Food Chemistry 197 (2016) 1153–1159

Contents lists available at ScienceDirect

Food Chemistry

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

Polyphenols, antioxidants, and antimutagenic effects of *Copaifera langsdorffii* fruit

Ângela Giovana Batista [a], Angélica Simões Ferrari [b], Débora Cristina da Cunha [b], Juliana Kelly da Silva [a], Cinthia Baú Betim Cazarin [a], Luiz Claudio Correa [d], Marcelo Alexandre Prado [a], Luciano Bruno de Carvalho-Silva [b], Elizabethe Adriana Esteves [c], Mário Roberto Maróstica Júnior [a,*]

[a] School of Food Engineering, University of Campinas (UNICAMP), P.O. Box 6121, 13083-862 Campinas-SP, Brazil
[b] School of Nutrition, Federal University of Alfenas, MG (UNIFAL-MG), Brazil
[c] Nutrition Department, Federal University of Vales do Jequitinhonha e Mucuri (UFVJM), Diamantina, Minas Gerais, Brazil
[d] Brazilian Agricultural Research Corporation, Embrapa Tropical Semi-arid, Petrolina, Pernambuco 56302-970, Brazil

ARTICLE INFO

Article history:
Received 17 April 2015
Received in revised form 21 October 2015
Accepted 17 November 2015
Available online 18 November 2015

Chemical compounds studied in this article:
Gallic acid (PubChem CID: 370)
(−)-Epicatechin (PubChem CID: 72276)
(+)-Catechin (PubChem CID: 964)
(−)-Epicatechin gallate (PubChem CID: 367141)
Isoquercitrin (PubChem CID: 5280804)

Keywords:
Copaiba
Gallic acid
Micronuclei
Antioxidant capacity

ABSTRACT

Copaifera langsdorffii (copaiba) is a Brazilian exotic fruit, poorly studied regarding its bioactive composition. The aim of this study was to determine bioactive compounds, antioxidant and antimutagenic activities of copaiba pulp. The samples were extracted with different solvents in order to analyze polyphenol compounds (Folin Ciocalteau and HPLC–DAD), total flavonoids (reaction with $AlCl_3$) and antioxidant capacity (FRAP, ORAC, DPPH). The copaiba fruit showed high polyphenol content and antioxidant capacity. Phenolic compounds, such as gallic acid, epicatechin gallate, catechin, epicatechin and isoquercitrin, were identified in the copaiba pulp. Despite the antioxidant capacity, the highest dose of copaiba showed no antimutagenic effects in the *in vivo* study. The dose which showed antimutagenic activity was 100 mg kg^{-1}.

1. Introduction

Copaiba (*Copaifera langsdorffii*) is a typical plant from Brazil. It has leguminous fruits, which could be harvested from August to September, when the plants are almost completely without leaves. However, the frequency of its fructification is irregular, with alternating years of good production and years without crops (Sebbenn et al., 2011).

The resin oil of copaiba trunk is known by its cicatrizing, antipsoriatic, anti-genotoxic effects and antioxidant capacity (Gelmini et al., 2013; Paiva, Cunha, et al., 2002; Paiva, Gurgel, et al., 2002; Paiva et al., 2004). The extract of copaiba leaf also contains polyphenols with antioxidant and antitumor properties (Costa-Machado, Bastos, & de Freitas, 2013; dos Santos et al., 2010). However, studies of the fruit are limited.

The leguminous fruit of copaiba offers a dried pulp, which covers the seed. A recent paper showed that its intake had hypoglycemic and hypocholesterolemic effects in healthy rodents (Esteves et al., 2011). These data excite interest in the unknown bioactive compounds of copaiba pulp that could be used against degenerative and metabolic chronic diseases.

Polyphenols are a large group of secondary metabolites, widespread in the plant kingdom. They have the ability to quench and scavenge free radicals due to their ability to donate electrons and hydrogen atoms. However, their antioxidant effect depends on their stability in various systems, as well as the number and position of hydroxyl groups in the molecule (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999).

