



## Original Article

# *Pterodon pubescens* oil nanoemulsions: physicochemical and microbiological characterization and *in vivo* anti-inflammatory efficacy studies



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## ABSTRACT

*Pterodon pubescens* (Benth.) Benth., Fabaceae, fruits have been investigated for their anti-inflammatory and antinociceptive activities, and have demonstrated effectiveness in inflammatory conditions. A physicochemical and microbiological stability study was conducted to investigate two nanoemulsion-based delivery systems of two different hydrophilic surfactants (polyethylene glycol-40H castor oil or polyethylene glycol-40 castor oil). The nanoemulsions, containing *P. pubescens* oil, lecithin, hydrophilic surfactant and water, were analyzed for droplet size distribution, polydispersity index, pH, consistency index, stability against centrifugal force, and active content/vouacapan derivatives. The physicochemical characteristics were followed for 365 days. The nanoemulsion system was evaluated for anti-inflammatory activity by using with a peritonitis model, immediately after preparation and after 365 days of storage at 25 °C. The stability study demonstrated that proper storage (25 °C) preserved the characteristics of the nanoemulsion containing 7.5% polyethylene glycol-40H castor oil, 5% lecithin, and 5% *P. pubescens* oil. Further, it ensured a shelf life of 365 days as a phytotherapeutic formulation. In the peritonitis assay induced by carrageenan, nanoemulsion prepared with polyethylene glycol-40H castor oil (125 mg/kg) reduced leukocyte migration, even after 365 days of storage (25 °C), highlighting its potential for the treatment of inflammatory diseases. However, further studies are needed to confirm its clinical effectiveness.

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## Introduction

Several phytotherapeutic formulations have been used as effective alternatives for the treatment of diseases (Parveen et al., 2015). The anti-inflammatory (Coelho et al., 2005; Hoscheid et al., 2013; Pascoa et al., 2015) and antinociceptive activities (Nucci et al., 2012; Servat et al., 2012) of the oil from *Pterodon* sp. fruits have been demonstrated. However, to date, drugs containing this oil are not available on the market (Hoscheid and Cardoso, 2015). Similar to several drugs, drugs containing *Pterodon* sp. fruit oil are associated with low bioavailability (due to instability, low permeability, and low solubility) (Parveen et al., 2015). Thus, in recent years, pharmaceutical industries have focused on improving the permeability and bioavailability of poorly water-soluble compounds (Alam et al., 2012). Nanoemulsions (NE) have been

shown to increase the therapeutic efficacy of several drugs and improve physical and chemical stability (Parveen et al., 2015).

As regards the interface of the NE, lecithin uses has been described, because have a unique set of properties including biocompatibility, biodegradability and low to absent toxicity (Washington, 1996). Systems stabilized by phospholipids have appropriate carriers as drug delivery systems (Baspinar and Borchert, 2012; Schuh et al., 2014). Polyethylene glycol castor oil (PEG) derived are nonionic surfactants, which are being used increasingly in oral, topical and parenteral pharmaceutical formulations; particularly suitable for the production of liquid preparations containing volatile oils, vitamins and other hydrophobic substances (Ran et al., 2001). O/W emulsions' are often stabilized using ionic and nonionic ethoxylated surfactants. As a rule, such disperse systems are stable against flocculation due to steric and electrostatic stabilization (Koroleva and Yurtov, 2012). Product stability refers to the physical and chemical integrity of the dosage unit and where appropriate, the drug's ability to maintain protection against microbiological contamination. An ideal product should be fully characterized (physically, chemically, and

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microbiologically) at baseline and over the desired shelf life (Alam et al., 2012).

Droplet size is an important factor when assessing the stability of emulsion systems. Changes in droplet size are generally due to aggregation and coalescence thorough of micelles (Petsev et al., 1995). Coalescence and molecular diffusion (also called Ostwald ripening) in NE systems are responsible for controlling the aging process. Ostwald ripening is often the dominant aging process, which occurs when a molecular or macromolecular stabilizer is present in sufficient amount in the interface and consists of spontaneous diffusions of small droplets to form larger droplets (Kong and Park, 2011).

Rheological behavior is essential in stability studies. A reduction in viscosity during the storage of a kinetically unstable emulsion leads to the displacement of the droplets, which subsequently collide with each other, resulting in coalescence thorough. Thus, changes in flow behavior with respect to time are important and can provide essential information regarding the stability of the system (Badolato et al., 2008).

Chemically, a change in the pH of formulations may indicate degradation and/or ionization of one or more of the constituents of the formulation. Moreover, these chemical transformations reflect the system incompatibility and can lead to toxic effects when administered to patients (Alam et al., 2015).

Taking advantage of the lipid characteristics of the extract of *Pubescens pubescens* fruits, our group previously developed and characterized NE containing *P. pubescens* oil (Hoscheid et al., 2015). Following our previous study, we evaluated the physical, chemical, and microbial stability, and anti-inflammatory potential of NE based-formulation of *P. pubescens* oil.

