was 0.9970, indicating the strong relationship between the analytical response (peak area) and the concentration of the β-caryophyllene in the sample.

$$\textit{Peak area} = 102.18 \times \textit{Concentration} + 177.19 \quad (2)$$

It was obtained a well-defined and pure peak at a retention time of 23.75 min (Figure No. 2). The content of β-caryophyllene in the EOC was 51.52 ± 1.20% (Table No. 1).

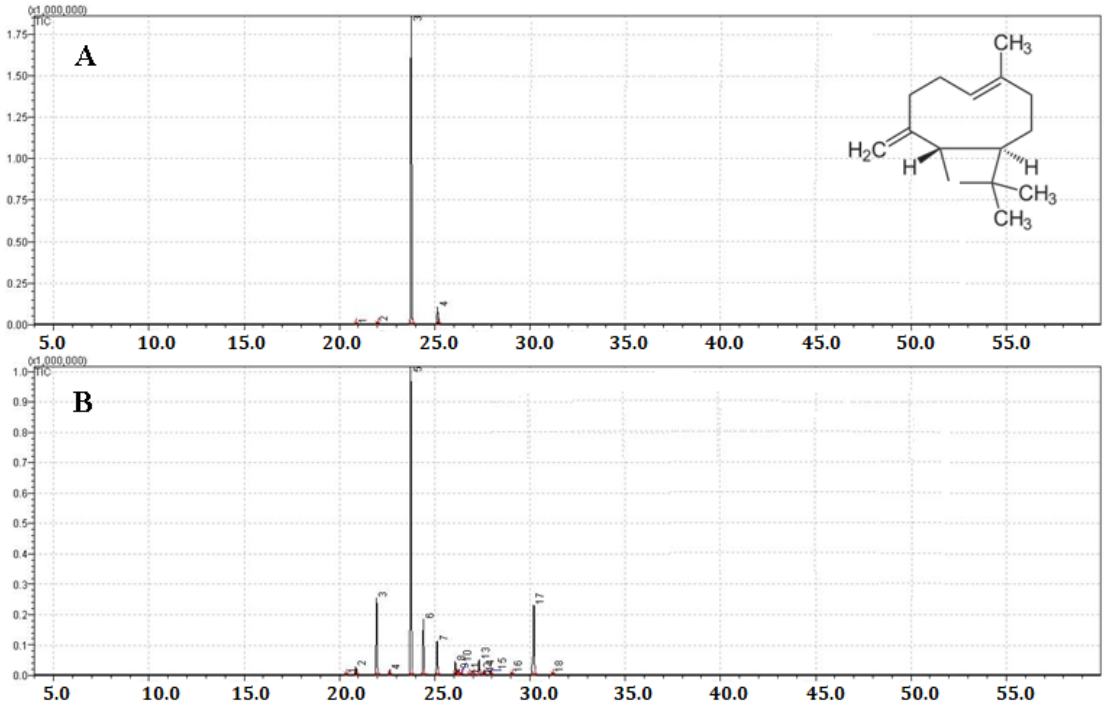

Figure No. 2
GC chromatograms for the quantitation of β-caryophyllene in the essential oil of *Copaifera* spp. A, chromatogram of the β-caryophyllene standard; B, chromatogram of the EOC added with standard showing the same retention time at 23.75 min

Required hydrophilic-lipophilic balance (HLB)
The variation in particle size obtained during the determination of the HLB required for the nanoemulsification of EOC is shown in Figure No. 3. The smallest particle sizes (82 and 101 nm) were obtained using HLB values of 11 and 12, respectively. However, 24 h after preparation, the nanoemulsion obtained using a surfactant with an EHL of 12 showed a high polydispersity index (0.241) and phase separation.

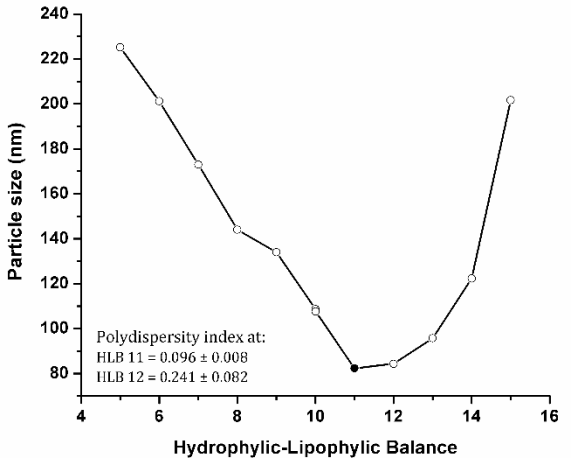

Figure No. 3
Particle size variation of nanoemulsions obtained during the process for determining the HLB required by essential oil of *Copaifera* spp

Selection of the optimal surfactant system

Three surfactant systems composed of pairs of non-ionic surfactants were evaluated to select the better one for preparing the nanoemulsion with the smallest particle size and the greatest homogeneity (smallest polydispersity index), as well as the highest modular value of zeta potential. Nonionic surfactants (Tween/Span) were used because they have low toxicity and are well tolerated orally at up to 15% in formulations (Rowe, 2009; USP, 2014). The effect of the three-surfactant systems on the main properties of the EOC-loaded nanoemulsions is shown in Table No. 3.

