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# Phytochemical constituents, antioxidant activity, cytotoxicity and osmotic fragility effects of Caju (*Anacardium microcarpum*)

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ABSTRACT

*Anacardium microcarpum* is consumed as infusion in the Brazilian folk medicine for the treatment of various diseases in which free radicals assaults have been implicated in their etiology. However, there is no scientific report on this traditional claim. Thus, we investigated for the first time, its antioxidant activity as well as its potential toxic effects on human leukocytes and erythrocytes *in vitro*. In addition, qualitative and quantitative analyses of the classes of phytochemicals in the various fractions from *A. microcarpum* were carried out by HPLC–DAD. The results demonstrated that $IC_{50}$ (for DPPH radical scavenging) varied from $27.88 \pm 1.60$ (AcOEt fraction) to $32.86 \pm 3.05$ μg/mL (EtOH fraction), and all the fractions strongly inhibited $Fe^{2+}$ (10 μM)-induced lipid peroxidation in rat brain and liver homogenates. All the fractions were not cytotoxic to leukocytes and were able to prevent against 2 mM $H_2O_2$-induced cytotoxicity. Moreover, the fractions did not have any effect on human erythrocytes osmotic fragility, suggesting that *A. microcarpum* infusion can be consumed safely. Preliminary phytochemical analysis exhibited the presence of phenolics and flavonoids compounds as major phytochemical groups. Taking together, our results indicate that the popular use of *A. microcarpum* for preventive or therapeutic agents in pathologies where cell oxidative stress is implicated has a scientific basis.



## 1. Introduction

The growing interest in plant-derived extracts, instead of pure compounds have generated considerable interest of researchers in finding information on the biological activities and toxicity of various plants traditionally used in folk medicine to treat diseases (Lantto et al., 2009; Oke and Aslim, 2010; Kuete et al., 2011; Awah et al., 2012; Kamdem et al., 2013). This is due to the fact that their health-promoting effects might involve several active compounds in the extracted material, and that they are generally regarded as safe (Dastmalchi et al., 2007; Lantto et al., 2009).

The genus *Anacardium* is represented by eleven (11) species in which *Anacardium occidentale* is widely known due to its nutritional and economic values. *A. microcarpum* (family: Anacardiaceae) known as Caju, is one of the plant species of this genus. The plant is native from Brazil, especially in the Northeastern region. Its fruits contain high level of vitamin C, sugars, phenolic compounds and minerals (calcium, iron and phosphorous). Anacardic acid which is

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used in the chemical industry for the production of cardanol (used for resins, coatings and frictional materials), has been reported to be the main component of both *A. occidentale* and *A. microcarpum* (Agostini-Costa et al., 2004; Alexander, 2008; Tedong et al., 2010). The infusions of *A. microcarpum* barks is used in Brazilian folk medicine as a tonic for the treatment of inflammation, rheumatism, tumor and infectious diseases, in which free radicals and/or reactive oxygen/nitrogen species (ROS/RNS) assaults have been implicated in their etiology.

Substantial evidences from the literature indicate that free radicals and/or ROS/RNS may cause cell and tissue damage by interacting with cell membranes and organelles. For instance, by-products of lipid peroxidation have been shown to cause profound alterations in the structural organization and functions of the cell membrane such as loss of membrane fluidity, loss of essential fatty acid, increased permeability to ions, leading to the release of cell and organelle contents (Van Ginkel and Sevanian, 1994; Gutteridge, 1995; Petrescu et al., 2001). Therefore, the potential of plant extracts or natural antioxidants that may help the organism to modify the oxidative status in disease conditions is of utmost importance.

In spite of the beneficial effects of plant extracts, exposure to phytotherapeutic agents however, can represent a serious public health problem (Secco, 1990; De Moraes et al., 2006) since several of them can be toxic to cells (ex. leukocytes and erythrocytes) (Bent and Ko, 2004; Shi et al., 2008). They can exert their toxicity by promoting excessive fluidity or denaturation of the membranes (Penha-Silva et al., 2007; De Freitas et al., 2008). Some authors have attempted to screen the potential toxic and beneficial effects of plants extracts (Yen et al., 2001; Ajaiyeoba et al., 2006; Oke and Aslim, 2010; Kuete et al., 2011; Awah et al., 2012; Kamdem et al., 2013) using several cell types. As part of our interest in the efficacy and safety of *A. microcarpum*, we evaluated its antioxidant activity and potential toxicity in human leukocytes and erythrocytes.