## Materials and methods

### Chemicals

Polyethylene glycol (PEG)-40 castor oil/sorbitan oleate and PEG-40 hydrogenated castor oil/sorbitan oleate (PEG-40H) were kindly provided by Oxiteno (São Paulo, Brazil). Purified soybean lecithin (Phospholipon 90G) was obtained from Lipoid (Ludwigshafen, Germany). Ultra-purified water (Milli-Q<sup>®</sup> Plus, Millipore Corporation, Billerica, MA, USA) was used for preparation of all aqueous solutions. Sabouraud dextrose agar and Tryptic soy agar (TSA) were purchased from BD<sup>®</sup> (Difco<sup>™</sup>, Dickinson and Company, Bedford, Massachusetts, USA).  $\lambda$ -Carrageenan, used as a phlogistic agent to induce inflammation, was obtained from Sigma-Aldrich (Auckland, New Zealand).

### Plant material and oil extraction

*Pterodon pubescens* (Benth.) Benth., Fabaceae, fruits were obtained from Nossa Senhora do Livramento, Mato Grosso, Brazil (15°89' S; 56°41' W). The taxonomic identity was confirmed by Dr. Germano Guarim Neto, and a voucher specimen (no. 20502) was deposited in the Herbarium of the Universidade Estadual de Maringá.

Oil extraction was performed as previously reported (Hoscheid et al., 2012). Briefly, *P. pubescens* oil were extracted with ethanol by turbo extraction (Ultra-Turrax UTC115KT, IKA Works, Wilmington, NC, USA) and partitioned with hexane. The organic solvent was evaporated in a vacuum rotary evaporator (Büchi<sup>®</sup> R-210, Flawil, Switzerland) at 40 °C until the solvent evaporated completely.

### NE preparation

NE was prepared at the optimal conditions that have been previously described (Hoscheid et al., 2015). The oil phase consisting

of *P. pubescens* oil (5%, w/w) and a lipophilic emulsifier (soybean lecithin 5%, w/w) was previously homogenized and injected into the aqueous phase consisting of water and a hydrophilic emulsifier (PEG-40H or PEG-40, 7.5%, w/w), in a high-speed shear apparatus (IKA<sup>®</sup> T10 basic, Wilmington, DE, USA) at 14,500 rpm for 15 min. The pH was adjusted to 7.4 with NaOH solution (1 M).

### Stability study

The study was performed according to the Cosmetic Stability Guide (Anvisa, 2004). NE (10 ml) was packed in glass containers (with 15 ml capacity) with pressure cap, and kept in incubation chambers (BINDER<sup>®</sup>) at different storage temperatures and conditions: room temperature (25 ± 1 °C), intermediate temperature with humidity (30 ± 1 °C with 75% relative humidity), and high temperature with humidity (40 ± 1 °C with 75% relative humidity). At pre-determined intervals (30, 60, 90, 120, 150 and 180 days for NE stored at 30 and 40 °C; and 30, 60, 90, 120, 150, 180, 270 and 365 days, for NE stored at 25 °C), samples were removed from the storage and physicochemical characteristics were evaluated. The free *P. pubescens* oil was stored under the same conditions and temperatures to compare the chemical stability of the free oil to that of the NE. Samples removed from the incubation chambers were maintained at room temperature (25 °C) and analyzed immediately. The withdrawal timetable of the samples was strictly followed. Droplet size distribution, polydispersity index (PDI), morphology, pH, consistency index, physical stability against centrifugal force, and vouacapan content of the NE were evaluated.

### Assessment of stability by physicochemical characterization

#### Droplet size distribution and morphology

The average droplet size and PDI were determined by dynamic light scattering (DLS) in a particle analyzer (Nanoplus nano/zeta particle analyzer, Georgia, USA). NE was diluted with ultrapure water (pH 7.4) in the ratio of 1:10, to minimize the multiple scattering effects prior to each experiment. The analyses were carried out in triplicate to determine the average values. The size was expressed in nm.

The morphology and formation of aggregates were assessed by transmission electron microscopy (TEM) (JEOL JEM 1400 Transmission Electron Microscope, Peabody, MA, USA). NE was placed on formvar/Carbon 400 mesh copper grid (Ted Pella, Redding, CA, USA) and negatively stained with 2% phosphotungstic acid. After 24 h were observed at 120 kV (magnification 10k).

#### pH

A calibrated potentiometer (TECNAL, São Paulo, Brazil) was used to measure the pH of the NE samples at 25 ± 1 °C.

#### Consistency index

Rheometry was conducted using a MARS II (Haake<sup>®</sup>) controlled stress rheometer (Thermo Fisher Scientific Inc., Newington, Germany) in flow mode with controlled shear rate (CR), at 25 ± 1 °C, and in conjunction with parallel steel cone-plate geometry (35 mm, 2° angle, and separated by a fixed distance of 0.052 mm). Flow curves were measured over a range of shear rates (0–250 s<sup>-1</sup>). The shear rate was increased over a period of 150 s, maintained at the upper limit of 10 s, and then decreased over a period of 150 s. The consistency index was analyzed according to the Ostwald-de-Waele equation (power law).

#### Sedimentation behavior

The physical stability of the NE was examined in a multisampling analytical centrifuge (LUMiSizer<sup>®</sup>, LUM GmbH, Berlin, Germany).