In vivo studies, using Brazilian exotic fruits for both treatment and prevention of diseases, have shown that polyphenol

* Corresponding author at: Departamento de Alimentos e Nutrição, Rua Monteiro Lobato, 80, Cidade Universitária, Campinas-SP, Brazil.
E-mail address: mario@fea.unicamp.br (M.R. Maróstica Júnior).

http://dx.doi.org/10.1016/j.foodchem.2015.11.093
0308-8146/

compounds from these fruits are associated with improvements in oxidative stress due to increased antioxidant status, decreased lipid peroxidation, tissue preservation and increased activity of the endogenous antioxidant enzyme system (Batista et al., 2014; Prior et al., 2003). Furthermore, several fruits have shown interesting antimutagenic effects in rodents (Carvalho-Silva et al., 2012; Leite-Legatti et al., 2012; Malta et al., 2012), which could be associated with the presence of polyphenols. In this context, the aim of this study was to investigate the polyphenol composition of copaiba pulp fruit, its antioxidant capacity and its antimutagenic activity. This is the first study to explore polyphenols and antioxidants in copaiba fruit.

2. Materials and methods

2.1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH); (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), and 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), were all obtained from Sigma–Aldrich (São Paulo – Brazil). Cyclophosphamide (CP) was purchased from Europharma (São Paulo, Brazil). Randomly methylated β-cyclodextrin (Trappsol; RMCD) was acquired from CTD Inc. (High Springs, FL).

HPLC Standards (benzoic, cinnamic, and gallic acid standards) were purchased from Chem Service (West Chester, USA). (+)-Catechin, (−)-epicatechin, (−)-epicatechin gallate, procyanidin B1, procyanidin B2, quercetin (dihydrate), isoquercitrin, and rutin standards were obtained from Extrasynthese (Genay, France). p-Coumaric acid was purchased from Sigma (United Kingdom).

2.2. Samples

Ripe copaiba fruits were collected from the city of Serro, São Gonçalo do Rio das Pedras district in the Northwestern region of Minas Gerais State, Brazil, at a latitude of 18°24′00″S and longitude of 43°29′00″ West of Greenwich. The fruits (approximately 160 units) were cleaned and pulps were extracted (0.55 g per each fruit pulp). The materials were dried in a forced air circulation oven at 60 ± 5 °C for 48 h, ground to a powdered flour, homogenized, packed in polyethylene bags and frozen at −18 ± 5 °C.

2.3. Proximate composition

Total nitrogen was determined using a NDA 701 Dumas Nitrogen analyzer (Velp Scientifica, Usmate, MB, Italy). Moisture and ash were analyzed according to standard methods (IAL, 1985), and lipids by a Soxhlet apparatus using petroleum ether as solvent. Carbohydrates were calculated by difference.

2.4. Polyphenols and antioxidant power determinations

2.4.1. General

All the absorbance and fluorescence readings for the analyses were determined in a Synergy HT, Biotek microplate reader (Winooski, USA) with Gen5™ 2.0 data analysis software.

2.4.2. Extractions

The extracts were made in duplicate as follows:

For ethanol extract, copaiba pulp (10 g) was extracted by successive maceration with ethanol at room temperature to obtain a polar extract, after solvent evaporation and freeze-drying (Leite-Legatti et al., 2012). The dried extract (48.97% yield) was resuspended in water for the analysis, except for HPLC (resuspended in the mobile phase).

For 60% ethanol extract, 15 ml of 60% ethanol solution were added to 1 g of sample, mixed and kept in a water bath during 60 min at 70 °C. The sample was filtered and the supernatant transferred to a 25 ml volumetric flask. The procedure was repeated with 10 ml of 60% ethanol solution and the supernatants were combined and stored in amber flasks at 4–8 °C (Spagolla, Santos, Passos, & Aguiar, 2009).

For 80% methanol extract, the sample was weighed (1 g) and extracted with 20 ml of 80% methanol (MeOH) at 37 °C for 3 h in a shaking water bath. Later, it was centrifuged (2000*g*, 10 min), and the supernatant made up to 25 ml and stored in amber flasks at 4–8 °C. A hydrolysis of the glycoside flavonoids was performed in the MeOH extract as described in a previous study (Batista et al., 2014).

