Journal of Ethnopharmacology 170 (2015) 66–71

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Acute and sub-acute oral toxicity of Brazilian red propolis in rats

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ARTICLE INFO

Article history:
Received 30 December 2014
Received in revised form
4 May 2015
Accepted 4 May 2015
Available online 13 May 2015

Keywords:
Red propolis
Acute toxicity
Sub-acute toxicity
Isoflavone
Hematological parameters
Biochemical parameters

ABSTRACT

Ethnopharmacological relevance: Propolis is a bee product widely used in folk medicine due to its numerous pharmacological properties. However, samples from different regions can differ in chemical composition, effectiveness, and side effects. Despite the widespread use of Brazilian red propolis, which is an isoflavone-rich variety, its toxicity has not been carefully studied.
Aims of the study: To assess the acute and sub-acute toxicity of the hydroethanolic extract of red propolis (HERP) administered orally to rats.
Materials and methods: HERP for the acute (300 mg/kg) and sub-acute (10, 100 and 200 mg/kg) toxicity studies was administered orally to rats according to OECD Guidelines 420 and 407, respectively. Clinical signs were identified, and hematological and biochemical analyses were performed. Water and food uptake as well as body and organ weights of animals were recorded.
Results and conclusions: The acute study revealed no lethal effects at 300 mg/kg of HERP, but toxic signs were observed, as HERP had an LD50 of more than 300 mg/kg, indicating a warning. The most toxic signals in sub-acute studies were observed in males at a dose of 200 mg/kg HERP. These results suggest estrogen-like activity, possibly from the isoflavones in HERP.

1. Introduction

The recorded use of propolis in folk medicine dates back to about 300 BC. Its widespread use is related to numerous properties, such as antibacterial, antiseptic, anti-inflammatory, antifungal, and anesthetic activities (Kuropatnicki et al., 2013). However, the chemical composition of propolis is highly dependent on edaphoclimatic conditions, and differences could influence biological activity and the toxic responses to propolis (López et al., 2014).

In the last decade, many studies have investigated the biological activities of red propolis, which is a variety found in northeastern Brazil (Daugsch et al., 2008) and other developing countries such as Cuba (Piccinelli et al., 2011) and Venezuela (Trusheva et al., 2004). The botanical origin of red propolis from Alagoas (Northeast of Brazil) was reported by Daugsch et al. (2008), as species of Leguminosae family (*Dalbergia ecastophyllum*). Studies on hydroalcoholic extracts of Brazilian red propolis have shown antioxidant (Frozza et al., 2013), antitumor (Pinheiro et al., 2014), healing (Almeida et al., 2013), and antiparasitic activities (Morsy et al., 2013), but its safety has not been evaluated properly.

Red propolis has a specific chemical composition, and new compounds never reported in other propolis varieties, such as vestitol and neovestitol (Piccinelli et al., 2011; Bueno-Silva et al., 2013), 3,4,2′,3′-tetrahydroxychalcone, C-glycoside (Righi et al., 2011), biochanin A, liquiritigenin, isoliquiritigenin, formononetin, and medicarpin (Alencar et al., 2007; Piccinelli et al., 2011; Frozza et al., 2013; López et al., 2014) have been detected in red propolis. These chemical markers have antimicrobial (Daugsch et al., 2008), cytotoxic against tumoral cells (Li et al., 2008), anti-inflammatory (Bueno-Silva et al., 2013), anti-atherogenic, and anti-angiogenic (Daleprane et al., 2012) properties.

Although commercial propolis extracts include compounds recognized as safe substances and have popular health status, these new propolis varieties represent an important source of unreported bioactive compounds with unknown pharmacological and toxicological actions (Czyżewska et al., 2014). In the present study, we assessed the toxic effects of orally administering a

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http://dx.doi.org/10.1016/j.jep.2015.05.009
0378-8741/

hydroethanolic extract of red propolis (HERP), using international protocols to study acute (OECD, 2001) and sub-acute toxicity (OECD, 2008) in rats.

2. Materials and methods

2.1. Hydroalcoholic extract of red propolis (HERP)

Propolis was collected in Marechal Deodoro/Alagoas/Brazil (S–9°44′36″ and W–35°52′3″) in June 2013. The extraction was performed using propolis samples (2 g) and 70% ethanol (25 mL) at room temperature for 1 h in an ultrasound bath. After extraction, the mixture was filtered, and the solvent was evaporated to produce HERP. The yield of extraction was 46% (w/w).

Chromatography analysis was conducted according to Alencar et al. (2007) with minimal modifications. Briefly, a chromatograph equipped with a DGU-20A3 degasser, a SIL-20A auto-sampler, two LC-20AD pumps, an SPD-M20Avp photodiode array detector with a CBM-20A interface, and a reverse-phase Phenomenex Luna C18 column (C-18, column size 4.6 mm × 250 mm; particle size, 5 μm) was used.

HERP was dissolved in methanol (high performance liquid chromatography [HPLC] grade; Merck, Darmstadt, Germany) (0.2 mg/ml) and filtered with a 0.45 μm filter (Millipore-HVHP, Milford, MA, USA). A 20-μl aliquot of HERP was injected into the HPLC system with a solvent flow rate of 1 mL/min. The column was eluted with a linear gradient of acetic acid: water 1% v/v (A) and methanol (B). The elution started with 40% B (10 min), followed by 50–60% B (to 20 min), 65–70% B (to 35 min), and finishing with 70–80% B (to 45 min).