This analyzer simulates phase separation processes under gravitational forces (Cadddeo et al., 2014) by means of infrared light emitting in the infrared length of the cells, during centrifugation and transmission through the sample; hence, separation kinetics can be studied under accelerated conditions. Scans are repeated over time, each producing a curve, and all curves are superimposed on a graph to evaluate stability over time. Migration of particles, due to centrifugal force, leads to variation in the local concentration of particles and therefore, variations in the transmission will occur, indicating system instability since the overlap of transmission profiles over time highlights the quality of the colloidal dispersion (Ng et al., 2013). The NE were centrifuged at  $4000 \times g$  force for 13 h and 44 min at  $20^\circ\text{C}$ , conditions established by the equipment for evaluating the equivalent to three years of physical stability.

#### *Vouacapan content*

The recovery and chemical stability of vouacapan derivatives were investigated using a previous validated method (Hoscheid et al., 2015) of gas chromatography coupled to mass spectrometry in the selected-ion monitoring (SIM) mode (GC–MS–SIM) (Thermo Electron Corporation DSQ II; TLC, Thermo Fisher, Boston, MA, USA). Aliquots of NE for each time and temperature analysis were frozen and lyophilized. To the oily residue,  $1500 \mu\text{l}$  of chloroform was added and  $1 \mu\text{l}$  was injected. Vouacapanes were quantified using the calibration curve. The recovery was calculated by dividing the experimental concentration by the theoretical concentration and then multiplying by 100.

#### *Statistical analysis*

All determinations were performed in triplicate with two samples for each time and temperature, and represented as the means  $\pm$  standard deviations. The effects of storage duration and temperature were statistically analyzed using two-way analysis of variance (ANOVA) with the R-3.2.2 software.

#### *Microbiological stability*

The number of viable pathogens in NE was determined according to the standards set by the Brazilian Pharmacopoeia V (Farmacopéia Brasileira, 2010). The evaluation was performed using the pour plate technique. Briefly, fresh NE was diluted with sterile saline in the ratio of 1:10 and then 1 ml was transferred to Petri dishes. The solution was homogenized in culture medium (15 ml), allowed to solidify, and then incubated in refrigerated BOD incubators (Biogenic Oxygen Demand, TE-390 model, TECNAL, Brazil). The culture media employed were as follow: Sabouraud dextrose agar for fungi and yeasts, and TSA Agar for bacteria. Storage conditions were as follow: 7 days at  $28^\circ\text{C}$  to determine the presence of fungi and 48 h at  $35^\circ\text{C}$  for total heterotrophic bacteria.

To determine microbiological stability, NE was reevaluated after 365 days of storage at  $25^\circ\text{C}$ . All analyses were performed in triplicate.

#### *Stability assessed by the anti-inflammatory effect of the NE*

##### *Animals*

The experimental procedures were approved by the Ethics Committee of the Universidade Estadual de Maringá (protocol number 3968190615). The peritonitis experiment was conducted with Swiss mice (weighing 30–40 g). Mice were housed under controlled conditions ( $22 \pm 2^\circ\text{C}$  with a 12 h light/12 h dark cycle and free access to food and water).

#### *Anti-inflammatory activity of the NE*

The anti-inflammatory activity was evaluated by carrageenan-induced peritonitis. Pharmacological experiments were conducted with the NE that showed the best performance during the stability study, i.e., NE containing 7.5% of PEG-40H castor oil as the hydrophilic surfactant.

Groups of mice ( $n = 5$ ) were administered intramuscularly with different doses of NE (31.25, 62.50, 125, and 250 mg/kg). Sterile saline was used as the control. After 1 h, animals received an intraperitoneal carrageenan injection ( $500 \mu\text{g}/\text{animal}$ ). After 4 h, the animals were anesthetized with xylazine ( $10 \text{ mg}/\text{kg}$ ) and pleural exudates were collected and the number of leukocytes was determined using the Neubauer chamber.

The anti-inflammatory activity of NE was evaluated after 365 days of storage at  $25^\circ\text{C}$ , at a dose of 125 mg/kg, corresponding to a decrease of 60% in leukocyte migration.

#### *Statistical analysis*

Data were expressed as the mean  $\pm$  standard error of the mean (SEM). Results were statistically analyzed using the GraphPad software (GraphPad Software, Inc., San Diego, CA). Analysis of variance (ANOVA) post hoc Tukey's test was used to compare the compare.  $p < 0.05$  was considered statistically significant.

