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Pomegranate seed oil nanoemulsions improve the photostability and *in vivo* antinociceptive effect of a non-steroidal anti-inflammatory drug

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ABSTRACT

The combination of pomegranate seed oil and ketoprofen in nanoemulsions aiming to improve the antinociceptive effect was evaluated according to the writhing test and Complete Freud's Adjuvant induced paw inflammation in mice. The formulations showed adequate characteristics and improved ketoprofen's photostability against UVC radiation exposure. The dialysis bag technique showed that 100% of the drug was released from the nanoemulsions after 3 h and the oil amount had no influence on the releasing. Furthermore, time- and dose-response curves were obtained to determine the antinociceptive effect of the formulations. In the post-test, the nanoemulsion containing ketoprofen significantly reduced abdominal constrictions in time-response curve, showing effect up to 12 h while the free ketoprofen showed effect up to 3 h. In addition, the blank nanoemulsion presented a reduction of abdominal constriction up to 1 h of pre-treatment. Regarding the dose-response curve, the free ketoprofen presents effect at 0.5 mg/Kg dose and nanoemulsion at 1.0 mg/Kg dose. Time- and dose-response curves were performed to determine the antinociceptive effect in inflammatory pain. After the evaluation of mechanical allodynia testing at the Von Frey Hair, the free ketoprofen showed effect up to 6 h while nanoemulsions presented effect up to 10 h. Moreover, acute toxicity was performed with ALT and AST activity evaluations and urea levels. After 7 days of treatment, no toxic effects for nanoemulsions were found. In conclusion, ketoprofen-loaded pomegranate seed oil nanoemulsions presented adequate characteristics and a high antinociceptive activity in the animal models tested.

1. Introduction

Pain and inflammation are complex and multifactorial processes that exert an essential role in the physiological homeostasis. The first acts as a warning system emphasizing the presence of any potential danger while the second is related to cellular and molecular events aiming the body's protection and regeneration [1]. Regarding their protective character, in some situations both processes could become deleteriously and, as a consequence, trigger several injuries, as observed in many chronic inflammatory diseases as rheumatoid arthritis (RA) [2]. The RA is characterized by a polyarticular inflammation and gradual joint destruction commonly affecting women over 50 years old [3]. Disease-modifying antirheumatic drugs as methotrexate and chloroquine, are employed to delay RA progression; however, they fail as pain relievers. In contrast, nonsteroidal anti-inflammatory drugs (NSAIDs) are prescribed to alleviate the pain, but they can promote many adverse effects and are not effective in controlling the disease progression [4,5].

Over the past years, the interest in natural products, especially concerning the use of vegetable oils, has increased considerably due to their biological potentials. The presence of the conjugated fatty acids and antioxidant compounds in their composition can be responsible for the related pharmacological properties [6–9]. In general, vegetable oils are rich in substances that absorb

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radiation in the ultraviolet region; and due to this property, they are considered potential materials to stabilize photolabile drugs [10,11]. In folk medicine, pomegranate and their preparations have been reported as effective for the treatment of colic, colitis, diarrhea, dysentery, as well as having properties as anthelmintics, astringents, diuretics and antispasmodics [12]. In this context, pomegranate seed oil (PSO) has attracted interest due to its rich conjugated fatty acids composition, polyphenols and estrogen compounds that might support its anti-inflammatory, antioxidant and antitumor properties [13].

Aiming the development of therapeutic alternatives for pain and inflammation management, especially in the matter of chronic conditions, this study proposes a novel association between PSO and ketoprofen (KP). Additionally, KP is a NSAID that belongs to the propanoic acid's class and presents analgesic, anti-inflammatory and antirheumatic activities through inhibition of COX activity and consequent reduction of prostaglandin production [14]. However, the main limitation to the long term therapeutic application of KP is linked to harmful effects on the gastrointestinal tract that could be attributed to direct contact of the drug with the gastric mucosa and its systemically action after absorption [15,16].

In order to study this association, we report in this work the development of nanoemulsions in view of the benefits attributed to this type of nanostructured delivery system. Nanoemulsions (NEs) are considered kinetically stable systems and present advantages compared to conventional emulsions due to their smaller droplet size. These systems may promote modified drug release, increased gastrointestinal distribution [17] and drug protection against chemical and enzymatic degradation [18]. Besides, instabilities phenomena as sedimentation, creaming, flocculation and coalescence are also prevented due to the electrostatic or sterical protection given by the surfactants on their surface [19,20]. The choice of the oil that composes the oily phase is extremely important because its properties could affect some of the NE's characteristics such as the droplet size [21]. Natural oils like soybean oil [22], grape seed [7] and castor oil [23] have been already used to prepare NEs.

