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Original Article

Physicochemical, biological and release studies of chitosan membranes incorporated with *Euphorbia umbellata* fraction

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ABSTRACT

Formulations containing chitosan incorporated with methanolic fraction of *Euphorbia umbellata* (Pax) Bruyns, Euphorbiaceae, were studied aiming future applications of this new material as medicine. In order to investigate potential interactions between chitosan and the methanolic fraction (10, 50 and 100% in relation to the amount of chitosan) physicochemical characterization was performed by scanning electron microscopy, density, differential scanning calorimetry, thermogravimetry, X-ray diffraction, Fourier-transform infrared spectroscopy and colorimetry techniques. The phenolic compounds released from the chitosan membranes were evaluated using the Folin-Ciocalteau quantification method; antioxidant and antimicrobial activity were also studied. Increasing amounts of the methanolic fraction added to polymeric matrix produced different numbers of pores on the surface of the membranes, changes in the calorimetric, spectroscopic and crystalline properties as well as color changes, when compared to the inert membrane. These changes can be attributed to chemical interactions that occurred between the structure of the chitosan and the phenolic compounds present in the studied fraction. The matrix samples incorporated with 50 and 100% of the methanolic fraction presented different release profiles of phenolic compounds from the membranes (controlled manner) and promoted antioxidant and antimicrobial activity.

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Introduction

Chitosan is a biodegradable, biocompatible, non-toxic polymer, with a cationic and hydrophilic character. It is composed of the linear monomer units (1,4)-2-acetamido-2-deoxy-D-glucopyranose, and 2-amino-2-deoxy-D-glucopyranose (Dias et al., 2013). Its solubility in water occurs in acidic medium due to the protonation of the amino group in the second carbon atom of glucosamine (Senel and Mcclure, 2004). It is characterized by containing three different functional groups in its structure: amine, acetamide and hydroxyl. The amine grouping has a cationic character that favors its ability to interact or react with different compounds (Abdel-Rahman et al., 2016).

Chitosan has gained growing interest recently because it is a promising natural substance that can be used in the biomedical field and in the chemical, pharmaceutical and food industries (Abdel-Rahman et al., 2016). Due to the great capacity of accelerating wound healing, chitosan has been widely used for the topical treatment of wounds (Coqueiro and Di Piero, 2011; Abdel-Rahman et al., 2016).

Chitosan is capable of stimulating cellular proliferation and migration; providing proteins for healing; strengthening the formation of tissue; acting as a barrier against microorganisms; minimizing skin deformation; stimulating natural blood coagulation; absorbing fluids (exudates); guiding the reorganization of the cellular histoarchitecture of wounds; and blocking nerve endings, thereby reducing pain (Freitas et al., 2011; Prichystalová et al., 2014; Benhabiles et al., 2012).

The polymeric characteristic of chitosan allows it to generate porous structures that can be used in the formulations of

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dressings and controlled-release drug products such as membranes, nanoparticles and microspheres (Denkbas and Ottenbrite, 2006; Dias et al., 2013). The membranes are prepared by evaporating a chitosan dispersion that is dispersed in acetic acid on a support, which results in the formation of a flexible and resistant film (Leceta et al., 2013; Wang et al., 2013). Chitosan membranes associated with other polymers and incorporated with different substances such as antibiotics, have beneficial effects in the control of infection and promotion of wound healing (Bernkop-Schnürch and Dünnhaupt, 2012).

Phenolic compounds are a group of substances with different degrees of chemical complexity, which have the ability to neutralize reactive oxygen species (ROS) and have antioxidant activity that is often related to a wide range of biological effects (Quideau et al., 2011). Recent studies have reported a strong correlation between the amount of phenolic compounds present in plant extracts and their biological activities (Timmers et al., 2015; Nađpal et al., 2016). Some studies have evaluated the addition of plant extracts that are rich in phenolic compounds to chitosan membranes in order to explore the biological benefits of this mixture (Martel-Estrada et al., 2015).

Euphorbia umbellata (Pax) Bruyns, (synonymous: *Synadenium grantii* Hook. f., *Synadenium umbellatum* Pax, *Synadenium umbellatum* var. *puberulum*), is popularly known in Brazil as *janaúba* and *leitosinha* and has been used in folk medicine for its anti-inflammatory, anti-ulcer, homeostatic and angiogenic properties. Previous ethnopharmacological studies have demonstrated the antioxidant and anti-inflammatory activities of stem bark extracts and have related these effects to the presence of phenolic compounds. Additionally, data shown that crude bark hydroalcoholic extract of *E. umbellata* (CBE) and its methanolic fraction (MF) did not cause gastric lesions (300 mg/kg/day, oral administration, 5 days (CBE), or 200 mg/kg, oral administration, single dose (MF)) or renal and hepatic function changes (100 mg/kg/day, oral administration, 5 days) revealed by serum biomarkers (urea, creatinine, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase). Furthermore, no changes in animal behavior (food or water intake), weight or organs (macroscopic evaluation of the liver, spleen, kidneys) were observed with the doses evaluated (Munhoz et al., 2014; Minozzo et al., 2016).