## **Results and discussion**

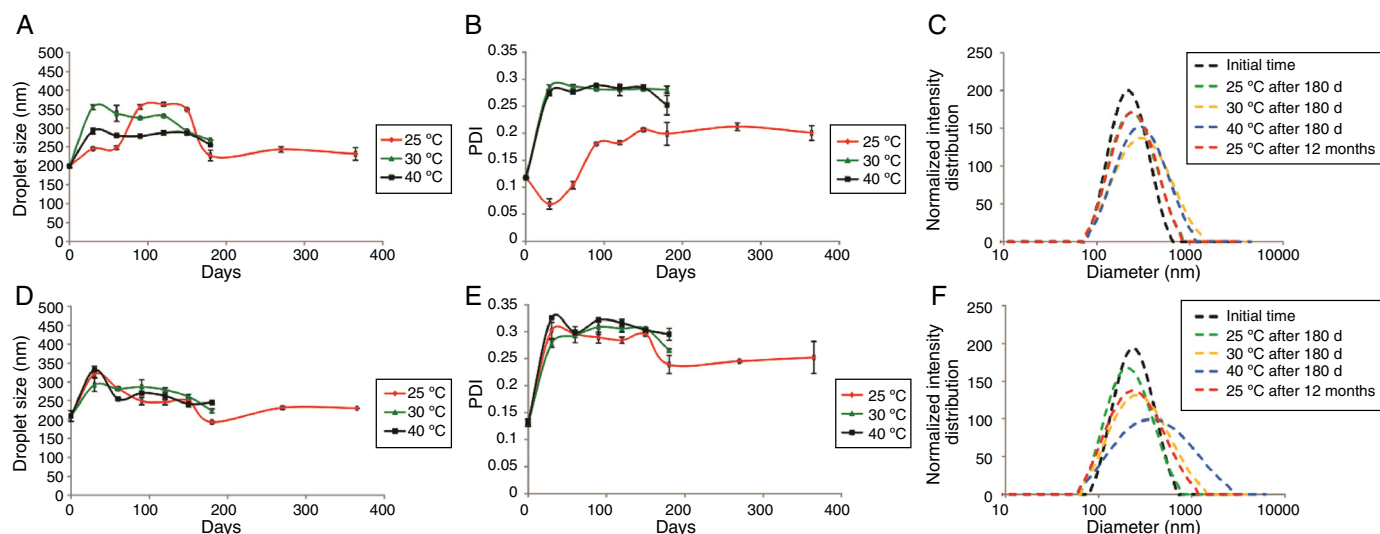
#### *Physicochemical stability evaluation*

The purpose of stability testing is to ensure the quality of the active compound or the pharmaceutical product, over time, under the influence of several factors, such as environment, temperature, humidity, and light. Further, stability study establishes a product shelf life and recommends storage conditions. An ideal product should be physically and chemically characterized (Ali et al., 2014). Our optimized NE was characterized by droplet size, PDI, morphology, pH, consistency index, physical stability against centrifugal force, and vouacapan content, at different times and temperatures.

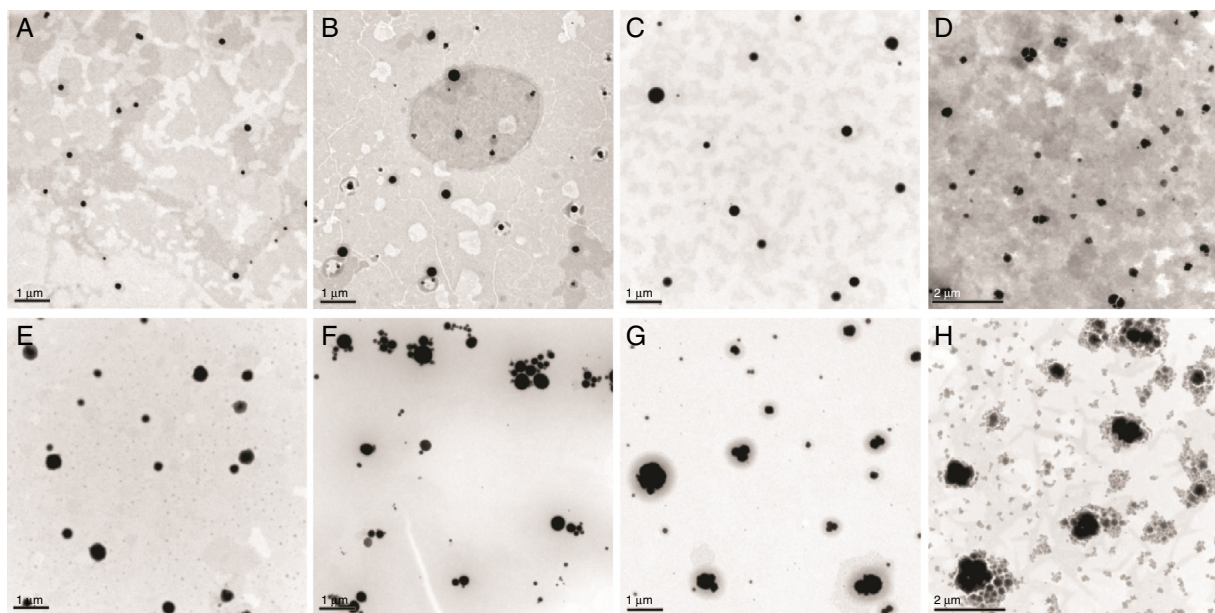
From a practical standpoint, it is important that NE remain physically stable during storage. Therefore, we determined the changes in droplet size over time, at different temperatures (Fig. 1). In general, a unimodal distribution profile was observed for all NE, with slight increase in the average droplet size and PDI as a function of time, and the increase in droplet size was the highest in formulations stored at 30 and  $40^\circ\text{C}$  (Fig. 1C and F). The increase in droplet size and PDI were lower in formulations containing 7.5% PEG-40H castor oil (Fig. 1B), which showed PDI less than 0.3 throughout the test period. Taking into consideration the long duration of the study, this result indicates a consistent system and reflects the overall stability of this formulation (Junyaprasert et al., 2009; Galindo-Alvarez et al., 2011; Li and Ge, 2012).

Fig. 2 shows the morphology of the droplets by TEM and indicates that the droplets were not perfectly spherical, i.e. exhibit anisotropy, which is a common feature of soft warts. Furthermore, the photomicrographs demonstrated that aggregation (Fig. 2F) and coalescence thorough were pronounced (Fig. 2G and H) in the NE prepared with PEG-40 castor oil. On the other hand, NE prepared with PEG-40H castor oil showed signs of aggregation just after 365 days of storage (Fig. 2D).