Table No. 3
Effect of the surfactant system (Tween-Span classes) on the main properties of the EOC-loaded nanoemulsions

Surfactant system	Day	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Conductivity (mΩ/cm)
Tween 20-Span 20	1	108.10 ± 1.96	0.329 ± 0,009	-35.00 ± 1.47	0.018 ± 0,004
	7	125.33 ± 4.51	0.366 ± 0,111	-30.26 ± 5.65	0.142 ± 0,097
Tween 60-Span 60	1	208.50 ± 3.01	0.253 ± 0.005	-33.70 ± 0.42	0.014 ± 0.006
	7	209.41 ± 26.47	0.292 ± 0.094	-26.93 ± 3.69	0.084 ± 0.077
Tween 80-Span 80	1	99.47 ± 0.64	0.224 + 0.010	-37.10 ± 0.26	0.009 ± 0.000
	7	126.31 ± 1.69	0.146 + 0.024	-37.10 ± 0.26	0.008 ± 0.000

The surfactant system Tween 80:Span 80 provided a nanoemulsion with small particle size and a low polydispersity index, as well as an appropriate zeta potential value (-37.10 mV). This nanoemulsion was evaluated for 72 h and no changes in its properties were observed. Thus, the surfactant system Tween 80/Span 80 was selected to optimize the amount of surfactant (ratio surfactant: oil: water ratio) to be used in the EOC-loaded nanoemulsion.

Surfactant: Oil: Water ratio

The ternary phase diagram for evaluating the effect of the SOW ratio on nanoemulsion' properties is shown Figure No. 4. Nanoemulsions with a smaller particle size and higher zeta potential (modular value) were obtained when using 10 and 13% of surfactant and EOC of 10 and 15% (Figure No. 4, light gray area). Nanoemulsions with SOW 13:10:77 and 13:15:72 (%, w:w:w) showed smaller particle sizes and polydispersion indices (Table No. 4).

To select the better nanoemulsion for the evaluation of the biological activity and the shelf stability, both nanoemulsion were observed for three days measuring (every day) the particle size and zeta potential (Table No. 4).

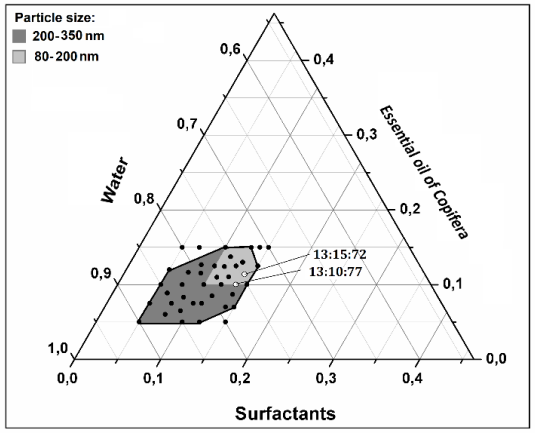

Figure No. 4
Ternary phase diagram representing the optimization process of the surfactant: oil: water ratio of the EOC-loaded nanoemulsions

Table No. 4
Physicochemical properties of the selected nanoemulsions obtained during the evaluation the effect of the Surfactant: Oil: Water ratio

Day	S:O:W (w:w:w)	Particle size (nm)	Polydispersity index	Zeta potential (mV)
	13:10:77	203.50 ± 1.83	0.329 ± 0.010	-20.00 ± 0.55
1	13:15:72	174.30 ± 1.01	0.136 ± 0.010	-23.60 ± 0.17
	13:10:77	213.50 ± 3.25	0.346 ± 0.079	-18.23 ± 1.65
2	13:15:72	173.60 ± 1.21	0.136 ± 0.069	-24.33 ± 1.21
	13:10:77	244.47 ± 4.62	0.360 ± 0.025	-17.15 ± 1.87
3	13:15:72	174.55 ± 1.32	0.133 ± 0.041	-24.60 ± 1.10

Effect of the temperature

The change in particle size and zeta potential of the EOC-loaded nanoemulsion produced by the temperature variation from 5 to 75°C are shown in Figure No. 5. Particle size was almost unaffected, remaining between 75 and 80 nm all the time. The polydispersity index tended to decrease as the temperature increase (showing an improvement of the homogeneity). In contrast, the zeta potential kept stable from 5 to 45°C, but showed a marked increase onwards 45°C, inverting the charge to a positive value (17.50 mV) at 55°C. Above 55°C, large variations in the zeta potential were observed, indicating nanoemulsion instability.

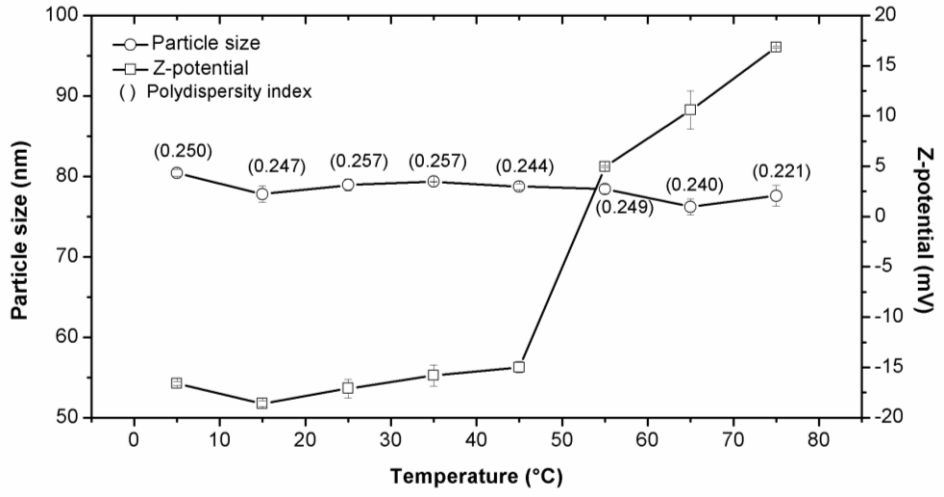

Figure No. 5
Effect of the temperature on particle size and zeta potential of the EOC-loaded nanoemulsion

