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Acute, subacute toxicity and mutagenic effects of anacardic acids from cashew (*Anacardium occidentale* Linn.) in mice

Ana Laura Nicoletti Carvalho[a,*], Raquel Annoni[a], Paula Regina Pereira Silva[a], Primavera Borelli[b], Ricardo Ambrósio Fock[b], Maria Teresa Salles Trevisan[c], Thais Mauad[a,d]

[a] *Experimental Atmospheric Pollution Laboratory (LPAE), Department of Pathology, São Paulo Medical School, University of São Paulo, Av Dr Arnaldo, 455, Room 1155, 01246-903 São Paulo, SP, Brazil*

[b] *Experimental Hematology Laboratory, Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, University of São Paulo, Av Prof Lineu Prestes, 580/17, 05508-900 São Paulo, SP, Brazil*

[c] *Department of Organic and Inorganic Chemistry, Federal University of Ceará, CP 12200, 60451-970 Fortaleza, CE, Brazil*

[d] *National Institute for Integrated Analysis of Environmental Risk (INAIRA), National Council for Scientific and Technological Development (CNPq), Ministry of Science and Technology, for the Development of National Institutes of Science and Technology (MCT), Av Dr Arnaldo, 455, Room 1220, 01246-903 São Paulo, SP, Brazil*

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ABSTRACT

Aim of the study: Anacardium occidentale Linn. (cashew) is a Brazilian plant that is usually consumed *in natura* and is used in folk medicine. Anacardic acids (AAs) in the cashew nut shell liquid are biologically active as gastroprotectors, inhibitors of the activity of various deleterious enzymes, antitumor agents and antioxidants. Yet, there are no reports of toxicity testing to guarantee their use *in vivo* models.

Materials and methods: We evaluated AAs biosafety by measuring the acute, subacute and mutagenic effects of AAs administration in BALB/c mice. In acute tests, BALB/c mice received a single oral dose of 2000 mg/kg, whereas animals in subacute tests received 300, 600 and 1000 mg/kg for 30 days. Hematological, biochemical and histological analyses were performed in all animals. Mutagenicity was measured with the acute micronucleus test 24 h after oral administration of 250 mg/kg AAs.

Results: Our results showed that the AAs acute minimum lethal dose in BALB/c mice is higher than 2000 mg/kg since this concentration did not produce any symptoms. In subacute tests, females which received the highest doses (600 or 1000 mg/kg) were more susceptible, which was seen by slightly decreased hematocrit and hemoglobin levels coupled with a moderate increase in urea. Anacardic acids did not produce any mutagenic effects.

Conclusions: The data indicate that doses less than 300 mg/kg did not produce biochemical and hematological alterations in BALB/c mice. Additional studies must be conducted to investigate the pharmacological potential of this natural substance in order to ensure their safe use *in vivo*.

1. Introduction

Cashew, *Anacardium occidentale* Linn. is a tropical tree native to northeast Brazil. Cashew apples (pseudofruit) and nuts can be consumed *in natura* and converted into various nutritional products (juice, tea, jam and beverages), (Kubo et al., 1993; Trevisan et al., 2006). Cashew nut shell liquid (CNSL) is used in industrial applications such as food preservatives, paints, cements and for gasoline stabilization; as such, it is an important commercial product in several tropical countries (Paramashivappa et al., 2001; Trevisan et al., 2006; Narasimhan et al., 2008).

In addition, this plant has been widely used in folk medicine in Brazil, India and Africa to treat inflammation, gastrointestinal diseases and hypertension (Mota et al., 1985; Cavalcante et al., 2003; Konan and Bacchi, 2007b). Several studies have evaluated the biological effects and pharmaceutical potential of cashew tree extracts and parts. For instance, pre-treatment with 200 mg/kg of the methanol extract of *Anacardium occidentale* stem bark completely protected against lipopolysaccharide-induced septic shock in Swiss mice (Olajide et al., 2004). Hydroethanolic extract from cashew leaves, which are rich in polyphenols, inhibited gastric lesions induced by HCl/ethanol in female rats (Konan and Bacchi, 2007b). Finally, a mixture of condensed and hydrolysable tannins from

Abbreviations: AAs, anacardic acids; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; b.w., body weight; CAJ, cashew apple juice; CNU, cashew nut oil; CNSL, cashew nut shell liquid; ECPs, polychromatic erythrocytes; MNPCEs, micronucleated polychromatic erythrocytes; MNU, N-methyl-N-nitrosourea; ROS, reactive oxygen species.