Colloidal particles must collide to cause aggregation. The collision may be caused by Brownian motion or generated by shear field. In both cases, if the energy of the collision impact is greater than the energy of the electrical barrier and/or mechanical energy, the particles will aggregate, otherwise the particles will move away



**Fig. 1.** Average droplet size, PDI, and normalized intensity distribution of NE prepared with 7.5% PEG-40H (A, B, and C) and 7.5% PEG-40 (D, E, and F) respectively, at different time and temperature of storage.



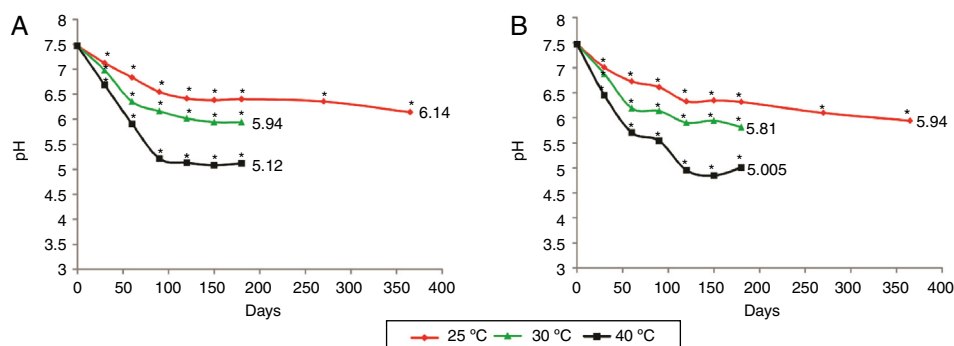
**Fig. 2.** TEM image, at 120 kV (magnification 10k) of NE prepared with 7.5% of PEG-40H (A, B, C, and D) and PEG-40 (E, F, G, and H) at the start of the experiment, after 180 days at 25 °C and 40 °C, and after 365 days storage at 25 °C, respectively.

from each other (Han et al., 2004). Aggregation does not imply an increase in droplets size, but is a coalescence through precursor, which together with Ostwald maturation induces increase in droplet size (Kong and Park, 2011). Furthermore, according to Li and Ge (2012), the solubility of nonionic surfactant changes as the temperature increase, and high temperatures can lead to the breakage of hydrogen bonds at the surfactant surface, resulting in loss of stability and facilitates aggregation and coalescence through (Fig. 2G).

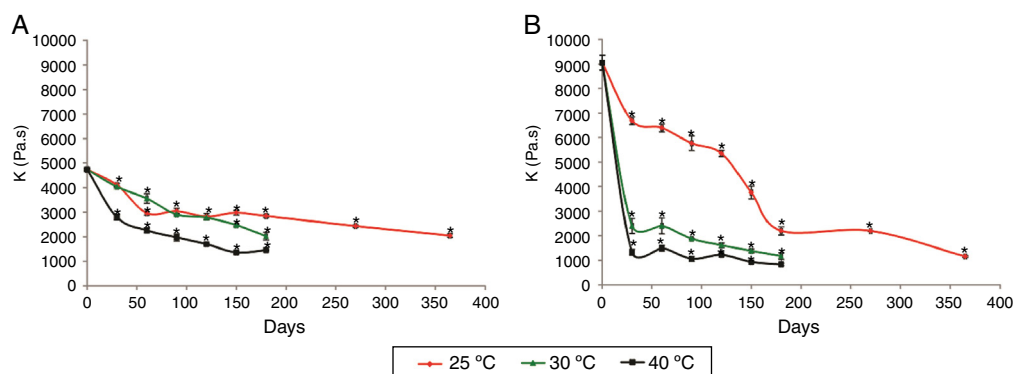
It is important to monitor pH to evaluate the stability of emulsions since pH change indicates the occurrence of chemical reactions and may compromise the final product quality. It can be seen that regardless of the surfactant, the reduction in pH showed similar kinetics (Fig. 3), with significant differences observed only

among 0, 30, and 60 days, and statistically similar values were observed after 60 days (25 °C). According to the Cosmetic Stability Guide (Anvisa, 2004), it is important that during formulation packaging of stability study, the volume of the formulation is not at the maximum volume of the bottle and maintains a gap of approximately one third of the container capacity for possible gas exchange.

A reduction in pH can be observed in emulsions containing plant oils due to the hydrolysis of the esters of fatty acids into free fatty acids (Martini, 2005; Bernardi et al., 2011). Moreover, high temperatures can destabilize NE via lecithin hydrolysis (Bernardi et al., 2011). Thus, pH decline during the first 60 days can be due to oil phase oxidation or triacylglycerides hydrolysis, owing to the presence of oxygen in the bottles. Acidification was more pronounced



**Fig. 3.** pH values of NE at different time and temperature of storage. (A) Formulations prepared with PEG-40H. (B) Formulations prepared with PEG-40. \* $p < 0.05$  compared to the initial time with Tukey's test.



**Fig. 4.** Consistency index (K) of NE at different time and temperature of storage. (A) Formulations prepared with PEG-40H. (B) Formulations prepared with PEG-40. \* $p < 0.05$  compared to the initial time with Tukey's test.

as the temperature increase but this did not affect the overall quality of the NE containing PEG-40H castor oil, since at ideal storage conditions (25 °C), pH remained above 6.0, which is an acceptable level for parenteral formulations (Ghosh and Jasti, 2004).

