Industrial Crops and Products 82 (2016) 171–178

Contents lists available at ScienceDirect

Industrial Crops and Products

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

Total phenolic content, antioxidant activity and pre-clinical safety evaluation of an *Anacardium occidentale* stem bark Portuguese hypoglycemic traditional herbal preparation

Sofia Encarnação[a], Cristina de Mello-Sampayo[a], Nuno A.G. Graça[a], Luís Catarino[b,c], Isabel B. Moreira da Silva[a], Beatriz Silva Lima[a], Olga Maria Duarte Silva[a,*]

[a] Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisbon, Portugal
[b] Department of Plant Biology, Faculty of Sciences, Universidade de Lisboa, Edifício C2, Piso 4, Campo Grande, 1749-016 Lisboa, Portugal
[c] Research Centre in Biodiversity and Genetic Resources (CIBIO/InBio), Campus Agrário de Vairão, Universidade do Porto, 4485-601 Vairão, Portugal

ARTICLE INFO

Article history:
Received 9 June 2015
Received in revised form 22 October 2015
Accepted 1 November 2015
Available online 19 December 2015

Keywords:
Cashew tree bark
Genotoxicity
Herbal medicines
Plant raw material
Repeated dose toxicity
Total phenolic content

ABSTRACT

Anacardium occidentale L. (cashew tree) has been traditionally used to treat type 2 diabetes. Hereby we present results of free radical scavenging activity assay and *in vivo* toxicity and genotoxicity tests on red and white cashew stem bark Portuguese traditional herbal preparations (CSBTHPs) used to control type 2 diabetes, standardized on the basis of the total phenolic content.

A 2-week repeated dose toxicity study was performed with three doses (40.2, 127, 402 mg/kg/day) of each CSBTHP, administered by gavage to CD-1 mice ($n = 28$). Micronucleus test and comet assay were performed in CD-1 mice ($n = 18$) which received a single dose of 2000 mg/kg (p.o.) of each CSBTHP or cyclophosphamide 50 mg/kg (i.p.) or water (p.o.).

The total phenolic content was 58.0 ± 0.4 mg gallic acid equivalents (GAE)/g cashew stem bark (CSB) in white CSBTHP and 51.3 ± 1.6 mg GAE/g CSB in red CSBTHP. Both red and white CSBTHPs exhibited concentration-dependent radical scavenging activity with IC_{50} values of 180.7 ± 6.7 μg/mL and 143.8 ± 2.8 μg/mL, respectively. No treatment-related effects on relative organ weights, biochemical parameters and food intake were observed in the repeated dose toxicity study with both CSBTHPs. In the mice micronucleus test both CSBTHPs showed absence of bone marrow suppression and a similar frequency of micronuclei in immature erythrocytes between treated and negative control groups. The comet assay revealed both CSBTHPs to be non-genotoxic.

Concluding, both red and white CSBTHPs, standardized on the basis of the total phenolic content, revealed to be sources of natural antioxidants and devoid of a genotoxic risk. The daily oral administration of doses up to 402 mg/kg of those CSBTHPs did not induce relevant signs of toxicity in mice.

1. Introduction

Throughout human history, plants have been a good source of medicines, providing many novel prototype bioactive molecules (Salim et al., 2008). Nowadays, a great part of conventional drugs are derived from medicinal plants and intensive investigations are ongoing concerning the use of natural herbal health remedies (Lahlou, 2013).

Popularly known as cashew tree, *Anacardium occidentale* L., is native to South America and belongs to the Anacardiaceae family, which includes about 75 genera and 700 species (Mitra et al., 2007). It is an evergreen tree growing 10–15 m high with a short, irregular shaped trunk (Yahia, 2011). Nowadays it is cultivated in large crops in many countries such as Brazil, Côte d'Ivoire, India, Mozambique, Nigeria and Vietnam (FAO, 2013). Different parts of the plant (mainly leaf and stem bark) have been extensively used as traditional herbal medicines, covering a wide range of health benefits in countries from all over the world, but mainly from American and African continents (Brandao et al., 2008; Cano and Volpato, 2004; Iwu, 2014; Ling, 2006). The leaf is traditionally used as an

Abbreviations: CSB, cashew stem bark; GAE, gallic acid equivalents; IE, immature erythrocytes; ME, mature erythrocytes; THPs, traditional herbal preparations.