Effect of pH

The particle size of EOC-loaded nanoemulsion showed good stability in the pH range between 2.5 and 10 (Figure No. 6). The isoelectric point of the nanoemulsion was detected at pH 1.94. The zeta potential remained practically constant from pH 2.5 to 6.0; however, above pH 6, the zeta potential decreased to -10.2 mV at pH 10.

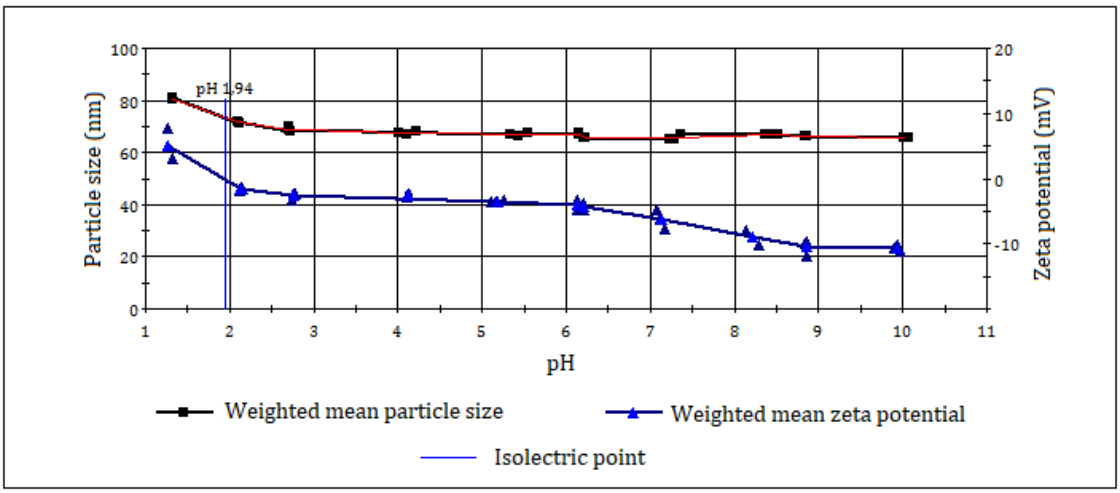

Figure No. 6
Effect of pH on particle size and zeta potential of the EOC-loaded nanoemulsion

Rheology

Different mathematics models were evaluated to select the best model for fitting the rheological dataset. The statistical descriptors of the evaluated models are presented in Table No. 5. The Oscar-de Waele model (The low power) was the better descriptor with the higher R^2 value (0.9898) and a lower *p*-value (0.0025) of the Fisher test.

Table No. 5
Mathematical models with the statistical descriptors for the evaluation of the rheological behavior of the EOC-loaded nanoemulsion

Mathematical model	Statistic model	R^2 value	Fisher test F, p-value
Newton	$\tau = 0.0142 \cdot \Upsilon$	0.9902	135.43; 0.0458
Oscar -de Waele	$\tau = 1.7977 \cdot \Upsilon^{0.4953}$	0.9999	149.82; 0.0025
Herschel-Burkley	$\tau = 0.0021 + 1.7310 \cdot \Upsilon^{0.5120}$	0.9908	128.75; 0.0332

τ, shear stress; τ_0, yield stress; Υ, shear rate; η, viscosity; n, flow index; k, consistency index

The rheological behavior of the EOC-loaded nanoemulsion is shown in Figure No. 7. A classical pseudoplastic behavior (shear-thinning) is well-described by the Oscar-de Waele equation (Figure No. 7A). The nanoemulsion exhibited thixotropic characteristics, with a decrease in viscosity as the shear rate increased (Figure No. 7B, viscosity curve).

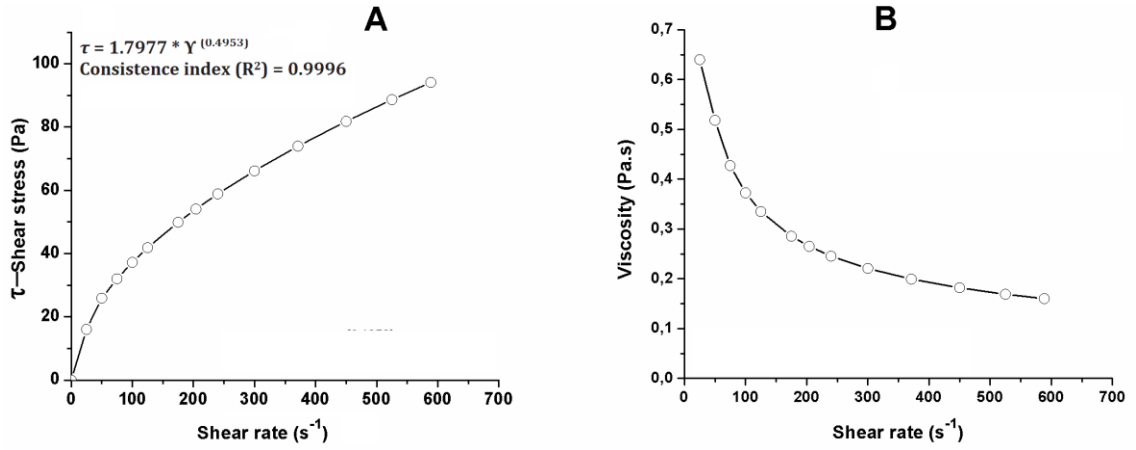

Figure No. 7
Rheological properties of the *Copaifera* spp. essential oil-loaded nanoemulsion.
A - Flow curve; B - viscosity curve