* Corresponding author. Tel.: +55 11 30617173; fax: +55 11 30628098.
E-mail addresses: analauranc@yahoo.com.br (A.L.N. Carvalho), rqannoni@yahoo.com.br (R. Annoni), preginahpv@usp.br (P.R.P. Silva), borelli@usp.br (P. Borelli), hemato@usp.br (R.A. Fock), trevisan@ufc.br (M.T.S. Trevisan), tmauad@usp.br (T. Mauad).

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the bark of *Anacardium occidentale* L. showed anti-inflammatory activity (Mota et al., 1985). Antimutagenicity and antigenotoxicity studies performed with methanolic extracts of the cashew stem bark reinforced the potential therapeutic properties of this plant (Barcelos et al., 2007a,b).

Cashew apple, nut (raw and roasted) and CNSL contain a range of alkyl phenols such as anacardic acids (AAs), cardanols and cardols. The highest levels of AAs were detected in CNSL (353.6 g/kg), followed by cashew fiber (6.1 g/kg) and roasted cashew nut (0.65 g/kg) (Trevisan et al., 2006). Cashew apples and fiber contained mainly AAs, whereas CNSL contained an abundance of cardanols and cardols in addition to AAs (Trevisan et al., 2006).

CNSL is a cheap and renewable by-product obtained during cashew nut processing (Paramashivappa et al., 2001; Rodrigues et al., 2006). As a unique, natural source of unsaturated long-chain phenols, CNSL is being used in insecticidal, fungicidal and medicinal applications. For instance, in the hypoxanthine/xanthine oxidase assay, CNSL is a potent scavenger of reactive oxygen species (ROS) (Trevisan et al., 2006). Anacardic acids have been described as the main active compound in CNSL, and evidence suggests that the phytyl side-chain, along with the phenolic ring system (as salicylic acid), drives its great antioxidant capacity (Trevisan et al., 2006). Cavalcante et al. (2003) measured protection of DNA damage from ROS and showed that fresh cashew apple juice (CAJ) has higher antioxidant capacities than the processed juice (cajuína). Interestingly, there was a correlation between antioxidant properties and the AAs content in CAJ (17.9 mg/100 g) and in cajuina (0.41 mg/100 g).

AAs have other diverse biological effects including the following: (1) antimicrobial activity against methicillin-resistant *Staphylococcus aureus*, *Streptococcus mutans* and anti *Helicobacter pylori* (Muroi and Kubo, 1996; Kubo et al., 1999; Kubo et al., 2003; Green et al., 2007), (2) gastroprotection (Morais et al., 2010), and (3) inhibition of enzymes such as lipoxygenase (Shobha et al., 1994; Ha and Kubo, 2005), tyrosinase (Kubo et al., 1994), cyclooxygenase (Grazzini et al., 1991; Paramashivappa et al., 2003; Ha and Kubo, 2005) and histone acetyltransferases (Sun et al., 2006; Dekker and Haisma, 2009). Sung et al. (2008) have demonstrated that AAs modulate the nuclear factor-κB signaling pathway through a variety of stimuli and suggested that AAs could be a therapeutic option for cancer prevention or treatment.

In order to investigate the clinical potential of this natural substance, pharmacokinetic, pharmacodynamic and toxicity testing still has to be performed in animal models. To the best of our knowledge, no toxicity or mutagenicity tests using AAs *in vivo* have been performed. In the present study, acute and subacute toxicity of AAs is investigated via BALB/c mice.

2. Materials and methods

This study was approved by the Ethical Committee of São Paulo University Medical School (protocol number: 0114/2007).

