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A new biodegradable polymeric nanoparticle formulation containing *Syzygium cumini*: Phytochemical profile, antioxidant and antifungal activity and *in vivo* toxicity

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ABSTRACT

Syzygium cumini seeds were used for the preparation of an aqueous extract (ASc) and of polymeric nanoparticles containing ASc (NPASc) with the aim to evaluate and compare their *in vitro* efficacy against the complications of Diabetes *mellitus* (DM) and the *in vivo* toxicity. NPASc were produced by the emulsification/evaporation solvent technique, employing poly-ε-caprolactone, a biocompatible polymer. The antioxidant activity of both ASc and NPASc was evaluated by the scavenging of DPPH radicals and by the ferric reducing antioxidant power assay (FRAP). The *in vitro* efficacy of both formulations against oxidized LDL particles (ox-LDL) and fungal species was also assessed.

NPASc presented properties compatible with nanometric systems, and chromatogram analysis demonstrated that the composition of *S. cumini* was not affected. The antioxidant properties of the extract were also maintained in the new formulation. Indeed, both formulations showed high protection against ox-LDL. The antifungal activity of NPASc against *Candida guilliermondii* and *Candida haemulonii* was superior to that observed for the ASc. No acute toxicity was observed in the *Artemia salina* lethality assay and in rats. These findings highlight the possibility of expanding the use of *S. cumini* to ameliorate the chronic complications of DM. The lack of toxicity of the nanoparticles indicates that NPASc might be a safe candidate for drug delivery systems.

1. Introduction

Diabetes *mellitus* (DM) is a heterogeneous group of metabolic disorders mainly characterized by hyperglycemia which affects over 10% of the world population, representing a fast growing medical problem (ADA, 2013; SBD, 2015). Several studies have demonstrated that diabetes-induced oxidized low-density lipoprotein (ox-LDL) or its products can affect several components of the atherogenic process, including lesion initiation and thrombosis (Younis et al., 2009; Jin et al., 2011). Moreover, diabetes-mediated changes in immune status may render patients more prone to infections such as those caused by fungi (Delamaire et al., 1997; Sun et al., 2012; Fraga-Silva et al., 2015). The most commonly fungal pathogens found in diabetic patients are *Candida* spp., *Cryptococcus* spp., *Aspergillus fumigatus* and *Rhizopus oryzae* (Kauffman et al., 2011). DM requires continuing medical care, ongoing patient self-management and support to help patients optimize metabolic control, prevent and manage complications in order to improve and maximize their quality of life. There are several drugs of plant origin containing substantial amounts of antioxidant substances such as flavonoids with fewer side effects, and that can be useful for the management of DM (Ayyanar et al., 2013). Therefore, it is

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necessary to develop a traditionally adapted, but more advanced, complementary and alternative drug formulation to treat the several symptoms of DM and its complications.

The use of nanotechnology for medical therapy has dramatically increased over the past few decades, especially because this technology has the potential to improve treatment strategies against many important diseases (Pautler and Brenner, 2010). However, the use of plant extracts is much less reported due to the complexity of this type of biological matrix (Ribeiro et al., 2015). Among the nanostructured systems, polymeric nanoparticles are one of the most promising nanocarriers being developed. Manufactured with a mean diameter in the range of 10–1000 nm, polymeric nanoparticles are mainly composed of synthetic biodegradable polymers such as the poly-ε-caprolactone (PCL). Indeed, studies have reported PCL as the polymeric matrix of choice because of its low cost, biocompatibility and US Food and Drug Administration approval (Rao and Geckeler, 2011; Woodruff and Hutmacher, 2010).

Nanodosage forms can provide a number of advantages for herbal drugs, including enhancement of solubility and bioavailability, reduction of toxicity, increase of therapeutic index, improvement of stability, controlled delivery and protection from physical and chemical degradation. At the same time, the toxicity of nanoparticles is a very important point in scientific research due to the nanometer size of these structures that can provide facilitated diffusion between tissues and promote their accumulation (Fadeel and Garcia-Bennett, 2010). Nonetheless, a limited number of studies have been conducted on this issue.

Herbal drugs have gained popularity both in developing and developed countries because of their natural origin and lesser side effects. *Syzygium cumini* (L.) Skeels is an evergreen tropical tree of the Myrtaceae family. All parts of the plant have been widely used as an alternative medicine in various diseases. Seeds in particular are an important source of phenolic and flavonoid compounds, therefore possessing a promising role in regulating DM (Ayyanar et al., 2013). In fact, recent studies from our group have shown that the extract obtained from different parts of *S. cumini* demonstrated beneficial effects against DM *in vitro* and *in vivo* (De Bona et al., 2010, 2011, 2014; Bitencourt et al., 2015).