Fig. 4 shows the consistency index of the NE. Increasing shear gradients were applied to the formulations to determine the flow parameters and ensure the quality of the formulations under stress conditions. A reduction in the consistency index was observed in the NE that were subjected to high temperatures and an inverse proportional relationship was demonstrated. In most emulsions, the consistency index decreased at high temperature due to the activation energy generated through heat, which is necessary to break the hydrogen bonds responsible for the association of molecules and facilitated movement (Sinko, 2008).

Moreover, it was found that the viscosity decreased over time in all formulations. This fact can be explained, in part, by the PDI of the droplets. The wider distribution of droplet size, the lower the viscosity, compared to systems containing narrower PDI (Sinko, 2008). In this study, we observed that the PDI increased during storage, which may be attributed to the reduction in consistency index.

It is believed that viscous emulsions are more stable than less viscous emulsions, due to the delay in flocculation and coalescence thorough. However, the stability of emulsion depends on other factors, which can be assessed by the flow behavior of the NE as a function of time. For all samples, thixotropic behaviors were observed, with a small area of hysteresis. As the samples aged, all NE containing PEG-40H castor oil showed the same behavior, while NE with PEG-40 castor oil showed rheopectic behavior (data not shown). Thus, to promote stability, which is more important

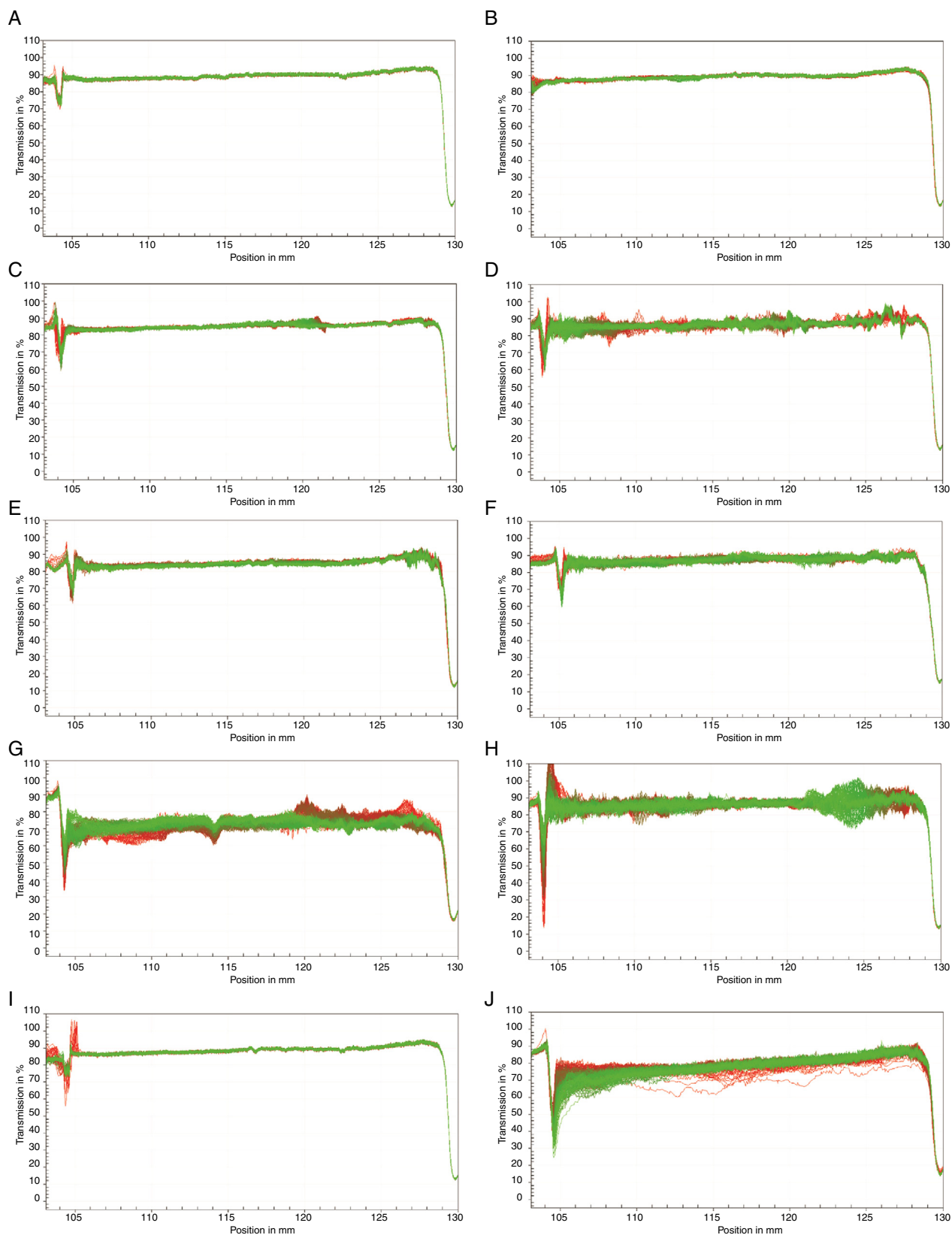
than developing a high viscosity formulation, is to achieve ideal characteristics for the system. Viscosity alone does not indicate stable emulsions; it is only a small influence of the overall O/W emulsions' stability (Sinko, 2008). The NE prepared with PEG-40H castor oil (Fig. 4A) showed a lower initial consistency index, due to hydrogenation on PEG chains, and lower rate of change of the consistency index compared to those of the NE with PEG-40 castor oil, which is indicative of greater stability of this system.