Nanoemulsion stability

The shelf stability of nanoemulsion containing 15% of EOC, 13% surfactant (Tween 80-Span 80), and 72% water (SOW 13:15:72, %) for 180 days stored at 25°C and at 65% environmental humidity is shown Figure No. 8. The figure shows the particle size distribution with the particle diameter, polydispersity index, and the zeta potential value over time (0, 15, 45, 90, and 180 days). After 6 months of storage, the particle size remained practically unchanged (98-99.5 nm). The polydispersity index decreased from 0.220 at time 0 to 0.175 after 180 days. Similarly, zeta potential remained stable between -24.45 and -26.11 mV.

The content of β-caryophyllene remained statistically equal over time, showing 7.42 ± 0.14% at time 0, and 7.30 ± 0.23% after 180 days (t-test=0.65, p=0.5489).

The consistency index (k) (Oscar-de Waele model) of the EOC-loaded nanoemulsion over 180 days is shown in Figure No. 9. A decrease was observed from day 0 to day 15; however, after 15 days, the variation of K over time did not show statistically significant variation (F=22.35; p=0,9501).

Images of the essential oil-loaded nanoemulsion at time 0 and at 180 days after the nanoemulsion preparation are shown in Figure No. 10.

Anti-inflammatory activity

The anti-inflammatory effect of the copaifera essential oil-loaded nanoemulsion and the whole essential oil is presented in Figure No. 11. Neither the saline solution injection nor the blank nanoemulsion induced inflammatory processes in the animals' paw. In contrast, the zymosan injection induced strong paw inflammation, which was reduced by the treatment with the essential oil-loaded nanoemulsion and in small amount by the treatment with the pure essential oil (Figure No. 11). The anti-inflammatory effect produced by the nanoemulsion (84.58 ± 7.35%) was not statistically different from the effect produced by diclofenac (93.54 ± 9.21%) at p<0.05.

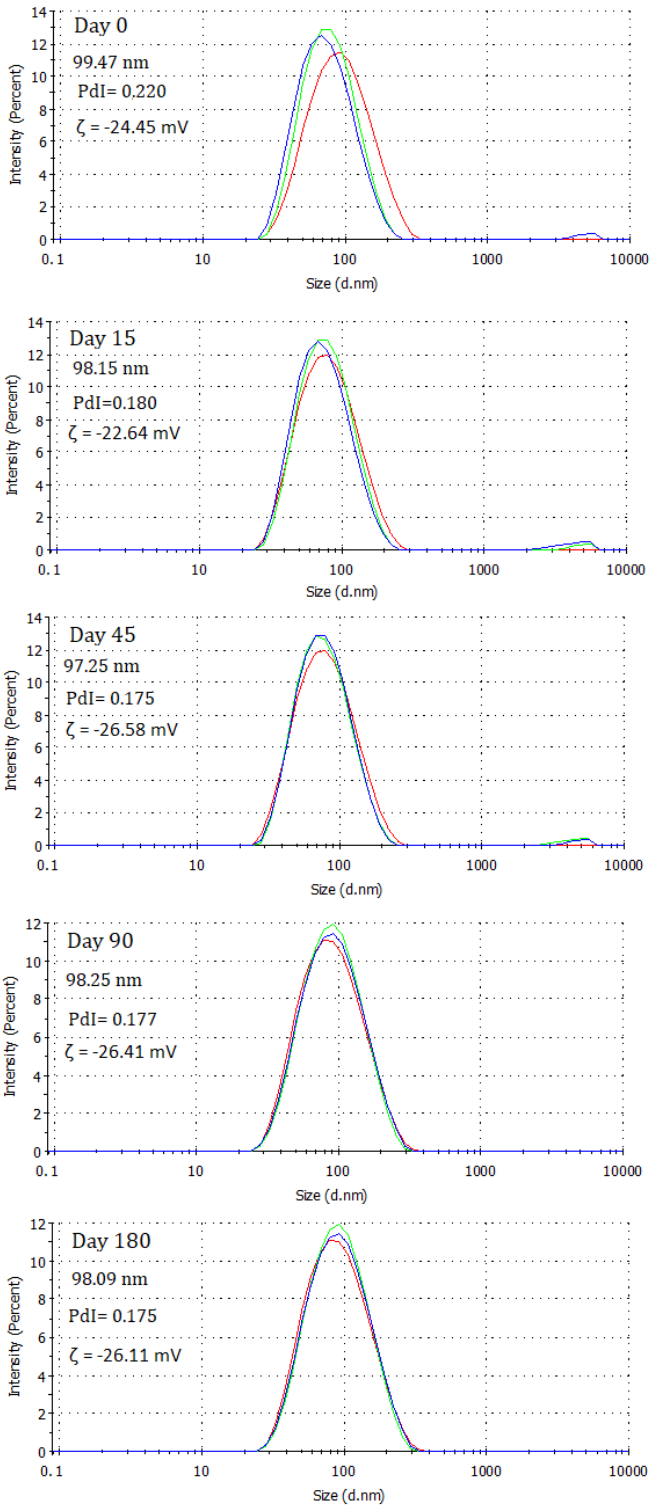

Figure No. 8
Particle size distribution of the EOC-loaded nanoemulsions during storage for 180 days