Considering the potentialities of nanoparticles and the paucity of data on the use of *S. cumini* against the chronic complications of DM, this study was aimed to develop PCL nanoparticles containing an aqueous seed extract of *S. cumini* (ASc) and to evaluate their antioxidant and antifungal activities *in vitro* and toxicity *in vivo*.

2. Materials and methods

2.1. Chemicals

Ethyl acetate, methanol, acetonitrile and acetic, gallic, chlorogenic, caffeic and ellagic acids were purchased from Merck (Darmstadt, Germany). Polysorbate 80 (Tween 80®), PCL, Sorbitan monooleate (Span 80®), 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), thiobarbituric acid, catechin, epicatechin, quercetin, isoquercitrin, quercitrin, kaempferol and rutin reference standards were acquired from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

2.2. Plant material and extract preparation

S. cumini seeds were collected at Santa Maria, Rio Grande do Sul, southern Brazil (29°43′22″S and 53°43′47″W). They were identified by the Laboratory of Botanic and Pharmacognosy of the Federal University of Santa Maria (UFSM) and a voucher specimen (SMDB 14.001) was deposited in the herbarium of the Institution. Eighty grams of the seed powder were extracted with 400 mL of distilled water for 1 h under reflux (Prince et al., 1998). The yield of the extract was 6.2% w/w.

2.3. HPLC-DAD phytochemical analysis

Reverse phase chromatography analyses were carried out under gradient conditions using a C_{18} column (4.6 mm × 150 mm, 5 μm). The mobile phase was composed of water containing 2% acetic acid (A) and acetonitrile (B), and the composition gradient was: 13% of B until 10 min and changed to obtain 20%, 40%, 50%, 60%, 70% and 100% B at 20, 30, 40, 50, 60 and 80 min, respectively. The ASc was analyzed at 10 mg/mL concentration. The presence of 11 antioxidant compounds was investigated, namely gallic, chlorogenic, caffeic and ellagic acids and catechin, epicatechin, quercetin, quercitrin, isoquercitrin, kaempferol and rutin. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of commercial standards. The flow rate was 0.7 mL/min, the injection volume 50 μL and the wavelength was 254 nm for gallic acid, 280 nm for catechin and epicatechin, 327 nm for chlorogenic, ellagic and caffeic acids and 365 nm for rutin, isoquercitrin, quercitrin, kaempferol and quercetin. The samples and mobile phases were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. The chromatography peaks were confirmed by comparing their retention time with those of reference standards and by DAD spectra (200–500 nm). Calibration curves for each substance were: gallic acid, $Y = 13973x + 1095.6$ ($r = 0.9993$); catechin, $Y = 11840x + 1178.2$ ($r = 0.9998$); epicatechin, $Y = 12542x + 1412.7$ ($r = 0.9991$); chlorogenic acid, $Y = 11864x + 1252.8$ ($r = 0.9994$); caffeic acid, $Y = 13178x + 1267.2$ ($r = 0.9999$); ellagic acid, $Y = 12681x + 1164.9$ ($r = 0.9998$); rutin, $Y = 13077x + 1265.4$ ($r = 0.9992$); isoquercitrin, $Y = 11927x + 1306.2$ ($r = 0.9996$); quercitrin, $Y = 13470x + 1293.7$ ($r = 0.9994$) and quercetin, $Y = 12693x + 1176.0$ ($r = 0.9997$). All chromatographic operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

2.4. Preparation of the nanoparticle suspension containing ASc (NPASc)

Nanoparticles were prepared by the emulsification/evaporation solvent method described by Quintanar-Guerrero et al. (1998), with modifications. Briefly, 10 mg of ASc were dissolved in an aqueous phase containing 1% polysorbate 80. An organic phase (ethyl acetate) containing 1% PCL and 1% sorbitan monooleate was also prepared. Both phases remained under moderate stirring at 40 °C. After 60 min, the aqueous phase was added in the organic phase, forming the primary emulsion. This emulsion was kept under strong magnetic stirring during 20 min and then a second aqueous phase containing 2% polysorbate 80 was added in the primary emulsion. After 20 min, the emulsion was transferred to a high shear mixer (Marconi, MA-102/PLUS) and stirred at 6000 rpm during 10 min. Then, ethyl acetate was eliminated and water was concentrated by evaporation under reduced pressure to achieve 10 mL final volume. For comparison purposes, blank nanoparticles (NPb) were prepared.