2.4.3. Polyphenols determination

2.4.3.1. Folin–Ciocalteau method. Total phenolic content was determined by the Folin–Ciocalteau method (Swain & Hillis, 1959). Water, Folin–Ciocalteau reagent and sodium carbonate were added to the extract (at proportions of 16:1:2:1) and, after 2 h in the dark at room temperature, the absorbances of samples and standard curve were read at 725 nm. The results were expressed as gallic acid equivalents (mg GAE g^{-1}).

2.4.3.2. Yellow flavonoids. The colorimetric method of determination of total yellow flavonoids (Zhishen, Mengcheng, & Jianming, 1999) was done as follows: previously water-diluted extracts, water, 5% sodium nitrite, 10% aluminum chloride and 1 M sodium hydroxide solutions were added to the tubes at 10:50:3:6:20 proportion and they were filled with water. There was a precipitation after the addition of NaOH solution to the copaiba extract, and, due to this, the mixes were centrifuged at 2000*g*, 10 min. A calibration curve was constituted, using catechin and read at 510 nm. The results were expressed as catechin equivalents (mg CE g^{-1}).

2.4.3.3. HPLC analysis. The analysis of polyphenolic compounds in the EtOH extract was performed, using an HPLC system (Waters e2695 Separation Module Alliance, Milford, MA, USA) equipped with a quaternary solvent pump and an automatic injector. For the phenolic determination, a diode array detector (DAD) (Waters model 2998) and a fluorescence detector (FLD) (Waters model 2475) were employed. Acquisition and processing of data were carried out, using the Waters Empower™ 2 software (Milford, MA, USA).

The extracts were filtered through a 0.45 μm nylon membrane (Allcrom-Phenomenex, USA). The injection volume was set as 10 μl. The Gemini NX C-18 column (150 mm × 4.6 mm × 3 μm) (Phenomenex, USA) was maintained at 40 °C to optimize the phenolic compound separations, as recommended in previous work (Natividade, Correa, de Souza, Pereira, & Lima, 2013). The mobile phase consisted of a gradient mixture of solvent A (0.85% phosphoric acid solution) and solvent B (acetonitrile), with a flow-rate of 0.5 ml min^{-1}. The gradient was started with 100% of solvent A and adjusted for 93% of solvent A and 7% of solvent B in 10 min, 90% of solvent A and 10% of solvent B in 20 min, 88% of solvent A and 12% of solvent B in 30 min, 77% of solvent A and 33% of solvent B in 40 min, 65% of solvent A and 35% of solvent B in 45 min, and 100% of solvent B in 55 min (Natividade et al., 2013).

The standard solutions were injected for identification of the wavelengths of the absorption of the compounds and their respective retention times (RT). The fluorescence detector was used at 320 nm emission for identification of the following compounds: (+)-catechin, procyanidin B2, and (−)-epicatechin. The diode array detector was employed at three wavelengths, these being 280 nm for identification of the gallic acid, (−)-epigallocatechin gallate,

(−)-epicatechin gallate and procyanidin B1, 320 nm for the other phenolic acids, and 360 nm for flavonols.

The polyphenols in the 60% EtOH, 80% MeOH and hydrolyzed extracts of copaiba were determined as described in previous work (Batista et al., 2014).

2.4.4. Antioxidant measurements

2.4.4.1. FRAP method. The ferric reducing ability of the extracts was determined, using the FRAP method (Rufino et al., 2010). The FRAP reagent was prepared in the dark, using 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ in a 40 mM HCl solution and 20 mM $FeCl_3$ (at proportions of 10:1:1). The sample or standard solutions, water and FRAP reagent were mixed and incubated in a water bath for 30 min at 37 °C. The samples and trolox standard curve were read at 595 nm.