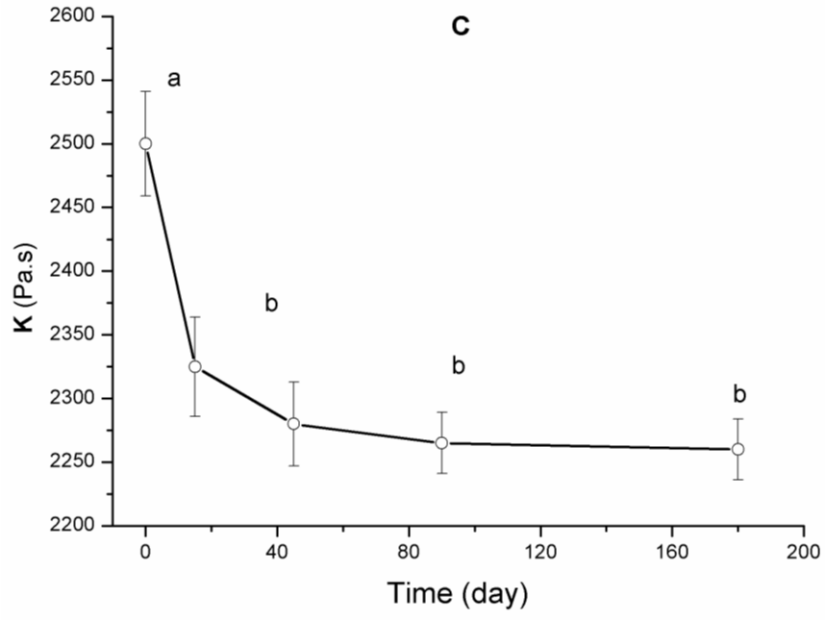

Figure No. 9
Variation in the consistency index (k) of EOC-loaded nanoemulsion over time.

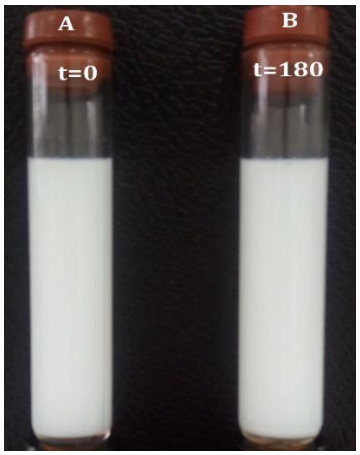

Figure No. 10
Photograph of the EOC-loaded nanoemulsion at time 0 and time 180 days after the preparation

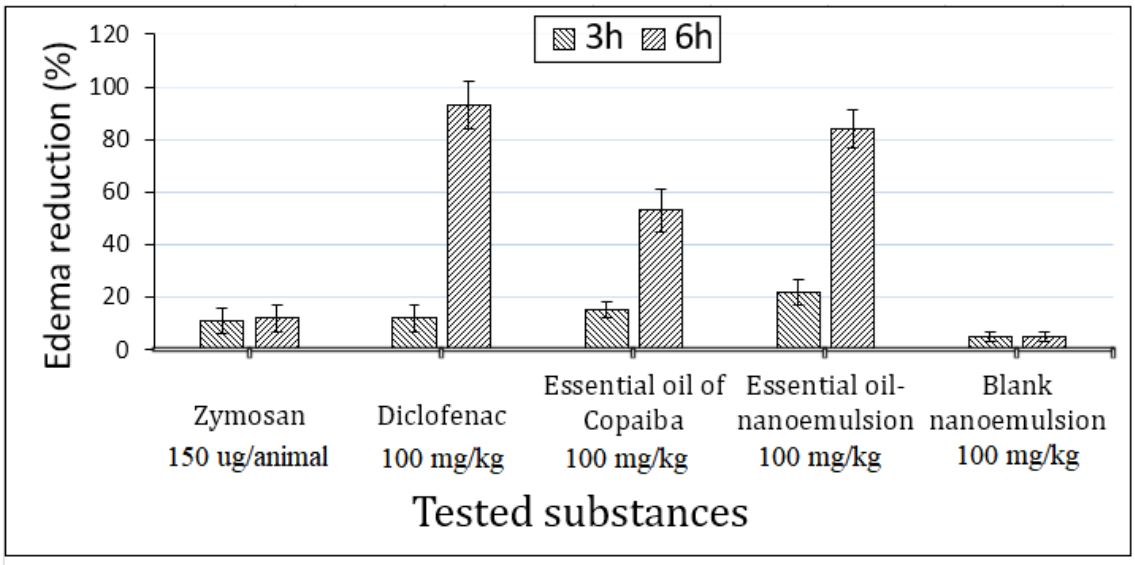

Figure No. 11
Anti-inflammatory effect of *Copaifera* spp. essential oil-loaded nanoemulsion and the whole essential oil in zymosan-induced paw edema. Different letters in the columns mean statistically significant difference at $p<0.05$

Acute oral toxicity

The highest concentration administered (2000 mg/kg) did not induce death in the animals. Abnormal signs were not observed in the mobility, appearance, color of the mucosae, and the eyes of the animals. In the experiment, the animals consumed water and feed normally. The body weight in both groups increased normally, with no statistically significant differences between them (Table No. 6). Similarly, no signs of toxicity were observed in the internal organs of animals (heart, kidneys, liver, lungs, and pancreas). In both groups, it was observed a normal color and appearance, like those shown by the control group. No statistically significant differences in the relative weight of the internal organs of both groups were observed (Table No. 6).

