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Original Article

Immunomodulatory and toxicological evaluation of the fruit seeds from *Platonia insignis*, a native species from Brazilian Amazon Rainforest

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ABSTRACT

The "bacuri" (*Platonia insignis* Mart., Clusiaceae) is a native tropical fruit from the Brazilian Amazon and Northeast Regions. Its seeds are used to treat inflammatory diseases, diarrhea and skin problems in traditional medical practices. Regarding its widespread medicinal uses, it is important to evaluate the biological and toxicological potential of this species. This way, the aim of this study was to investigate the *in vitro* cytotoxic and immunomodulatory effects of the hexanic extract of *P. insignis* seeds, as well as its *in vivo* acute oral toxicity. The biological evaluation was performed by the determination of cytotoxic (MTT and hemolysis assay) and immunomodulatory (phagocytic capacity, lysosomal volume and nitrite production) activities of EHSB in murine peritoneal macrophages. In addition, the oral acute toxicity was evaluated using female Wistar rats treated with EHSB (2.0 g/kg), in accordance with the OECD 423 Guideline. The EHSB showed low toxicity for macrophages in the MTT test (CC_{50} value: 90.03 μg/ml), as well as for erythrocytes, which caused only 2.5% hemolysis at the highest concentration. A strong immunomodulatory activity was observed by a markedly increase of the NO production, phagocytic ability and lysosomal volume. On the other hand, it was not observed deaths or changes in the clinical and behavioral parameters in the toxicological evaluation. This manner, the present study contributes to the knowledge about the immunomodulatory and toxicological properties of the *P. insignis*. This may provide perspectives for the evaluation and development of effective and safe phytomedicines created from the Brazilian local biodiversity.

Introduction

The Brazilian Amazon region has the richest biodiversity around the world. There is about a million species of animals and plants in this area, which represents half of the species in the entire planet. This biodiversity represents a strategic reserve for the survival of humanity (Agra et al., 2008). Its importance is represented by the considerable reserve of food and medicinal plants. Natural products are an important source of research that aimed the discovery of new substances with pharmacological activities (Butler, 2004).

The species *Platonia insignis* Mart., Clusiaceae, popularly known as "bacuri", is a native species from the Brazilian Amazon Region. This plant is distributed naturally in all states of North and Northern Region of Brazil, more precisely in the states of Pará, Mato Grosso, Maranhão and Piauí. The fruit pulp of *P. insignis* is widely used in the food industry as raw material for production of juices, ice cream, sweets and beverages. Its seeds are used in the production of soap or butter (Agra et al., 2008; Costa-Júnior, 2011; Ferreira, 2008; Moraes and Gutjahr, 2009; Shanley and Medina, 2005).

There are various studies reporting the antioxidant (Lima et al., 2007; Rufino et al., 2010), wound healing (Santos Júnior et al., 2010), leishmanicidal (Costa-Júnior et al., 2013a) and anticonvulsant activities (Costa-Júnior et al., 2010) of the extract of the fruit seeds of *P. insignis*. In addition, the decoction of the fruit seeds is

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used to treat diarrhea in traditional medical practices (Agra et al., 2008). Also, the oil and yellow latex are used to treat bites of spiders, snakes, in the treatment of skin diseases, otitis, rheumatism and arthritis due to wound healing, antitumor, antimicrobial, antioxidant and cytotoxic properties (Costa-Júnior, 2011; Ferreira, 2008; Moraes and Gutjahr, 2009; Shanley and Medina, 2005).

Moreover, the family Clusiaceae represents a rich source of polyisoprenylated benzophehones and xanthones which are responsible for several biological activities (Acuña et al., 2009; Diderot et al., 2006; Kumar et al., 2013). Previous studies of our group have reported the identification of high content of xanthones (alpha- and gamma-mangostin) in *P. insignis* (Costa-Júnior et al., 2013a). In addition, the isolation of the garcinielliptone FC, a polycyclic polyprenylated acylphloroglucinol, from the hexane extract of *P. insignis* (Costa-Júnior et al., 2011) have been reported to present a wide range of biological activities, such as antioxidant (Costa-Júnior et al., 2012), leishmanicidal (Costa-Júnior et al., 2013b), vasodilator (Arcanjo et al., 2014) and anticonvulsant (Silva et al., 2014).

Immunomodulators are a class of drugs that causes a non-specific stimulation of immunological defense mechanisms. The decrease of immunity due to the exposition of human body to different stressful factors represents the main target of these drugs and their benefits. Hence, the immunological stimulation or suppression may be useful in maintaining a health state (Wagner and Proksch, 1985). Furthermore, several medicinal plants have demonstrated the modulation of NO release and phagocytic capability in macrophages. This fact reinforces the immunomodulator profile of medicinal plants and their involvement with several other biological properties, such as anti-inflammatory and antinociceptive (Rosa et al., 2014).

The wide pharmacological spectra of fruit seeds of *P. insignis* together with the absence of toxicological studies about this plant are the main support to the immunomodulatory and toxicological evaluation done in this work. Also, this study confirms the importance of this species as a source of bioactive compounds.