The kinetic demixing was analyzed by a transmission profile sequence using a multisampling analytical centrifuge (Fig. 5), which allows optical characterization of any type of dispersion in a more rapid and sensitive manner than with the naked eye.

After 180 days of storage at different temperatures and 365 days at 25 °C, a series of smooth curves in a centrifugal field were observed and showed minimal changes, which confirmed the physical stability of colloidal dispersions.

Degradation of free oil and oil contained in the NE are shown in Fig. 6. Rapid degradation of vouacapans was observed with the free oil at all tested temperatures, resulting in yields of 19.16% after 30 days at 40 °C and 18.70% after 120 days of storage at 25 °C. In addition, NE prepared with 7.5% PEG-40 castor oil suffered more accelerated degradation compared to those prepared with PEG-40H castor oil. The kinetic model of degradation was non-linear and suggests that complex reactions occurred owing to the variability of the sesquiterpenes, and linear and cyclic diterpenes. Thus, a more detailed and thorough analysis of the degradation model is required.

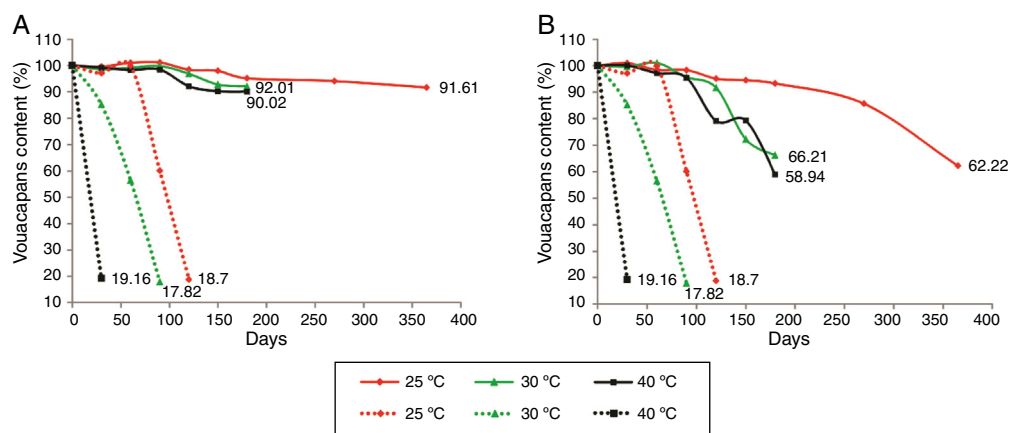
However, pharmaceutical formulations are considered stable if they maintain an active level  $\geq 90\%$  of the initial concentration (Glass and Haywood, 2006). The results indicated that the systems



**Fig. 5.** Physical stability against centrifugation force of NE prepared with PEG-40H and PEG-40 castor oil, at the initial time (A and B respectively), after 180 days of storage at 25 °C (C and D, respectively), after 180 days storage at 30 °C (E and F, respectively) and 40 °C (G and H, respectively), and after 365 days storage at 25 °C (I and J, respectively).

were influenced by storage temperature and the chemical stability of the formulations at 40 °C was lower than those at 25 and 30 °C were. However, NE containing PEG-40H castor oil showed slow degradation and maintained vouacapans content greater than

90% after 180 days at 30 °C (content: 92.01%) and 365 days at 25 °C (content: 91.60%), which indicates a chemical balance between the formulation and the lipid components. This highlights the stability of the NE during the desired period.



**Fig. 6.** Vouacapans content (%) in NE at different time and storage temperatures. (A) Formulations prepared with 7.5% of PEG-40H (solid lines) and free *Pterodon pubescens* oil (dashed lines). (B) Formulations prepared with 7.5% of PEG-40 (solid lines) and free *P. pubescens* oil (dashed lines).

The instability of NE is primarily due to the interactions between active contents and excipients via oxidation, hydrolysis, photolysis, and thermolysis (Alam et al., 2015). The low degradation of vouacapans in NE containing PEG-40H castor oil indicates low risk of chemical instability and highlights the importance of preparation, storage, and transportation at appropriate conditions to preserve stability and bioactivity.

#### Microbiological stability

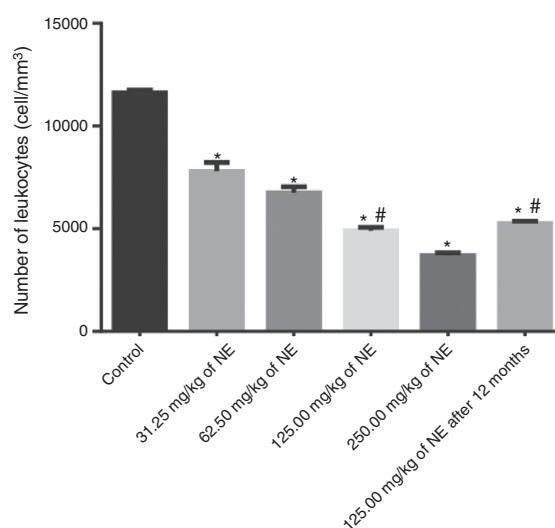
Parenteral drugs need to be sterile to ensure the stability of the product and prevent detrimental effects to the patient. Microbial contamination is associated with the loss of therapeutic efficacy due to degradation of the active ingredients, or changes in physical and chemical parameters (Baumer et al., 2011). Therefore, to confirm microbiological stability, fresh NE (24 h after preparation) and NE stored for 365 days at 25 °C, were evaluated for viable pathogens. All NE were subjected to microbiological analysis and showed absence of bacterial and/or fungal. These results demonstrate that the NE were well prepared.