2.4.4.2. DPPH method. The analysis of DPPH-IC_{50} was conducted as follows (Rufino et al., 2010): an aliquot of DPPH solution (0.06 mM in methanol) was added to water-diluted extracts in the proportion of 40.4:1. In order to determine the time of reaction, kinetic readings were obtained each minute at 515 nm until the stabilization of the absorbance. The reading time of copaiba extracts in the DPPH analysis was 10 min. Five concentrations of the extracts ($Abs_{samples}$) were used for calculations of inhibition and the solvents of the extractions were used as controls ($Abs_{control}$). After the calculation of inhibition (Eq. (1)), the concentration of copaiba fruit pulp required in order to reduce DPPH radical by 50% (IC_{50}) by linear regression was calculated. The results were expressed as g l^{-1}.

$$\% \text{ Inhibition} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100 \quad (1)$$

2.4.4.3. H-ORAC method. The hydrophilic-ORAC (oxygen radical absorbance capacity) (Davalos, Gomez-Cordoves, & Bartolome, 2004) test was carried out by adding phosphate buffer (PB pH 7.4)-diluted sample extracts or standard solutions, PB-diluted fluorescein and AAPH (at proportion of 1:6:3, respectively) to black microplates. Trolox was used as a standard and the microplate reader with fluorescent filters set as follows: excitation wavelength, 485 nm; emission wavelength, 520 nm. ORAC values were expressed as micromole trolox equivalent (μmol TE) by using the standard curves for each assay. The linearity between the net area under the curve and the concentration was checked for the samples and the fluorescence readings were used to make the appropriate calculations.

2.4.4.4. L-ORAC method. The lipophilic-ORAC analysis was based on a previous study (Prior et al., 2003) with modifications. Copaiba pulp was extracted by successive maceration with dichloromethane at room temperature to obtain a non-polar extract, as described previously (Leite-Legatti et al., 2012). After solvent evaporation and freeze-drying, the non-polar freeze-dried extract (1.21% yield) was resuspended in acetone. The test was carried out by adding 7% RMCD in 50% acetone solution diluted sample extracts or standard solutions, fluorescein and AAPH diluted in PB (pH 7.4), at proportion of 1:6:6, to black microplates. Trolox was used as a standard and a microplate reader with the same fluorescent filters, as in H-ORAC. The linearity between the net area under the curve and the concentration was checked.

2.5. Animal study

2.5.1. General

Newly weaned male Swiss mice were obtained and maintained under controlled conditions of temperature (22–24 °C), light (12 h light/12 h dark), and humidity (45–65%), with food and water *ad libitum*. The mice used for experimental research had body weights between 25 and 35 g. The Ethics Committee of the Federal University of Alfenas approved this study under protocol #236, in accordance with the *Sociedade Brasileira de Ciência em Animais de Laboratório* – COBEA.

2.5.2. Micronucleus test

The micronucleus test was performed (MacGregor et al., 1987) in order to investigate the protective effect of copaiba pulp against the clastogenicity induced by cyclophosphamide (CP). Fruit aqueous solution was administered by orogastric gavage for 15 consecutive days, at concentrations of 30, 100 and 300 mg kg^{-1} body weight (bw), selected on the basis of our previous acute toxicity studies in mice, which was found to be above 1000 mg kg^{-1} bw.

For gavage administration, copaiba fruit powder was diluted with distilled water, and a new dilution was performed every 3 days, taking into account the aforementioned concentrations, and each animal received 0.18 ml of the solution/day.

Experiments were performed according to distribution groups: G1 = 0.9% NaCl + CP (positive control); G2 = 0.9% NaCl (negative control); G3 and G4 = copaiba, 30 mg kg^{-1} bw; G5 and G6 = copaiba, 100 mg kg^{-1}; G7 and G8 = copaiba, 300 mg kg^{-1}. The groups G1, G3, G5 and G7 received intraperitoneal injections of 50 mg kg^{-1} bw of CP 24 h before the euthanasia, and the G2, G4, G6 and G8 received injection of 0.9% NaCl solution.

All animals were euthanized 24 h after treatment by cervical dislocations under anesthesia. For the conventional assessment of micronucleus frequencies, two slides for each animal were prepared (MacGregor et al., 1987). Briefly, femurs were dissected and cleaned of any adhering muscle, and bone marrow cells were flushed with bovine fetal serum into a centrifuge tube. The cells were stained with Leishman stain and centrifuged at 2000 rpm for 5 min, and the supernatant was removed. The slides were coded, and the cells were blindly scored by light microscopy at 1000 magnification. The frequency of micronucleated polychromatic erythrocytes (MNPCE) in individual mice was used as the experimental unit with variability (standard deviation) based on differences among animals within the same group. The percentage of reduction in the frequency of CP-induced DNA damage was calculated as follows:

$$\% \text{ Reduction} = [(\bar{y}A) - (\bar{y}B)/(\bar{y}A) - (\bar{y}C)] \times 100$$

where A = positive control group treated with CP; B = group treated with copaiba + CP; and C = negative control group.