2.1. Plant material

The cashews (*Anacardium occidentale* Linn.) were harvested at the Embrapa Tropical Agroindustry Experimental Station, located in Paraipaba, Ceará, Brazil during the 2007 season. The fruits were from a commercial cultivar (CCP-76), genetic material from which is maintained on the Embrapa's germplasm bank. The fresh cashew apples were manually separated from the nuts and were provided as a kind gift from Dr. Edy Sousa de Brito (Embrapa, Fortaleza, Brazil).

Cashew nut shell liquid (300 g) was obtained by heating (175 °C) the fruit (1 kg) in an oven for 45 min. The cashew nut oil (2 L) was obtained from cashew nuts subjected to Soxhlet extraction with hexane (3 h).

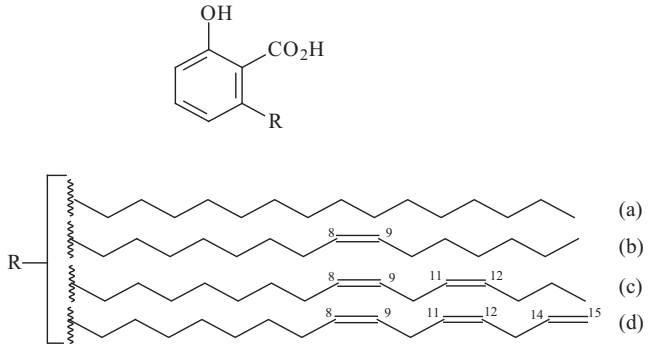

Fig. 1. Chemical structure of anacardic acids. Description of illustration: (a) $C_{22}H_{36}O_3$, (b) $C_{22}H_{34}O_3$, (c) $C_{22}H_{32}O_3$, (d) $C_{22}H_{30}O_3$.

2.2. Extraction and isolation of anacardic acids

Extracted CNSL (200 g) was dissolved in 5% aqueous methanol (1200 mL) and calcium hydroxide (100 g) was added while stirring. The mixture was kept at 50 °C stirring for 3 h. The supernatant solution was monitored using two-dimensional thin layer chromatography to test for the absence of anacardic acids. The precipitated calcium anacardate was filtered and washed with methanol. Calcium anacardate was then dissolved in distilled water acidified with 11 M HCl. The solution was extracted with ethyl acetate; the ethyl acetate layer was washed with distilled water and dried over anhydrous sulfate, then concentrated under reduced pressure to yield 120 g of AAs mixture, as described by Paramashivappa et al. (2001). All of the structures were established by comparing spectral and physical data with those previously reported in the literature (Trevisan et al., 2006) and by direct comparison with authentic samples. Fig. 1 depicts the structure of the AAs isolated from CNSL.

2.3. Animals

Male and female BALB/c mice (20–25 g) were obtained from the animal facility of São Paulo Medical School, University of São Paulo. All animals received care in compliance with the "Principles of Laboratory Animal Care" published by the National Institutes of Health (NIH publication #85-23, revised in 1985). Animals were housed in group-cages by sex at 22–26 °C with a 12-h/12-h light/dark cycle and received *ad libitum* water and commercial pellet food for small rodents from Nuvital (Nuvilab CR-1; Colombo, Brazil).

2.4. Anacardic acids toxicity tests

The protocols of oral acute and subacute toxicity were performed according to the United States Environmental Protection Agency Guidelines for Acute Oral Toxicity (2002) and Repeated Dose 28-day Oral Toxicity Study in Rodents (2000). For mutagenicity analysis, the acute micronucleus test was followed according to protocol described by MacGregor et al. (1987).

2.4.1. Oral acute toxicity

BALB/c mice were randomly divided into three groups (control, cashew nut oil, anacardic acids), with five males and five females in each group. A single, maximum dose of 2000 mg/kg body weight (b.w.) of AAs dissolved in 800 μL of cashew nut oil (vehicle) was orally administered (Konan et al., 2007a). The animals in the control (Ctrl) and the cashew nut oil (CNO) groups received the volume