Original Article

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


A R T I C L E  I N F O

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A B S T R A C T

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 (CC50 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 activity 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.

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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; Stanley 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...
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, 2008; Stanley and Medina, 2005).

Moreover, the family Clusiaceae represents a rich source of polyisoprenylated benzophenones 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 polyyclic polyprenylated acylphlorogluconol, 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 immunomodulatory stimulation or suppression may be useful in maintaining a health state (Wagner and Proshch, 1985). Furthermore, several medicinal plants have demonstrated the modulation of NO release and phagocytic capacity 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 Grazziella 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 (840 g) and then extracted with hexane (63%, v/v) in Soxhlet during 8 h. The extract was stored at 8 °C. A formation of a white precipitate, formed of tri-palmitin 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% FSB, 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 × 10⁶ 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% CO₂.

Afterwards 10 μl of MTT (3-[4,5-dimethylthiazole-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% CO₂. 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 C50 (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 3.12 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

Lysoosomal 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% CO₂, 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
lyosomal vesicle secretion. After 30 min, the absorbances were read at 550 nm (Absorbance microplate reader ELx800™, BioTek® Instruments, USA) (Bonatto et al., 2004).

**Phagocytic capacity**

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% CO2, 10 μl of zymosan solution was added (0.3 ml neutral red solution and 0.02 g zymosan not osmopson in PBS 3 ml), and the solution was incubated again for 30 min. Afterwards, 100 μl of Baker’s fixative (formaldehyde 4% v/v, sodium chloride 2% w/v, and calcium acetate 1% w/v in distilled water) were added in order to paralyze the process of phagocytosis, and after 30 min, the plate was washed with 0.9% saline in order to remove non-phagocytized neutral red and zymosan particles. Then, 100 μl of extraction solution was added to supernatant and after solubilization, the absorbances were read at 550 nm (absorbance microplate reader ELx800™, BioTek® Instruments, USA) (Bonatto et al., 2004).

**Nitric oxide (NO) production**

In 96-well plate, 2 x 10⁵ macrophages were added per well and incubated at 37 °C and 5% CO2 for 4 h aiming the cell adhesion. Then, EHSB (100, 50, 25, 12.5, 6.25 and 3.12 μg/ml) or positive control LPS (2 μg/ml) was added in culture medium and incubated again at 37 °C and 5% CO2 for 24 h. After this period, the supernatant was collected and transferred to another plate for measurement of nitrite. Then, Griess reagent (1:1) was added and the analysis was performed in ELISA plate reader at 550 nm. The standard curve was performed with 150 μM sodium nitrite in Milli-Q water at varying concentrations of 1, 5, 10, 25, 50, 75, 100 and 150 μM diluted in culture medium (Genestra et al., 2003).

**Acute oral toxicity in rats**

The toxicological evaluation of EHSB was performed by the Fixed Dose Procedure according to the Guideline 423 from the Organization for Economic Cooperation and Development (OECD, 2001) with some modifications proposed by Serafini et al. (2011). Female Wistar rats were orally treated with EHSB (2.0 g/kg, n = 5) or distilled water (n = 5). Shortly after administration, clinical and behavioral parameters (e.g. hyperactivity, aggressiveness, seizures, piloerection, ptosis, sedation, corneal reflex, writhings, vocalization, death) were evaluated during the first 8 h, and then these same parameters were observed daily for 14 days, including the body weight of these animals, as recommended by the protocol of recognition and evaluation of clinical signs by OECD (2000). After these 14 days of observation, the animals were euthanized and their blood serum samples were obtained in order to determine some biochemical parameters, as listed in Table 1.

In addition, the euthanized animals were submitted to necropsy and gross pathological evaluation of internal organs (lungs, heart, stomach, liver, brain and kidneys) for color, texture and consistency. Then, the relative weights of organs were obtained as follows: relative organ weight = [organ weight (g)] / 100 [animal weight on the day of necropsy (g)].

**Statistical analysis**

Values are expressed as mean ± standard error of the mean. Statistical analyses were performed by applying the Student’s t-test for non-paired samples or analysis of variance (ANOVA) Two-way followed by Tukey’s post-test or Dunnett for analysis of significance between groups. Values were considered statistically significant at p < 0.05. For all statistical analysis and plotting curves used the statistical program GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

**Results and discussion**

The cytotoxicity assays are essentials in the early stages of drug development, since they define the concentration to be used in later stages of evaluation (Elias et al., 1984). The MIT test is a colorimetric indicator of cell viability widely used. This test is able to assess cellular mitochondrial function by the enzymatic reduction of the tetrazolium salt by mitochondrial dehydrogenases in viable cells (Mossmann, 1983). In the evaluation of EHSB-induced cytotoxic effect against murine macrophages, a low cytotoxicity was observed, since it was able to reduce 50% of the viability of macrophages at the mean cytotoxic concentration (CC50) of 90.03 μg/ml, with confidence intervals between 70.72 and 114.6 μg/ml.