The chromatograms were recorded at 280 nm, and formononetin, daidzein, and biochanin A authentic standards (Sigma Co., St. Louis, MO, USA) were used as biomarkers López et al., 2014.

2.2. Biological assay

2.2.1. Animals

Male and female Wistar rats (age, 8–12 weeks; males; 250–350 g; females: 160–220 g) were used for acute and sub-acute toxicology studies. Five rats were housed per polycarbonate cage with free access to a normal diet and water. The rats were kept in a temperature controlled room (22 ± 3 °C), with a 12 h light/dark cycle and relative humidity of < 70%. The animals were acclimated to the laboratory conditions for 5 days prior to the experiments. All procedures were performed according to the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and approved by the Animals Ethics Committee of Tiradentes University (Protocol no. 010913).

2.2.2. Acute toxicity study

The experiment was conducted according to the protocols described in OECD Guideline 420 (OECD, 2001). The HERP extract was dissolved in 5% (v/v) Tween 80 aqueous solution and administered orally (300 mg/kg; HERP300) in a single dose. The control group (CTR) received only the Tween 80 solution. The rats were fasted overnight prior to dosing and 3 h after treatment. The groups (HERP300 or CTR_a) were comprised of female rats ($n=5$ each) and each received about 5 ml/kg (HERP 300 mg/mL or solvent, respectively).

The general behavior of the rats (changes in skin, hair, eyes, mucous membranes, and respiratory, circulatory, autonomic, and central nervous systems, abnormal behavior, motor activity, tremors, convulsion, salivation, diarrhea, lethargy, or sleep) was monitored continuously during the first 24 h (0.25, 0.5, 1, 2, 4, and 12 h) and daily until day 14 after dosing. The body weight of the rats was measured on days 1, 7, and 14 (OECD 420, 2001). All animals were euthanized by CO_2 inhalation at the end of the experiment, and their organs were excised and examined macroscopically.

2.2.3. Sub-acute toxicity study

The experiment was conducted according to the protocols described by OECD Guideline 407 (OECD, 2008). Wistar rats of both sexes were assigned randomly to four groups ($n=10$/group: five males and five females). The HERP extract was dissolved in 5% (v/v) Tween 80 aqueous solution and administered orally daily for 28 days at single doses of 10 (HERP10), 100 (HERP100), and 200 mg/kg (HERP200) (volume ~ 5 mL/kg), whereas the CTR_{sa} received only the solvent in the same volume.

The behavior and clinical signs of the rats, (same parameters observed in acute study, including death) as well as the estrous cycles (females) were observed daily. Body weight and water and food intake were recorded weekly. Additional satellite groups of four animals (two of each sex, control and highest dosage group–HERP200) were prepared to observe the reversibility, persistence, or delayed occurrence of toxic effects 14 days after the sub-acute treatment.

Estrous cycles were monitored cytologically by vaginal lavage. Vaginal samples were taken by flushing about 75 μL saline in and out of the vagina and placing a drop of exudate on a slide. Vaginal cytology was evaluated under light microscopy to determine the estrous cycle stage based on the predominant cell type: proestrus: presence of nucleated epithelial cells; estrus: cornified epithelial cells; diestrus: leukocytes. Regular cycling was characterized by short period, consisting of 2–3 days of diestrus (or metestrus), 1 day of proestrus, and 1–2 days of estrus.

On day 28, the animals were fasted 12 h prior to sampling of blood collected via the superior mesenteric artery into non-heparinized tubes for biochemical analyses and into EDTA tubes for hematological analyses. This procedure was conducted under anesthesia (100 mg kg^{-1} xylazine and 10 mg kg^{-1} ketamine, intraperitoneally). Full blood cell counts were determined using an automatic hematology analyzer (BC5380 Mindray, Shenzhen, PR China), and serum biochemistry tests were performed using a clinical chemistry analyzer (Abbott Architect C8000; Abbott Medical, Abbott Park, IL, USA), according to the manufacturer's instructions. Biochemical studies were carried out for liver function (alkaline phosphatase – ALP, aspartate aminotransferase – AST, and alanine aminotransferase – ALT), kidney function (urea, creatinine, and uric acid), and other biochemical parameters (glucose, triglycerides, cholesterol, protein, sodium, potassium, and albumin).

After blood sampling, the animals were euthanized under CO_2 inhalation, and the liver, kidneys, lungs, brain, spleen, heart, testes, and ovaries were excised, weighed, and examined macroscopically. Relative organ weights were calculated as (organ/body weight) × 100. Organs, such as the liver, kidneys, and spleen, were formalin-fixed, dehydrated, diaphanized, paraffin-embedded, and 5-μm thick sections of each sample were obtained and stained with hematoxylin/eosin for histological examination. The microscopic analysis of all tissue samples was carried out as a blind study. The tissue samples were evaluated regarding the architectural and cellular features of the parenchymal and stromal components of the organs, as well as the presence of inflammatory infiltrate, necrosis, or degenerative changes.

2.2.4. Statistics

All data are expressed as mean ± standard error. Student's *t*-test was used for data generated in the acute study, and two-way repeated-measures analysis of variance was used to determine differences between the groups in the sub-acute study.