2.6. Statistics

The polyphenols and antioxidant compounds of the different extracts were evaluated, using ANOVA and Tukey tests, considering $P < 0.05$. Pearson's coefficient was used to analyze the correlations ($P < 0.05$) (GraphPad Prism 5.0, GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

The polyphenols and antioxidant capacity of copaiba fruit were determined for the first time in this study. The macronutrients and contribution of the lipophilic compounds to the *in vitro* antioxidant capacity were also investigated. The results showed that the fruit of *C. langsdorffii* did not induce mutagenic damage, and that it had potential properties for pharmacological purposes, as seen in this study.

Regarding macronutrients, the copaiba pulp showed high amounts of carbohydrates and protein (Table 1).

Table 1
Proximate composition (g%) of copaiba fruit pulp (dry weight).

Composition	Mean ± SD
Moisture	3.63 ± 0.05
Protein[a]	5.51 ± 0.29
Lipids	4.20 ± 0.21
Ash	1.58 ± 0.001
Carbohydrates	85.08 ± 0.15

[a] $N \times 6.25$.

The contents of polyphenols were higher in the EtOH and 60% EtOH extracts, which were correlated with the highest flavonoid contents ($P < 0.01$, $r = 0.678$) (Fig. 1).

Gallic acid was the major compound among the 13 polyphenols identified (Fig. 2). However, phenolic acids, e.g. *o*-coumaric and benzoic acids, and flavanols, e.g. epicatechin gallate, were also found in important amounts in the EtOH extract, (Table 2). Procyanidins B1 and B2 were also identified (Fig. 2, Table 2).

The results for the EtOH and 60% EtOH extracts of copaiba showed higher amounts of gallic acid when compared to the MeOH extract. Nevertheless, the hydrolysis of MeOH extract improved the quantification of gallic acid in copaiba (Fig. 1). The hydrolysis of the MeOH extract also increased the quantification of catechin and epicatechin, showing higher values in comparison with the other extractions. The 60% EtOH extract showed higher values of epicatechin than those in the EtOH extract. However, the catechin was not detected in the first one, possibly because of the higher temperature, and time of extraction or the different polarities in the solvent extraction (Fig. 1).

Among flavonols in copaiba pulp, quercetin and its derivatives could be highlighted, isoquercitrin being the main compound among them (Table 2). The content of flavonoids in copaiba (measured in all extracts) was correlated with the antioxidant capacity (FRAP assay; $P < 0.0001$, $r = 0.8496$), and the extraction with EtOH showed the highest FRAP value (Fig. 1).

According to the DPPH-IC_{50} assay, the decreasing order of extraction of the antioxidant power of copaiba was EtOH > 60% EtOH > MeOH (Table 3).

The H-ORAC value in EtOH was similar to that in the 60% EtOH extract and higher than that in the MeOH; however, the hydrolysis of MeOH extract increased its antioxidant capacity (Table 3). The H-ORAC values was correlated with the amounts of gallic acid in all extracts of copaiba ($P < 0.01$, $r = 0.625$).

The extracts showed good linearity at the concentration range used in the ORAC method (Table 3). Low concentrations of copaiba pulp were necessary to improve antioxidant power by H-ORAC, which corroborated the results of DPPH-IC_{50} (Table 3).

The L-ORAC value of the copaiba extracted with dichloromethane (1.21% of yield) was 7.75 ± 0.17 μmol TE g^{-1} of extract (concentration range = 1.8–10 g l^{-1}; $y = -0.316x^2 + 5.767x + 8.277$; $r^2 = 0.966$).

The results of the antimutagenic assay suggested no significant variation in body weight and chow consumption among the experimental groups ($P > 0.05$) during the study period (Suppl. 1). The groups (G1) that received CP showed a statistically significant induction of chromosomal damage in immature erythrocytes (MNPCE) in comparison to G2 (0.9% NaCl), demonstrating the sensitivity of the assay.