Human erythrocytes represent a good preliminary model of toxicity, which is useful in the cytotoxic evaluation of new drugs. Also, it can be used as a possible indicator of damage against non-target cells. This assay allows us to evaluate the potential of drugs of promoting injury in the plasma membrane of red cells. This injury can happen by formation of pores or by total lysis of the cells (Costa-Lotufo et al., 2002; Lexis et al., 2006). The EHSB-induced cytotoxic effect against human erythrocytes promoted less than 3% of hemolysis at the highest tested concentration (400 μg/ml).

The immunostimulation can be very effective and useful when immune system is impaired. In this aspect, the herbal medicines have demonstrated an appropriate immunostimulatory potential for the prophylaxis and therapy of moderated infections (Wagner et al., 1995). Among thousands of natural products, the species Woodfordia fruticosa has been used in Ayurvedic medicine due to its immunostimulant potential. In this perspective, the potential of drugs in promote stimulation of macrophages by activation of phagocytosis and production of nitrogen and oxygen-reactive species have been related to immunomodulatory properties. Moreover, these agents can provide supportive therapy to conventional chemotherapy by activation of host defense mechanisms in the presence of an impaired immune responsiveness (Shah and Juevkar, 2010).

The lysosomal volume was assessed based on the retention of neutral red in lysosomes of murine macrophages, which was determined colorimetrically. It is noteworthy that EHSB promoted a significant increase of lysosomal volume by 8.6, 7.9 and 9.2% at the concentrations of 50, 25 and 12.5 μg/ml, respectively (Fig. 1). According to Ghazanfari et al. (2006), several plants can produce a
wide range of substances with antimicrobial and/or immunomodulatory potentials. This way, the activation of macrophages is necessary to establish control and progression of intracellular infections in an attempt to adapt to the surrounding environment (Jelipo et al., 2000). The treatment of macrophages with EHSB promoted a slight increase in the number and volume of the endocytic compartment vesicles. This may suggest an increase in the potential of defense of these cells.

Another parameter of macrophage activation was assessed by the cellular response to an external stimulus, which was estimated by the phagocytosis of zymosan particles stained with neutral red. The EHSB induced a significant increase of phagocytic capability of macrophages by 42.9%, 49.8%, 53.7% and 71.2% at the concentrations of 25, 12.5, 6.25 and 3.12 μg/ml, respectively (Fig. 2). Zymosan is a polysaccharide obtained from the cell wall of Saccharomyces cerevisiae in water-insoluble particles with a mean diameter of 3 μm. In vitro studies have demonstrated that zymosan has the ability to stimulate defense cells to induce Th1-type immune response, which includes an increase in the production of IFN-γ (Wei et al., 2011). When murine macrophages were stimulated with zymosan particles, an increase in phagocytic activity for all EHSB tested concentrations was observed. According to Schwartz (2007), many antimicrobial enzymatic systems are activated within phagocytic vesicles in order to digest pathogens and/or phagocytized substances.

The determination of NO production was performed indirectly by measurement of the nitrite produced in macrophages treated with the EHSB. A significant increase of NO production in macrophages was observed at all tested concentrations, and the higher effects were observed for EHSB 100 μg/ml by 12.4% and for the positive control LPS by 27.2% (Fig. 3). The nitric oxide produced by macrophages plays an important role in pathological processes, such as antimicrobial defense, inflammation and angiogenesis (Krishnatry et al., 2010; Macmicking et al., 1997). The cytotoxic effect against pathogenic microorganisms is indirectly performed by NO, which combines with O₂⁻ within phagolysosomes to produce peroxynitrite, a high reactive oxygen species with micobicide properties. This micobicide reaction produces nitrite and nitrate as its final product (Beutler, 2004).

The toxicological investigation of natural products aims to establish a safe and effective range of doses for new drugs and pharmaceutical formulations. Likewise, the EHSB did not induce any death or changes in clinical and behavioral parameters at the oral dose of 2.0 g/kg in rats during the 14 days of daily observation. The serum biochemical parameters were also investigated, and the results are in accordance with the references values observed for various standardized animal facilities (Santos et al., 2010). Interestingly, it was not observed any alteration in the tested parameters (Table 1). It means no signs of systemic toxicity in the tested concentration.

After EHSB acute oral treatment, a significant weight gain was observed along the 14 days of observation compared with the control group (Fig. 4). After the necropsy, no changes in color, texture or consistency was observed in internal organs, as well as their relative weights compared with the control group (Table 2). Additionally, no gastric lesions were observed in any treated group.

The seeds extract from P. insignis (EHSB) induced low cytotoxicity and immunomodulatory property, which is markedly important in activation of defense mechanisms. In addition, the EHSB did not
show any toxicological signs after acute oral treatment in rats. It demonstrates a safety profile for the medicinal use of this species. Thus, the present study provides important information about the species and the Brazilian Amazon biodiversity, as well as we raise up perspectives for evaluation and development of new safe and more effective herbal medicines.

Authors’ contributions

AKMFL, DDRA, RGR, KAFR, FFBP, CAP and JSCJ performed the experiments. DDRA, FAAM and AMGLC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. DDRA, KAFR, BQA, JSCL analyzed data. JSCJ and AMGLC contributed with reagents. DDRA and KAFR and BQA wrote the paper. DDRA revised the English proofwriting. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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