Based on the aforementioned information and considering that there is no scientific basis in the literature on the use of *A. microcarpum* in the management of free radicals related diseases as well as its potential toxic effect to human, the present study was designed to investigate for the first time: (i) the antioxidant activity of different fractions from *A. microcarpum* barks using two *in vitro* biochemical assays; (ii) the cytotoxicity of fractions from *A. microcarpum* in human leukocytes using the Trypan blue assay; (iii) the influence of different fractions from *A. microcarpum* on the osmotic fragility of human erythrocytes. In addition, the chemical characterization of the fractions was performed using high performance liquid chromatography coupled to diode-array detector (HPLC–DAD).

## 2. Materials and methods

### 2.1. Chemicals

All chemicals used including solvents were of analytical grade. Methanol, acetic acid, gallic acid, caffeic acid, ellagic acid and chlorogenic acid were purchased from Merck (Darmstadt, Germany). Quercetin, rutin, isoquercitrin, quercitrin, kaempferol, catechin and epicatechin were acquired from Sigma Chemical Co. (St. Louis, MO, USA). 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, malonaldehydebis-(dimethyl acetal) (MDA), thiobarbituric acid (TBA), sodium azide and hydrogen peroxide ($H_2O_2$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### 2.2. Plant collection and extractions

The stem barks of *A. microcarpum* were collected from Barrero Grande, Crato-Ceará (7°22′ S; 39°28′ W; 892 m sea level), Brazil, in November 2011. The plant material was identified by Dr. Maria Arlene Pessoa da Silva of the herbarium Caririense Dárdano de Andrade – Lima (HCDAL) of the Regional University of Cariri (URCA) and a voucher specimen was deposited (number 6702).

The fresh barks of *A. microcarpum* were macerated with 99.9% of ethanol and water (1:1, v/v) for 3 days. The suspension was filtered, solvent evaporated under reduced pressure and lyophilized to obtain 490 g of ethanolic extract (EtOH fraction). One hundred and fifty grams (150 g) of this was partitioned with ethyl acetate and methanol to obtain 12.5 g of ethyl acetate fraction (AcOEt fraction) and 105.23 g of methanolic fraction (MeOH fraction). All the fractions were stored in the freezer and re-suspended in water prior to experiments.

### 2.3. Identification and quantification of phenolic and flavonoid compounds by HPLC–DAD

Reversed phase chromatographic analyses were carried out under gradient conditions using $C_{18}$ column (4.6 mm × 150 mm) packed with 5 μm diameter particles; the mobile phase was made up of water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% of B for 2 min and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10, 20, 30, 40, 50 and 80 min, respectively, following the method described by Boligon et al. (2013) with slight modifications. *A. microcarpum* (MeOH fraction, EtOH fraction and AcOEt fraction) were analyzed at a concentration of 15 mg/mL. The presence of twelve compounds namely, gallic acid, caffeic acid, chlorogenic acid, ellagic acid, catechin, epicatechin, quercetin, isoquercitrin, quercitrin, rutin, kaempferol, and kaempferol glycoside were investigated. Identification of these compounds was performed by comparing their retention time and UV absorption spectra with those of the commercial standards. The flow rate was 0.7 ml/min, injection volume of 50 μL and the wavelengths were 257 nm for gallic acid, 280 nm catechin and epicatechin, 327 nm for caffeic, ellagic and chlorogenic acids, and 365 nm for quercetin, isoquercitrin, quercitrin, kaempferol, kaempferol glycoside and rutin. The samples and mobile phase were filtered through 0.45 μm membrane filter (millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.030–0.400 mg/mL for quercetin, quercitrin, isoquercitrin, rutin, epicatechin, catechin and kaempferol; and 0.050–0.350 mg/mL for gallic, chlorogenic, caffeic and ellagic acids. The chromatography peaks were confirmed by comparing retention time with those of reference standards and by DAD spectra (200–500 nm). All chromatography operations were carried out at ambient temperature and in triplicates. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviations of the responses and the slopes using three independent analytical curves, as defined by Sabir et al. (2013). LOD and LOQ were calculated as 3.3 and 10 $\sigma/S$, respectively, where $\sigma$ is the standard deviation of the response and $S$ is the slope of the calibration curve.

### 2.4. DPPH radical scavenging assay

Scavenging activity on DPPH free radicals by the fractions was assessed according to the method described by Kamdem et al. (2013). Twenty microliters of the fractions (1–400 μg/mL) was mixed with 100 μL of 0.3 mM DPPH in ethanol. The mixture was allowed to stand at room temperature for 30 min in the dark. Blank solutions were prepared with each test sample (20 μL) and 100 μL of water. The negative control was 100 μL of 0.3 mM DPPH with 20 μL of water, while ascorbic acid (1–50 μg/mL) was used as positive control. The absorbance was measured at 518 nm against each blank using ELISA microplate reader (SpectraMax, USA) and the