Thus, this study purposes the development of chitosan membranes incorporated with the methanolic fraction of *E. umbellata* extract as well as their physicochemical characterization and release profile, antioxidant potential and microbiological activity evaluation aiming future applications of this new formulation as topical treatment of wounds.

Materials and methods

Botanical identification of plant material and obtaining the extract and fractions

The plant material (*Euphorbia umbellata* (Pax) Bruyns, Euphorbiaceae) was collected in the region of Ponta Grossa (Brazil), altitude: 975 meters, latitude: 25°05′38″ S, longitude: 50°09′30″ W. A voucher specimen was prepared and sent to the Herbarium of the Municipal Botanical Museum of Curitiba (# 363509). The stem bark of this plant species was cut and submitted to drying at room temperature for 14 days. The dry material was milled and then 334 g was mixed with a hydroalcoholic solution (30:70 water:ethanol, v/v) at a ratio of 1:5 (w/v). This mixture was homogenized for 8 days for exhaustive extraction (solvent exchanged every 2 days). The resulting solution was collected, filtered, concentrated, lyophilized and stored under refrigeration (4 °C) until the moment of use (70 g of dried crude extract (CE), yield of 21%, w/w). Fractions were obtained by extraction in a Soxhlet apparatus and 40.32 g of CE of *E. umbellata* was mixed with 20.16 g of kieselgel 60 silica (35–70 mesh, Merck®), which was conditioned in a filter paper bag and sealed. This material was extracted using a gradient of increasing polarity (hexane, chloroform, ethyl acetate and methanol (PA, Synth®)). The volume of the liquid extractant used for each solvent was 750 ml and the extraction time was 24 h. The solvents were completely removed under reduced pressure and low temperature to obtain a viscous material; subsequently, water was added to the concentrate extracts and freeze-dried. Finally the lyophilized materials (powder) that were obtained were stored under refrigeration (4 °C) for further analysis. The yield of the fractionation/dry process was as follows: 1.67% for the hexane fraction; 5.46% for the chloroform fraction; 0.84% for the ethyl acetate fraction; and 92.03% for the methanolic fraction.

Determination of total phenolics

The analysis of the total phenolic content of the hexane fractions (HF), chloroform fraction (CF), ethyl acetate fraction (EAF) and methanolic fraction (MF) was performed according to the Folin-Ciocalteau method. The crude extract and the fractions were re-suspended in their respective solvents at a concentration of 20 mg/ml. Aliquots of 500 µl were added to 10 ml flasks and the final volume was adjusted with their respective solvents at a concentration of 1 mg/ml; then 200 µl of these dilutions were transferred to the test tube and combined with 7 ml of distilled water and 0.5 ml of Folin-Ciocalteau reagent (Dinâmica®). After 30 s, 2.5 ml of 10.6% sodium carbonate solution (w/v, PA, Biotec®) was added. The tubes were homogenized and placed in a water bath at 50 °C for 5 min. After this period, the tubes were cooled and the readings were performed using a spectrophotometer (Genesy 10S UV-Vis, Thermo Scientific®) at 715 nm (Munhoz et al., 2014). The blank was prepared in the same way as the samples, replacing the fractions by distilled water. The analytical curve was obtained, in triplicates, with standard solutions of gallic acid (98% purity, Merck®) at concentrations of 50, 100, 200, 400, 600 and 800 µg/ml. The results were expressed in mg of gallic acid per g of sample.

Preparation of chitosan membranes

A chitosan solution (Sigma–Aldrich®/medium molecular weight (190–310 Da) and 75–85% degree of deacetylation) was prepared at a concentration of 1.5% (w/v) in 1% (v/v) acetic acid solution under constant agitation for 60 min. The material was subsequently filtered in sterile gauze and was added to 0.1% filtered sodium benzoate. It was homogenized for 20 min and then 1.5% of PEG 400 (w/w) and 0.2% of Tween 80 (w/w) were added, maintaining agitation for a further 20 min (ultrasound bath). The pH of the solution was adjusted to 5.5. From this solution, 7.5 g was poured into a plastic support (60 × 15 mm Petri dishes) and placed in an oven at 55 °C (±5 °C) for 4 h, to dry. After this period the supports were cooled in a desiccator and the membranes that had been formed were removed from the support, packed and sealed in plastic package (IM – inert membrane). For the membranes containing MF, the preparation steps were the same, but different percentages of freeze-dried MF (10% (M10MF), 50% (M50MF) and 100% (M100MF) in relation to the amount of chitosan) were added to the solution of PEG 400 and Tween 80, stirred, and subsequently spilled over the chitosan solution that was prepared as described above. A physical mixture (PM) of the components of the eligible formulation was prepared and used in the experiments. All membranes (packed and sealed) were stored for 72 h at a controlled environment (desiccator) before physicochemical analysis. For color measurements membranes were stored for 0, 7, 14, 21 and 28 days in the same conditions.

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