Table No. 6
Bodyweight of animals and the relative organ weight in both groups during the acute oral toxicity evaluation

Time (Days)	Measured	Weight in percent		
		Group I	Group II	p-value
Day 1		22.45 ± 2.37	23.88 ± 1.66	0.4937
Day 7	Bodyweight	24.97 ± 2.11	25.53 ± 2.27	0.8573
Day 15		27.58 ± 1.57	28.91 ± 2.08	0.2521
	Kidney	0.65 ± 0.25	0.75 ± 0.14	0.3347
	Pancreas	0.33 ± 0.18	0.29 ± 0.36	0.4394
Day 15	Heart	0.35 ± 0.17	0.33 ± 0.21	0.5819
	Lungs	0.61 ± 0.22	0.55 ± 0.34	0,8421
	Liver	3.88 ± 0.54	4.01 ± 0.47	0.5222

Group I, control group; Group II, EOC-nanoemulsion. n=5, statistical difference of t-test at $p<0.05$

DISCUSSION

The crude oleoresin from distinct species of the *Copaifera* genus have distinct chemical compositions and pharmacological potencies, which have limited their use in pharmaceuticals (Gomes *et al*., 2007, 2010; Veiga *et al*., 2007). However, the composition of the essential oil extracted from the crude oleoresin (EOC) has almost the same constituents, no matter the species from which it is obtained (Freitas *et al*., 2007; Sousa *et al*., 2011).

The hydrodistillation process yielded 15.26% (457.8 g) of EOC, which can be considered an excellent result. Unfortunately, there is neither reports about the obtainment nor the standardization of processes for obtaining the essential oil of *Copaifera*. EOC appears as a clear, translucent, yellow liquid, with pleasant woody aroma, characteristic of the genus. EOC had a viscosity of 10.70 cPs, a pH of 6, a relative density of 0.894, and a refractive index of 1.502 (Table No. 1). These properties agreed with those reported by Freitas *et al*. (2006), for the EOC obtained from other species of the *Copaifera* genus. Thus, these parameters could be used preliminarily for quality control of the EOC.

Among the chemical constituents of EOC, β-caryophyllene is the main compound (Figure No. 1, Table No. 2). β-caryophyllene is also the main constituent of the essential oil obtained of all *Copaifera* species (Freitas *et al*., 2007; Veiga *et al*., 2007; Sousa *et al*., 2011; Leandro *et al*., 2012). β-Caryophyllene is a bicyclic sesquiterpene, present as a mixture of the trans-caryophyllene isomers, α-caryophyllene and β-caryophyllene oxide (Fidyt *et al*., 2016). A β-caryophyllene content of 51.52% in EOC was determined by gas chromatography (Figure No. 2). The high concentration of β-caryophyllene in COR and EOC can explain the anti-inflammatory utility of these Amazonian products (Dahham *et al*., 2015). However, the low water solubility of sesquiterpenes hampers their inclusion in pharmaceutical formulations (Fidyt *et al*., 2016), however, the EOC-loaded nanoemulsion developed in this work improve the water solubility of the EOC, facilitating its uses in formulations.

The HLBr for the optimal emulsification of EOC was used for selecting the type, composition, and the proportion of surfactant necessary for nanoemulsion formulation. Nanoemulsions with the most homogeneous particle size distribution (lowest polydispersity index) and the smallest particle sizes were obtained using surfactants with EHL values of 11 and 12 (Figure No. 3). As the nanoemulsion prepared using surfactants with an HLB value of 11 had a small particle size and a monomodal particle size distribution (polydispersity index, 0.096), the HLBr for EOC was established as 11. Conversely, the use of surfactant with an HLB value of 12, produced a nanoemulsion with a high polydispersity index (0.241), and a bimodal particle size distribution (graphs not shown). According to Griffin (1949), emulsions with good stability and small particle size are obtained when the HLB of the surfactant is equal to the HLBr of the oily phase. There is no reference in the literature of the determination of the HLBr of EOC.

For the development of the EOC-loaded nanoemulsion, three pairs of nonionic surfactants were evaluated with the compositions described in Table No. 3. Evaluation of the surfactant system that achieved the smallest particle size and smallest polydispersity index was performed to maintain good stability. The surfactant system Tween 80:Span 80 produced a stable nanoemulsion (126.31 nm) with excellent homogeneity (polydispersity index 0.146), a zeta potential of -37.10 mV, and a decrease in conductivity along the time. It seems that surfactants coated completely the EOC, providing good stability. The latter statement appeared true, because the smaller the radius of particles at the interphase and the thicker the interfacial layer formed by surfactants (Tween 20:Span 20 < Tween 60:Span 60 < Tween 80:Span 80) leads to a lower probability of deformation of the interfacial layer, the loss of thickness, and the droplet breakage to produce coalescence (Saifullah *et al*., 2016). The surfactant system Tween 80:Span 80 formed a thick absorption layer, to obtain nanoemulsions with greater thermodynamic stability.