Copaiba powder showed no mutagenic capacity when compared to the values of the group that received NaCl on the day of injury. The doses 30 mg kg^{-1} and 300 mg kg^{-1} of copaiba powder were not able to reduce CP-induced chromosomal damage despite the linear *in vitro* antioxidant capacity. On the other hand, the 100 mg kg^{-1} of copaiba powder showed a 38% reduction of micronuclei (Table 4).

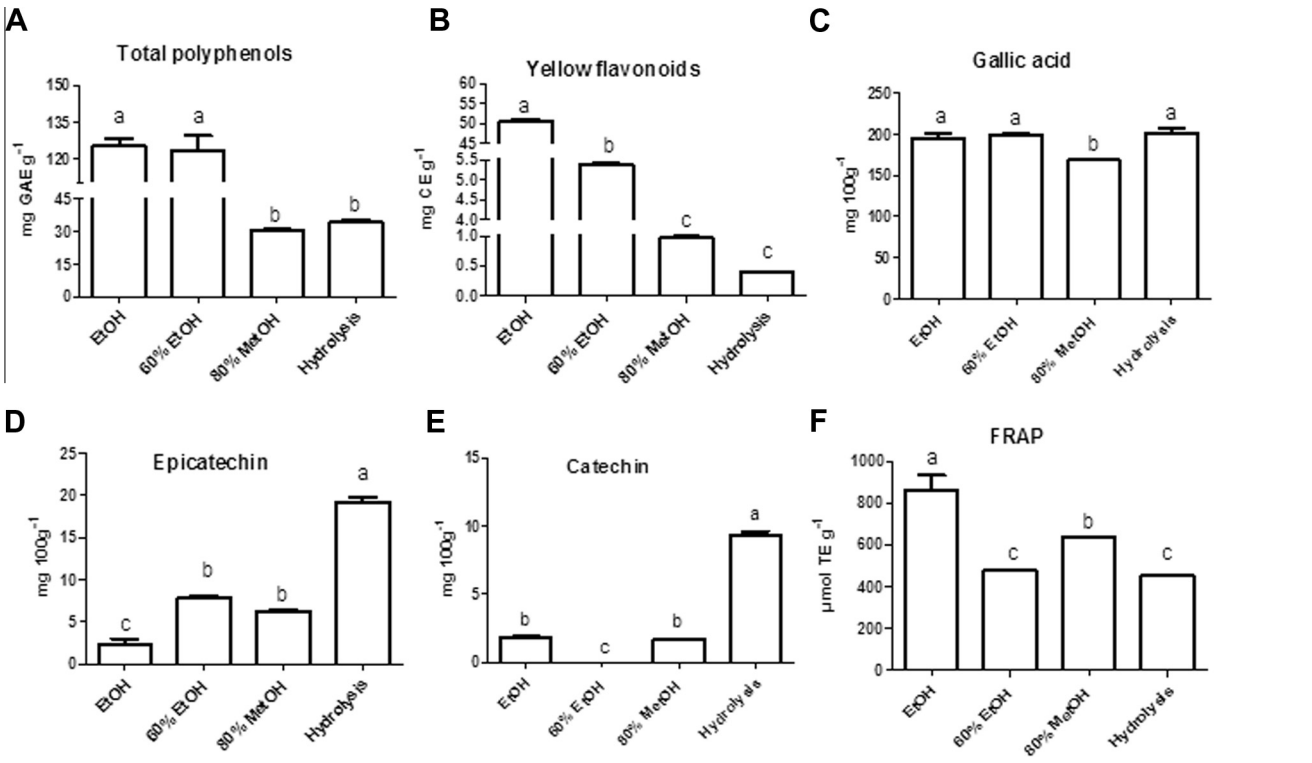

Fig. 1. Polyphenol contents and antioxidant capacity of copaiba fruit pulp. Polyphenols were assayed by Folin–Ciocalteau method (A); flavonoids assayed with $AlCl_3$ reaction (B); gallic acid (C), (−)-epicatechin (D), and (+)-catechin (E), assayed by HPLC; antioxidant capacity assayed by FRAP assay (F). Different letters in columns indicate statistical differences according to ANOVA and Tukey ($P < 0.05$).