* Corresponding author. Faculty of Pharmacy, Universidade de Lisboa, Department of Pharmacological Sciences, Laboratory of Pharmacognosy, Avenida Professor Gama Pinto, Lisboa 1649-003, Portugal.
E-mail addresses: odsilva@ff.ulisboa.pt, olgaduartesilva@gmail.com (O.M.D. Silva).

http://dx.doi.org/10.1016/j.indcrop.2015.11.001
0926-6690/

antiemetic and antidiarrheal agent and to treat diabetes, hypertension and headaches; the stem bark is used to treat external and internal wounds, stomach ache, cough, toothaches, diarrhea, hypertension, diabetes, hemorrhoids and sexual dysfunction; the root is used as an anti-inflammatory and antidiarrheal agent; the cashew apple juice is used to treat fever, scorpion and bee stings; powdered seeds are used as antivenom for snake bites; and the nut oil is used as antifungal and for healing cracked heels (Chabi Sika et al., 2013; Mitra et al., 2007).

The potential therapeutic activity of *A. occidentale* has raised interest among the scientific community, and has been the target of many chemical and pharmacological safety studies developed in the last few years. Concerning the cashew stem bark (CSB) different authors have identified the presence of flavonoids, alkaloids, tannins, saponins, phenols, terpenoids and coumarins (Alexander-Lindo et al., 2007; Chaves et al., 2010; Eliakim-Ikechukwu et al., 2010; Ojezele and Agunbiade, 2013). In related forms, phenol derivatives were identified by us as major constituents of the CSBTHPs used in Portugal (Encarnação et al., 2014) which are the object of the present study.

Data from *in vivo* pharmacological studies indicated that CSB enhances renal and brain functions (Ofusori et al., 2008), regenerates beta cells (Bassey et al., 2012) and possesses hypoglycemic (Alexander-Lindo et al., 2007; Eliakim-Ikechukwu et al., 2010; Ojewole, 2003; Olatunji et al., 2005), hypotensive and cardioinhibitory (Tchikaya et al., 2011), anti-inflammatory (Mota et al., 1985; Olajide et al., 2004; Vanderlinde et al., 2009), antidiarrheal (Yusuf et al., 2009), and antigenotoxic (Barcelos et al., 2007a) activities. *In vitro* studies demonstrated that CSB has antibacterial (Arekemase et al., 2011), antimalarial (Sha'a et al., 2014), antileishmanial (França et al., 1993) and free radical scavenging activities (Chaves et al., 2010). However, the published studies refer to different types of CSB extracts: aqueous (Ojewole, 2003), ethanolic (Bassey et al., 2012), hexanic (Alexander-Lindo et al., 2007) hydroethanolic (Eliakim-Ikechukwu et al., 2010) and methanolic extracts (Ojewole, 2003; Olatunji et al., 2005). The lack of extracts standardization does not allow the comparison between the obtained results. Variability is also verified in biological *in vivo* and *in vitro* models used to evaluate the safety and effectiveness.

In Portugal, CSB has been widely used for more than 30 years to treat type 2 diabetes, sometimes in combination with other plants, as unlicensed herbal medicinal products. The Portuguese traditional herbal preparations (THPs) are based on CSB aqueous extracts, which are used daily by oral administration (Silva, 2000). The raw plant material arises mainly from the African Portuguese speaking countries, namely Guinea-Bissau, in which two major types of *A. occidentale* are recognized, based on the color of false fruits—red or white. Both red or white CSB are used to prepare the CSBTHPs.

The essential role played by medicinal plants in the healthcare of much of world's population as well as the growing interest in this kind of natural sources have contributed to the development of industries based on plants for medicinal use. This industrialization requires a concerted research and development, in order to reassure that safe(r), effective, stable and reproducible formulations are produced (FAO, 1997).