Dias *et al*. (2014), prepared nanoemulsions using the whole oleoresin of *C. multijuga* Hayne using high-pressure homogenization and spontaneous emulsification methods. In both methods, the organic solvents used were medium-chain triglycerides, acetone, and/or a mixture of acetone: ethanol (50:50, v/v). From a technological perspective, the simpler the formula, the better the technological processes for scaling up and production. On the other hand, the use of organic solvents could produce undesirable residues and impact the bioavailability of nanoemulsified drugs. Different the abovementioned study, the EOC-loaded nanoemulsion was formulated without organic solvents. On the other side, in our work the homogenizing process was made in only one cycle at a low speed (5000 rpm) resulting in a

more reproducible method as compared with multiples cycles (six cycles) using high-pressure homogenization at 760 bars (Dias *et al*., 2014).

The ternary phase diagram obtained for optimizing the amounts of nanoemulsion components is shown in Figure No. 4. The changes in nanoemulsion properties produced by variations in the relative amounts of ingredients are shown in Figure No. 4. Nanoemulsions with SOW 13:10:77 and 13:15:72 had the smallest particle sizes (203.50 and 174.30 nm, respectively), with polydispersity indices of 0.329 and 0.136, respectively, Table No. 4). At 72 h after preparation, the nanoemulsion with SOW 13:10:77 increased its particle size and showed a bimodal size distribution (graph not shown) with a polydispersity index of 0.360, which is a sign of instability (Ostwald ripening). In contrast, the nanoemulsion with SOW ratio 13:15:72 showed a stable particle size and a reduction in polydispersity index from 0.136 to 0.133. This nanoemulsion also showed an increase in zeta potential (modular value). This behavior could be associated with the formation of a thick and dense interfacial layer of surfactants (13%) in a smaller volume of water (72%), which reduced the particle mobility and the interaction due to Brownian motion. These factors may have favored the absorption of the oily drops in the interfacial layer, reducing the possibility of Ostwald ripening, enhancing the thermodynamic and kinetic stabilization of the system (Rodriguez *et al*., 2020). Thus, the nanoemulsion containing 15% EOC, 13% surfactants (Tween 80:Span 80), and 72% of water was selected for stability studies and pharmaco-toxicological evaluation.

The effect of the temperature on nanoemulsion properties is shown in Figure No. 5. For particle size and size distribution, almost no variation was observed for a temperature up to 80°C. Thus, the surfactant system (Tween 80:Span 80) appeared to produce an effective steric barrier, avoiding the particles collision with the subsequent formation of new, larger droplets. However, above 45°C, the zeta potential decreased in modulus, with a charge inversion at 55°C, which is a sign of instability. The temperature enhanced the mobility of particles, increasing the interactions between the ions present in the double electric layer with counter-ions at the interphase, which could cause charge neutralization, and inversion of the charge above 55°C. This result suggested that, to increase the scale of production of the EOC-loaded nanoemulsion with prolonged shelf storage, and even for transportation of this product, the temperature should be maintained below 55°C.

The effect of pH on nanoemulsion properties is shown in Figure No. 6. EOC-loaded nanoemulsions showed good stability from pH 1 to 5, with practically no variation in particle size and zeta potential. Unlike other nanoemulsion systems (McClements, 2010, Teo *et al*., 2016), at pH values close to the isoelectric point (pH 1.94), the particle size and zeta potential of the EOC-loaded nanoemulsion showed good stability. This may have occurred because non-ionic surfactants, such as Tween and Span, tend to stabilize nanodroplets by forming a strong steric barrier (Jaworska *et al*., 2015), rather than the electric effect produced by pH on the double layer. This result suggested that a pH below 5 (from 1 to 5) did not affect the stability of EOC-loaded nanoemulsions. On the other hand, this fact suggests that the EOC entrapped in the nanoemulsion could be released in the intestine, however, this statement must be adequately assessed.

Changes in product rheology suggest variations in the internal structure that result in systems deterioration. The EOC-loaded nanoemulsion showed a non-Newtonian behavior (pseudoplastic/shear-thinning, Figure No. 7A) that was described by the Oscar-de Waele model (Table No. 5). The nanoemulsion showed thixotropy (Figure No. 7B), which is characteristic in systems with a volume-fraction of internal phase between 5% and 30% (Helgeson, 2016). It seems that the relatively high concentration of nanodroplets in the EOC-loaded nanoemulsion (≈28%) allows hydrodynamic and colloidal interactions among the droplets, modifying the viscosity, especially when subjected to different shear stresses (Figure No. 7B) at a constant temperature (25°C).

The stability of EOC-loaded nanoemulsions was evaluated in shelf storage for 180 days at 25 ± 2°C and 65% of ambient humidity, in a tightly closed amber flask. As shown in Figure No. 8, after 6 months of preparation, the nanoemulsion exhibited a narrow particle size distribution (polydispersity index 0.175) and low particle size (98.09 nm, Figure No. 8A), with almost no variation when compared with the size observed at time 0 (99.48 nm, Figure No. 8F). This behavior is a sign of good nanoemulsion stability. Coalescence and the phase separation of nanoemulsions with a volume-fraction of the internal phase between 5 and 30% occurred essentially due to van der Waals attractive forces (Tadros *et al*., 2011; Jaworska *et al*, 2015). The opposite effect (repulsion

and the maintenance of stability) occurs because of steric repulsions among the surfactants and/or through electrical repulsions caused by a high zeta potential (Tadros *et al*., 2011). It appears that using a pair of nonionic surfactants, such as sorbitan esters (*e.g.* Span 80) and its ethoxylated derivatives (e.g. Tween 80) provided a good steric stabilization, keeping the particle size and the size distribution practically constant throughout the stability study.

