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Original Article

Pterodon pubescens oil nanoemulsions: physiochemical 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 physiochemical and microbiological stability study was conducted to investigate two nanoemulsion-based delivery systems of two different hydrophilic surfactants (polyethylene glycol (PEG)-40H castor oil or PEG-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% PEG-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 PEG-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.

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 (NEs) 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 NES, 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® 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® (Difco™, Dickinson and Company, Bedford, Massachusetts, USA). λ-Carrageenan, used as a phlogistic agent to induce inflammation, was obtained from Sigma-Aldrich (Auckland, New Zealand).

Plant material and oil extraction

P. pubescens 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® 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® 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®) 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 NEs 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®) 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^{-1}). 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 NEs was examined in a multi-sampling analytical centrifuge (LUMiSizer®, LUM GmbH, Berlin, Germany). This analyzer simulates phase separation processes