Although previous studies have shown the safety of CSB extracts (Barcelos et al., 2007a, 2007b; Okonkwo et al., 2010), to the best of our knowledge there is no preclinical or clinical data characterizing red and white CSBTHPs used in Portugal as hypoglycemic agents. Hereby, two CSBTHPs standardized on the basis of the respective total phenolic content, according to the requirements established by the World Health Organization (WHO, 2004), were studied for their activity on free radical scavenging, as well as for the general toxicity and genotoxicity evaluation in mice, respectively, through a repeated dose toxicity test, a micronucleus test and a comet assay.

2. Materials and methods

2.1. Chemicals and reagents

Methanol was purchased from Fisher Chemicals® (Leicestershire, United Kingdom). Gallic acid standard was purchased from Fluka® (Buchs, Switzerland). Chloridric acid, dimethylsulfoxide (DMSO), disodium hydrogen, ethylenediaminetetraacetic acid (EDTA), ethanol, Folin–Ciocalteu reagent, Giemsa stain, potassium chloride, potassium phosphate, sodium carbonate, sodium hydroxide, were purchased from Merck® (Darmstadt, Germany). GelRed™ was purchased from Biotium, Inc. (Hayward, United States of America). Phosphate buffered saline (PBS), trizma base and isoflurane were purchased from Sigma–Aldrich® (Steinheim, Germany). Sodium chloride, triton X-100 and normal melting point agarose were purchased from BDH Prolabo (Leuven, Belgium). Low melting point agarose was purchased from Lonza® (Rockland, United States of America) and cyclophosphamide (Endoxan®) was purchased from Baxter® (Halle, Germany). All reagents used in the study were of analytical grade or high performance liquid chromatography grade.

2.2. Animals

Male CD-1 mice were obtained from Harlan Laboratories Inc. (Barcelona, Spain). Mice were housed at 4 or 5 animals/cage in a room maintained at 22 ± 3 °C and 50–60% relative humidity, under a 12 h light–dark cycle. Animals were fed with standard laboratory chow (4RF21 GLP; Mucedola srl, Milan, Italy) and water *ad libitum*. All animal experiments were carried out in accordance with the requirements of the Institutional Animal Ethical Committee, directive 2010/63/EU of the European Parliament and the Council of the European Union and the Portuguese Decree-Law No. 113/2013.

2.3. Plant material

CSB (white and red false fruit types) were collected and dried under shade in Guinea-Bissau in June 2012. The plant was identified by the collector Luís Catarino, at the LISC-Herbarium, Tropical Botanical Garden of IICT. After identification, voucher specimens of each sample were deposited at the same Herbarium (voucher numbers: white CSB collected at Dulombi, 11.858°N; 14.503°W: LC1924CJ; red CSB collected at Paiai, 11.836°N; 14.421°W: LC 1922 LC).

2.4. Preparation of extracts

Plant material was homogenized according to the standards of the 9th edition of the Portuguese Pharmacopoeia (Comissão da Farmacopeia Portuguesa, 2009). To obtain the THP, macerates were made with the plant material (dried CSB) and water (1:7 w/v). Different portions of white or red CSBTHPs were extemporaneously prepared during the study to ensure their chemical integrity.

2.5. Determination of total phenolic content

CSBTHPs total phenolic content was determined by a modified Folin–Ciocalteu method (Scalbert et al., 1989), previously validated in our laboratory. Two milliliters of Folin–Ciocalteu reagent (previously diluted with water 1:10 v/v) were mixed with 1.6 mL of sodium carbonate (75 g/L) and then 0.4 mL of the extract were added. After 2 h of incubation at room temperature, the absorbance of samples and standard solutions were measured at 765 nm, with the Hitachi U-2000 spectrophotometer (Tokyo, Japan). Increasing gallic acid concentrations (30, 60, 75, 90, 105, 120 mg/L) were used