The rheologic behavior of EOC-loaded nanoemulsions at 0, 7, 15, 45, 60, and 90 days was also evaluated as a stability criterion. The flow behavior over time provided valuable information regarding the stability of the nanosystem. The variation of the consistency index (k, from the Oscar-de Waele equation) over time is shown in Figure No. 9. A slight reduction in *k* was observed, which indicated the thixotropic behavior of the nanoemulsion. However, after day 15, the reduction in *k* over time was not statistically significant ($F=0.29$, $p=0.8341$). Sinko (2008), reported that for nanoemulsions, the polydispersity index increased as the viscosity was reduced. This fact could explain why the consistency index of the EOC nanoemulsion was not significantly different after day 15, which was most likely due to the polydispersity index that was generally constant.

Over time, the EOC-loaded nanoemulsion showed the same milky aspect observed in the freshly prepared product (Figure No. 10). The β-caryophyllene content was statistically unchanged ($t= 0.65$, $p=0.5489$) over time ($7.42 \pm 0.14\%$ at time 0; $7.30 \pm 0.23\%$ at 180 days), which indicates the good chemical stability of the nanoemulsion. It seems that the similar chemical structure of the surfactants (Tween 80:Span 80) resulted in a well-sealed nanomicelle encapsulating all the essential oil providing excellent physical and chemical stability for up to 6 months.

The main problem on the use of EOC and the crude oleoresin of *Copaifera* spp. for preparing pharmaceuticals is the poor water solubility. In this work, it was developed an EOC-loaded nanoemulsion, which, in addition to solving the low water solubility of EOC, reduced zymosan-induced paw-edema in mice. The anti-inflammatory potency of the nanoemulsion was stronger than that of EOC and was statistically equal to diclofenac (50 µg/kg). This effect could be produced by β-caryophyllene, a bicyclic sesquiterpene phytocannabinoid that is the main component of EOC (Fidyt *et al*., 2016). β-Caryophyllene has strong analgesic (Klauke *et al*., 2014; Quintans *et al*., 2016) and anti-inflammatory effects (Oliveira *et al*., 2018) owing to its structure as a selective agonist of the cannabinoid receptor 2 (CB2) (Gertsch *et al*., 2008). The anti-inflammatory effect of EOC and the EOC-loaded nanoemulsion (Figure No. 11) could be, at least in part, explained by the effect of β-caryophyllene in the CB2 receptor-dependent pathway for the reduction of inflammatory cytokines (Gertsch *et al*., 2018), and a high concentration in lipid membranes (Sarprieto *et al*., 2015). CB2 receptors agonists can ameliorate experimental arthritis, reducing INF-g, IL-17 cytokines, and bone damage (Fukuda *et al*., 2014). Although β-caryophyllene is found in high concentrations in some essential oils, it has poor water solubility, impairing its permeability in biological fluids (Sarpietro *et al*., 2015). This potential obstacle to the improvement of the *in vivo* anti-inflammatory properties of both EOC and EOC-derived β-caryophyllene was overcome in this work by the development of EOC-loaded nanoemulsions.

The acute oral toxicity was performed in mice to evaluate the *in vivo* toxicity of the nanoemulsion. At a dose of 2000 mg/kg, neither death of animals nor abnormal signs related to motility, appearance, and the color of mucosae were observed. The body weight in both experimental groups increased, with no statistically significant difference (Table No. 6). No signs of toxicity were observed in the internal organs (the heart, kidneys, liver, lungs, and pancreas), which showed normal color and appearance, like those in the control group. Similarly, the relative organ weight of the animals in both groups was not statistically different (Table No. 6), suggesting that the use of this product was safe in this animal model. Thus, the EOC-loaded nanoemulsion was shown to be a non-toxic substance in the acute oral toxicity test (OECD 423, 2001). However, more studies should be performed to ensure the safety use of this product.

CONCLUSIONS

A novel nanoemulsion loaded with essential oil of *Copaifera* spp. was developed, using 15% essential oil, 13% surfactants (Tween 80:Span 80, at HLB=11), and 72% Milli-Q water. The EOC-loaded nanoemulsion containing 7.42% of β-caryophyllene showed a particle size of 99.47 nm, a polydispersity index of 0.220, and a zeta potential of -24.45 mV. The system showed a classical pseudoplastic/thixotropic behavior, with excellent stability at pH between 1 and 5 and temperatures

between 5 and 55°C. In addition, the nanoemulsion properties remained practically constant, with excellent stability after shelf storage for 6 months. After 6 h, the anti-inflammatory effect of the nanoemulsion was more potent than the EOC, and similar to diclofenac 50 mg/kg. The nanoemulsion showed no oral acute toxicity (in mice) at 2000 mg/kg; hence, it can be considered nontoxic. The development of an EOC-loaded nanoemulsion will add value to both the COR and the EOC by increasing their potential for commercialization in several countries, owing to the suitability of this new form for use in the pharmaceutical industry.

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