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The incorporation of *Pterodon pubescens* fruit oil into optimized nanostructured lipid carriers improves its effectiveness in colorectal cancer

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ABSTRACT

The 2^3 full factorial design was used to optimize the development of nanostructured lipid carriers (NLCs) containing *P. pubescens* fruit oil by the melt-emulsification method. The effects of the type of solid lipid, concentration of Phospholipon® 80H and type of aqueous surfactant in parameters such as particle size, polydispersity index (PI), zeta potential (ZP), total content (TC), and encapsulation efficiency (EE) of vouacapans were evaluated. The preparation method of the NLCs was appropriate as the chemical profile of the oil remained unchanged before and after the development process. The studied factors proved to affect the NLCs quality factors at different significant levels. The NLC that presented the best results was the formulation F4, which was produced with Precirol® ATO 5, 0.5% Phospholipon® 80H, and PEG-40 hydrogenated castor oil/sorbitan oleate. The optimized formulation showed small and spherical particles of 94.47 ± 2.05 nm, a PI of 0.197 ± 0.003, ZP less than –30 mV, and excellent physical stability after dispersion analysis. In addition, high values (> 98%) of the TC and EE of vouacapans were obtained. X-ray diffraction, Fourier transform Raman spectroscopy and differential scanning calorimetry analysis were also performed. The results suggested that lipids may be partially recrystallized and less ordered in NLCs and that between the *P. pubescens* oil and lipid matrix an interaction may exist. The *P. pubescens* fruit oil presented *in vitro* cytotoxicity to HT-29 cells, with CC_{50} of 273.47 μg/mL, and was more cytotoxic when incorporated into NLCs (CC_{50} of 154.19 μg/mL), which justifies the use of such technology in the development of novel phytopharmaceutical products.

1. Introduction

Colorectal cancer is considered the third most common cause of death in the world. It is the third and fourth most common type of cancer in women and men respectively (Feng et al., 2015). The treatment of this illness involves surgery for the removal of the tumor and adjuvant therapy is often necessary, as local recurrence of the disease occurs in 3–50% of cases (Allardice et al., 1994). Antineoplastic chemotherapy is one of the main therapeutic modalities applied, in which the frequently used active principles include 5-fluorouracil, oxaliplatin, mitomycin, and doxorubicin (Feng et al., 2015). However, the low specificity and high toxicity of the active substances, propensity to induce resistance, and the presence of many side effects are problems that are frequently observed (Wong et al., 2007). Thus, owing to the prevalence and severity of the disease, the search for new substances is necessary.

Natural substances are excellent sources for the development of new medicines. A significant number of natural drugs are used to improve prognosis and treat several types of cancer, with minimal side effects (Kojima-Yuasa et al., 2015; Patel and Gogna, 2015).

Abbreviations: NLC, nanostructured lipid carrier; NLCs, nanostructured lipid carriers; SLNs, solid lipid nanoparticles; PpO, *Pterodon pubescens* fruit oil; TSL, type of solid lipid; PC, Phospholipon® 80H concentration; TAS, type of aqueous surfactant; TEM, transmission electron microscopy; XRD, X-ray diffraction; FT-Raman, Fourier transform Raman spectroscopy; DSC, differential scanning calorimetry; P80H, Phospholipon® 80H; PEG-40H, PEG-40 hydrogenated castor oil/sorbitan oleate; GC–MS, gas chromatography–mass spectrometry; SIM, single ion monitoring; DoE, design of experiments; RSM, response surface methodology; ANOVA, analysis of variance; R^2, coefficient of determination; D_{50}, average diameter of particles; PI, polydispersity index; DLS, dynamic light scattering; ZP, zeta potential; TC, total content of vouacapans; EE, encapsulation efficiency; CC_{50}, the concentration that was cytotoxic to 50% of the cells

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The *Pterodon pubescens* Benth. species (Fabaceae/Leguminosae), popularly known as "sucupira branca", is a native species of central Brazil, widely used in folk medicine for its antinociceptive (Coelho et al., 2005) and anti-inflammatory activities (Hoscheid et al., 2013). Previous studies have demonstrated the antiproliferative activity of *P. pubescens* fruits in several cell lines, which have been attributed to the presence of vouacapane diterpenoids (Oliveira et al., 2017; Vieira et al., 2008). Spindola et al. (2009) investigated the cytotoxic effect of several compounds isolated from *P. pubescens* seeds on HT-29 cells *in vitro*, and obtained promising results.

Currently, novel drug delivery systems, such as nanoparticles, have been used to target anticancer substances at the site of action with increased efficacy and reduced side effects compared with conventional dosage forms (Jung et al., 2017; Lu et al., 2017). Nanostructured lipid carriers (NLCs), a new generation of solid lipid nanoparticles (SLNs), are an alternative to traditional carriers, and are an aqueous colloidal dispersion with a biodegradable and low toxic solid lipid matrix with a certain content of liquid lipid, resulting in an unstructured lipid matrix (Beloqui et al., 2017; Vitorino et al., 2013).

NLCs present important qualities of SLNs (physical stability, protection against the degradation of labile compounds, controlled release, *etc.*), and also reduce some of their limitations, such as low encapsulation efficiency and drug expulsion during storage (Beloqui et al., 2017, 2016; Garcês et al., 2018). Furthermore, NLCs offer advantages with regard to biocompatibility and can be administered either by oral, parenteral, topical, pulmonary, and rectal routes (Beloqui et al., 2016; Müller et al., 2000). Several researchers have already incorporated chemotherapeutics into the lipid carriers, which have obtained satisfactory results (Serpe et al., 2004; Subedi et al., 2009; Wong et al., 2004). However, there are no reports on the encapsulation of *P. pubescens* fruit oil (PpO) in such type of system so far.