Materials and methods

Plant material

The fruit seeds of *Platonia insignis* Mart., Clusiaceae, were obtained from specimens located in the city of Barras, Piauí, Brazil (latitude −04°14′40″ and longitude −42°40′70″) in March of 2009, and the voucher specimen was deposited at Herbarium Graziella Barroso of Federal University of Piauí, Brazil, no. ICN TEPB 27,164.

Preparation of P. insignis *seeds extracts (EHSB)*

The EHSB was prepared according to Silva et al. (2014). Briefly, the *P. insignis* seeds were dried at 55° C, powdered (848 g) and then extracted with hexane (63%, w/v) in Soxhlet during 8 h. The extract was stored at 8 °C. A formation of a white precipitate, formed of tripalmitin and triolein (64 g, 7%), was observed (Bentes et al., 1987). After the removal of the precipitate, the supernatant was concentrated in a vacuum rotary evaporator. This yielded the hexane extract from the *P. insignis* seeds (EHSB, 534 g, 63%), which was used in the biological tests.

Animals

Female Wistar rats (200–250 g, $n = 5$ per group) and male Balb/c mice (25–30 g) were maintained under controlled conditions (24 ± 1 °C, 12 h light/dark cycle), with free access to food and water. After experimental procedures, female Wistar rats were euthanized by sodium thiopental (100 mg/kg, *i.p.*), and Balb/c mice were euthanized by cervical dislocation. All experimental protocols were approved by the Ethics Committee on Animal Experimentation of UFPI (no. 076/2010).

Cultivation and elicitation of mice peritoneal macrophages

Resident peritoneal macrophages were obtained from Balb/c mice. After euthanasia, the animals were immersed in 70% alcohol for 1 min to antisepsis, and then fixed in the supine position. In laminar flow, approximately 8 ml of PBS (sterile, pH 7.4, 4 °C) was administered into the abdominal cavity. Then, the solution containing peritoneal macrophages was transferred to a sterile tube on ice bath and then subjected twice to centrifugation at 1500 rpm for 10 min at 4 °C with successive cells washing by 0.9% sterile saline. Afterward, the supernatant was discarded and the cells were suspended in 2 ml RPMI 1640 containing 10% SFB, 10,000 IU/ml penicillin, and 10 mg/ml streptomycin. The macrophage count was performed in a Neubauer chamber, using the dye Trypan Blue analysis for cell viability.

MTT test

The cytotoxicity of EHSB was performed using the MTT assay in Balb/c murine macrophages. In 96-well plate, 1×10^6 macrophages per well were approximately incubated in 100 μl of RPMI 1640 medium (Sigma, St Louis, USA) and different concentrations of EHSB (100, 50, 25, 12.5, 6.25, 3.12 μg/ml), and then incubated for 48 h at 37 °C and 5% CO_2.

Afterwards 10 μl of MTT (3-[4,5-dimethylthiazo-2-yl]-2,5-diphenyltetrazolium bromide) diluted in PBS to a final concentration of 5 mg/ml was added (10% volume, *i.e.*, 10 μl of 100 μl for each well) and incubated for 4 h at 37 °C under 5% CO_2. The supernatant was discarded and 100 μl of DMSO was added in all wells. Then, the plate was placed under stirring for about 30 min at room temperature to complete dissolution of formazan. Then, the absorbances were read at 550 nm (Absorbance microplate reader ELx800™, BioTek® Instruments, USA). The results were expressed as CC_{50} (mean cytotoxic concentration for 50% of cells) with the confidence interval by non-linear regression (Reilly et al., 1998).

Hemolytic activity

This test was carried out according to Löfgren et al. (2008) with some modifications. The O^+-type human blood was withdrawn using anticoagulant (EDTA). The erythrocytes were washed three times (7 min at 165 × *g*) and resuspended to 5% erythrocyte solution in PBS (pH 7,4; NaCl 0,15 M) at the volume of 80 μl. Then, 20 μl of EHSB were added to a final volume of 100 μl (400, 200, 100, 50, 25, 12.5, 6.25 and 3.125 μg/ml), and they were incubated for 1 h at 37 °C and the reactions were stopped by adding 200 μl of PBS. The suspensions were centrifuged at 1000 × *g* for 10 min at room temperature. The supernatant was subjected to spectrophotometry at 540 nm to determine the hemolytic activity. The absence (negative control) or 100% of hemolysis (positive control) was determined by replacing the EHSB solution for an equal volume of PBS or Milli-Q sterile water, respectively. The percentage of hemolysis was obtained by comparison with the positive control (100% hemolysis).

Immunomodulatory assessment

Lysosomal volume. Peritoneal macrophages were plated and incubated with EHSB (100, 50, 25, 12.5, 6.25 and 3.12 μg/ml). After 24 h of incubation at 37 °C and 5% CO_2, 10 μl of a neutral red solution in 0.2% DMSO was added and incubated again for 30 min. Afterwards, the supernatant was discarded, the wells were washed with 0.9% saline at 37 °C, and then 100 μl of extraction solution [96% glacial acetic acid (1.0%, v/v) and ethanol P.A. (50%, v/v) in distilled water] was added in order to solubilize the neutral red present inside the