In a previous work [24], we developed PSO NEs loaded with KP and stabilized by pullulan, a polysaccharide which demonstrated a selective antiglioma activity by the significant decrease of C6 cell viability without exhibiting cytotoxicity to normal cells (fibroblasts). With the purpose of studying, in a more detailed way, the benefits provided by the PSO and KP nanoemulsification, the present work had as its objective to develop PSO NEs stabilized by polysorbate 80, a non-ionic synthetic surfactant. In order to evaluate the antinociceptive action, the model of abdominal constrictions induced by acetic acid injection, considered a chemical model of acute nociception, and the inflammatory pain induced by paw injection of Complete Freud's Adjuvant were the chosen animal models.

2. Materials and methods

2.1. Materials

The KP was obtained from Henrifarma (São Paulo, Brazil). PSO was purchased from Via Farma (São Paulo, Brazil). Span 80® (sorbitan monooleate) and Complete Freud's Adjuvant (CFA) were acquired from Sigma Aldrich (São Paulo, Brazil) and Tween 80® (polysorbate 80) was purchased from Delaware (Porto Alegre, Brazil). HPLC-grade methanol was acquired from Tedia (Rio de Janeiro, Brazil). Ultrapure water was obtained from Milli-Q® Plus apparatus. All other solvents and reagents were of analytical grade and used as received.

2.2. Methods

2.2.1. Analytical method

The analytical method was developed and validated by our previous published work [24]. It was employed a LC-10A HPLC system (Shimadzu, Japan) equipped with a LC-20AT pump, an UV–vis SPD-M20A detector, a CBM-20A system controller and a SIL-20A HT valve sample automatic injector. The separation was achieved using a Gemini C_{18} Phenomenex column (150 mm × 4.60 mm, 5 μm; 110 Å) coupled to a C_{18} guard column at room temperature. The Ketoprofen detection was performed at 254 nm and the isocratic mobile phase consisted of methanol and water pH 3.0 (70:30, v/v) at 1 mL/min flow rate.

2.2.2. Ketoprofen solubility determination in pomegranate seed oil

To evaluate the KP solubility in PSO, an excessive amount of KP was added to 1 mL of PSO in sealed vials ($n = 3$), which were submitted to a vortex mixer for 5 min and kept during 30 min in an ultrasonic bath. In the sequence, the samples were centrifuged at 3615 *g* for 10 min and an aliquot of the supernatant was diluted in 10 mL methanol. KP content was determined through the HPLC method.

2.2.3. Preparation and characterization of nanoemulsions

PSO NEs ($n = 3$) containing KP were prepared by the spontaneous emulsification method, following the conditions described in our previous report with some modifications [24]. Briefly, an oil phase containing PSO (1.5% w/v or 3.0% w/v), Span 80® (0.077 g) and KP (0.01 g) were solubilized in acetone (50 mL) and mixed with a Tween 80® (0.077 g) aqueous phase (50 mL). The magnetic stirring was kept during 10 min and then the organic solvent was eliminated by evaporation under reduced pressure to achieve a final volume of 10 mL, which corresponds to 1.0 mg/mL KP and 15 or 30 mg/mL PSO. The formulations were protected from light during preparation and storage. NEs were named NE PSOA KP (1.5% PSO) and NE PSOB KP (3.0% PSO). For comparison purposes, blank NEs were also prepared (NE PSOA B and NE PSOB B).

The total KP content in NEs was performed through HPLC analysis. An aliquot of 90 μL of the sample was diluted in methanol to a final volume of 10 mL then submitted to sonication for 30 min, and filtered through a 0.45 μm pore membrane. The encapsulation efficiency was determined by ultrafiltration/centrifugation technique, mean droplet sizes and polydispersity indexes (PDI) by photon correlation spectroscopy (Zetasizer Nanoseries, Malvern Instruments, UK), zeta potential (ZP) by microeletrophoresis, using the same instrument and pH values by directly immersing the electrode of a calibrated potentiometer (Model pH 21, Hanna Instruments, Brazil) in the formulations. The analysis of these parameters were performed at room temperature (25 ± 2 °C) in triplicate, following the methodologies standardized in our recent published study [24].

2.2.4. Photostability study

In order to evaluate the influence of the PSO amount in the KP photodegradation profile, a photostability study was performed by UVC radiation exposure. The experimental conditions were based on the protocol established in previous studies of our group [24,25]. At predetermined intervals, and through HPLC analysis, aliquots of 90 μL were withdrawn and diluted in methanol to estimate the KP concentration in each sample. Appropriate dark controls were simultaneously assessed to discard thermal degradation and the experiment was performed in triplicate.

2.2.5. In vitro release

Dialysis diffusion technique was performed to compare two formulations and determine the influence of the oil amount in