The formulation by design is an important tool in the development of a new product, which is based on the principles of Quality by Design (QbD), according to ICH. This is a very useful tool for obtaining the best possible formulation composition, as well as providing an understanding of the process and behavior of the product, based on sound science and quality risk management. The basic concept is that pharmaceutical products should be developed to meet the needs of patients through safety, efficacy and performance characteristics. In the case of nanomaterials, quality control and drug safety have become a very important issue currently being addressed. In the concept of QbD, mathematical models can be used to provide a scientific understanding of the phenomena in the development of a product and allow the prediction of the state of the system under certain conditions (Bastogne, 2017; Djuris and Djuric, 2017).

In the present study a multivariate data analysis approach was applied to optimize the manufacture of PpO-loaded NLCs. We have for the first time evaluated the effects of the type of solid lipids (TSL), the Phospholipon® 80H concentration (PC) and the type of aqueous surfactant (TAS) in selected NLC parameters. Transmission electron microscopy (TEM), X-ray diffraction (XRD), Fourier transform Raman spectroscopy (FT-Raman), and differential scanning calorimetry (DSC) analyses were carried out on the optimized formulation. In addition, the cytotoxicity of pure PpO and PpO-loaded NLCs were evaluated in HT-29 human colorectal adenocarcinoma cells *in vitro*. The results showed an enhancement in antitumoral activity when the oil was entrapped into the NLCs.

Therefore our study opens the scope for the use of lipid nanocarriers developed by medical and pharmaceutical industries to obtain improvements in the effectiveness of PpO biological activities, as well as to be tested for other drugs in the future.

2. Material and methods

2.1. Material

The fruits of *P. pubescens* were collected in August 2014 at Nossa Senhora do Livramento (Mato Grosso, Brazil). The species was identified by Dr. Germano Guarim Neto. Vouchers were deposited at the Herbarium of the State University of Maringá (num. 20502).

Compritol® 888 ATO (glyceryl behenate), Precirol® ATO 5 (glycerol distearate), Phospholipon® 80H (P80H) (hydrogenated soya bean lecithin), and PEG-40 hydrogenated castor oil/sorbitan oleate (PEG-40H) were kindly donated by Gattefossé (Saint Priest, France), Lipoid (Ludwigshafen, Germany), and Oxiteno (São Paulo, Brazil), respectively. Tween 80® (Vetec, Xerém, Brazil), HT-29 cell line (ATCC HTB38, USA), DMEM, penicillin/streptomycin and trypsin-EDTA 0.05% (Gibco™, USA), and Trypan blue (Acros Organics, USA) were also used. Other chemicals and reagents were of analytical grade.

2.2. Extraction of PpO

The extraction of PpO was performed following the methodology described elsewhere (Hoscheid et al., 2012). Briefly, the fruits were extracted with ethanol by turbo extraction (Ultra-Turrax UTC115KT, IKA® Works, Wilmington, USA), filtered, and concentrated using a vacuum rotating evaporator (Büchi® R-114, Flawil, Switzerland). The crude extract was partitioned with a mixture of water and hexane. The hexane fraction was concentrated using a vacuum evaporator at a temperature of 40 °C until the organic solvent was completely evaporated.

2.3. Gas chromatography–mass spectrometry analysis (GC–MS)

The constituents of PpO were analyzed by using a previously developed and validated GC–MS methodology (Hoscheid et al., 2015). A gas chromatograph with a quadrupole detector (Thermo Electron Corporation DSQ II, TLC, Thermo Fisher, USA) equipped with an HP-5 (30 m × 0.25 mm) was used. All samples were diluted in chloroform prior to analysis and 1 µL was injected into the equipment with a split ratio of 1:20. The chromatographic conditions were as follows: oven temperature program, increased from an initial temperature of 180 °C to 270 °C at 4 °C/min and held at 270 °C for 3 min; injector and detector temperatures, 270 °C; carrier gas, pressure, and flow-rate, helium, 0.7 bar, 1 mL/min. Mass spectra were obtained at an ionization energy of 70 eV and the scan rate was 0.5 scans/s and conducted in the mass/charge ratio range 50–650 *m/z*. Qualitative analysis was performed in complete scan mode and the quantification of furanoditerpenes with vouacapan skeleton, contained in the PpO, was conducted by monitoring the following selected ions using GC–MS-SIM (single ion monitoring) : 105, 109, and 175 (23.3–24.0 min); 131, 178, and 312 (24.0–25.8 min); and 105, 202, and 326 (25.8–26.5 min).

2.4. NLC development based on design of experiments (DoE) and response surface methodology (RSM)

A 3 factors and 2 level full factorial design (2^3) was carried out to optimize the development of NLCs loaded with PpO. The factors studied (one continuous and two categorical variables) were: X_1, the type of solid lipid (TSL), X_2, the P80H concentration (PC) and X_3, the type of aqueous surfactant (TAS). The full factorial design matrices with coded and noncoded values of each factor studied are summarized in Table 1. The trials were randomized to reduce the effects of unexplained variability.

The NLCs (F1–F8) were prepared by a melt-emulsification method (Souto et al., 2011). Briefly, the solid lipids (5%) and P80H (0.5 or 1%) were blended and melted at 85 °C. The PpO (2%) was incorporated in the melted oil phase to avoid an excessive exposure to high

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