The microbial quality of the final product can be affected by direct sources of contamination (water, raw materials, packaging material, and production environment) and indirect sources, resulting from cleaning equipment and surgical scrub and/or training operator (Eguchi, 2011).

Previous studies have confirmed the antimicrobial activity of oil extracted from *Pterodon* genus (Menna-Barreto et al., 2008; Dutra et al., 2009; Bustamante et al., 2010) suggesting that microbiological stability is related to the presence and antimicrobial property of *P. pubescens* oil.

#### Anti-inflammatory evaluation

During an inflammatory response, leukocytes circulating in the blood leave the intravascular compartment and migrate into the inflamed site via chemotaxis, to remove the injurious agent and promote tissue repair (Nourshargh and Alon, 2014). However, uncontrolled inflammatory response accompanied by severe leukocyte migration can lead to tissue damage. Therefore, the development and use of drugs that inhibit chemotaxis may be an interesting therapeutic strategy for the treatment of inflammation (Kummer et al., 2015). Thus, the anti-inflammatory properties of *P. pubescens* oil NE, prepared with PEG-40H castor oil, were evaluated using the peritonitis test induced by carrageenan in Swiss mice.



**Fig. 7.** Effect of the NE-based formulation of *Pterodon pubescens* oil, prepared with PEG-40H castor oil, on carrageenan-induced peritonitis (500 µg/i.p.) in Swiss mice. Values expressed as the mean ± standard error of the mean (n = 5 animals/group). \*p < 0.0001 compared to the control group (ANOVA, Tukey's test). #Do not show statistical difference between them.

The NE showed significant anti-inflammatory effect and drastically reduced inflammation in a dose-dependent manner compared to that of the control group (Fig. 7).

The results for the NE-based formulation of *P. pubescens* oil were in agreement with the findings of studies that demonstrated the anti-inflammatory activity of fresh oil (Cardoso et al., 2008; Hoscheid et al., 2015; Pascoa et al., 2015) and isolated compounds (Galceran et al., 2011) of *Pterodon* sp. fruits.

Since changes in evaluated physicochemical parameters are not necessarily related to the loss of therapeutic efficacy (Eguchi, 2011), evaluation of anti-inflammatory activity was performed to investigate the therapeutic efficiency versus the stability of NE. Thus, anti-inflammatory activity was reassessed after 365 days of storage at 25 °C, at a dose of 125 mg/kg.

After 365 days, we observed small reduction in the anti-inflammatory effect (6.92%) compared to that at the initial time, however, this reduction was not statistically significant, indicating that the therapeutic effect was preserved. Interestingly, it was observed that the percentage of reduction in anti-inflammatory activity, after 365 days, was relatively close to the percentage of vouacapans degradation (8.40%) in the same period (data shown in Fig. 6),

suggesting a relation between vouacapan derivatives content and therapeutic efficacy.

This study was the first to report the anti-inflammatory activity of a NE-based system of *P. pubescens* oil and the results encourage the progress of the research, since the system showed significant inhibitory effects on the migration of leukocytes and appears promising for the treatment of inflammatory diseases. Further studies should be conducted to complete the anti-inflammatory efficacy of this NE, and to evaluate its mechanism of action, possible toxic effects, and perform pre-clinical and clinical trials, in the near future.

## Conclusion

This study demonstrated that proper storage (at 25 °C) during transport and storage can preserve the contents present in the droplets of the NE, ensuring a physical, chemical, and microbial stability of 365 days, and potential anti-inflammatory activity of formulation prepared with 7.5% PEG-40H castor oil, 5% lecithin and 5% *P. pubescens* oil. On the other hand, the free oil showed rapid and drastic degradation. Furthermore, the system solved inconveniences related to low water solubility. Note that co-adjuvants were absent in the NE, such as antioxidants and preservatives, and the addition of these could extend the shelf life of the system. Finally, a significant inhibitory effect on leukocyte migration was demonstrated with the NE, by carrageenan-induced peritonitis in mice, which is an important process of the inflammatory response. Thus, this system may be useful in treating inflammatory diseases with excessive leukocyte migration. However, further investigations are needed to confirm this.

## Ethical disclosures

**Protection of human and animal subjects.** The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

**Confidentiality of data.** The authors declare that no patient data appear in this article.

**Right to privacy and informed consent.** The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

## Authors' contributions

JH, PMO and SAK (PhD student) contributed in running the laboratory work, analysis of the data and drafted the paper. PRNG and RKNC contributed to biological studies. MMSL contributed to rheometry analysis and contributed to critical reading of the manuscript. JH and MLCC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

## Conflicts of interest

The authors declare no conflicts of interest.

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