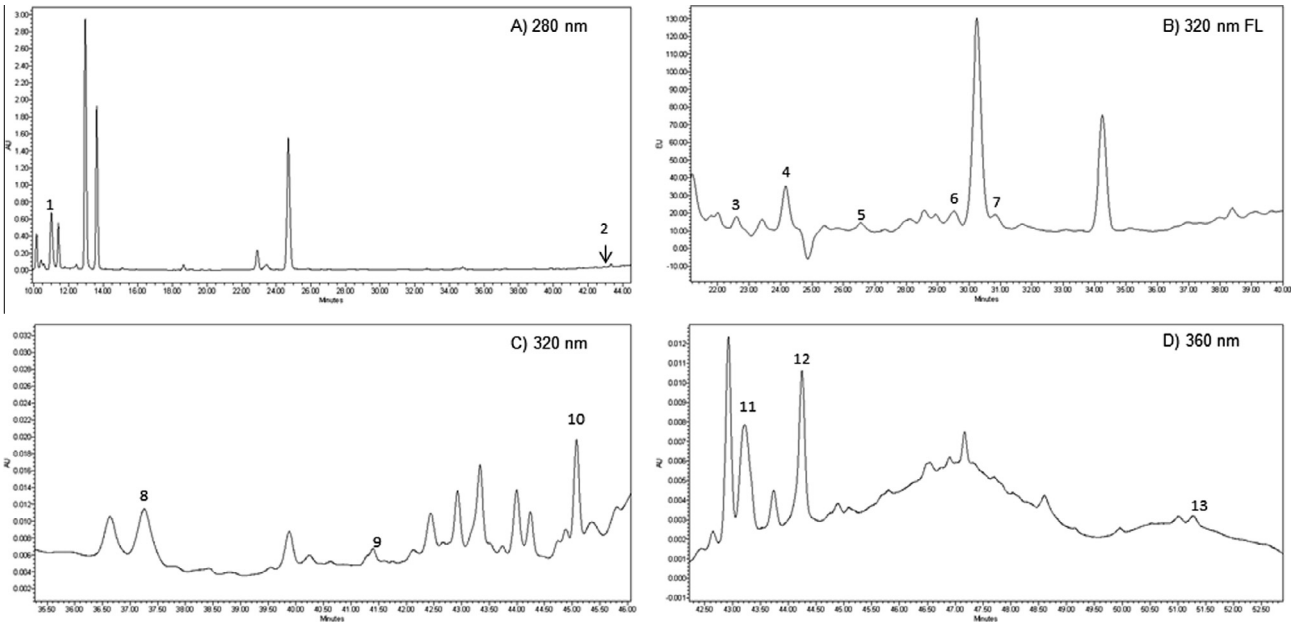

Fig. 2. Typical Chromatograms of the phenolic compounds identified in the EtOH extract of copaiba pulp. (A) 280 nm: 1. gallic acid (RT: 11.01 min); 2. (−)-epicatechin gallate (RT: 42.93 min); (B) 320 nm FL (fluorescence): 3. procyanidin B1 (RT: 22.60 min); 4. (+)-catechin (RT: 24.16 min); 5. benzoic acid (RT: 26.55 min); 6: (−)-epicatechin (RT: 29.53 min); 7. procyanidin B2 (RT: 30.83 min); (C) 320 nm: 8. *p*-coumaric acid (RT: 37.26 min); 9. cinnamic acid (RT: 41.39 min); 10. *o*-coumaric acid (RT: 45.08 min); (D) 360 nm: 11. rutin (RT: 43.21 min); 12. isoquercitrin (RT: 44.25 min); 13. quercetin (RT: 51.27 min).

Table 2
Polyphenols identified in the EtOH extract of copaiba fruit pulp by HPLC–DAD/FLD.

Polyphenols	(mg 100 g^{-1})
Phenolic acid	
p-Coumaric acid	1.49 ± 0.001
o-Coumaric acid	2.97 ± 0.26
Cinnamic acid	0.74 ± 0.26
Benzoic acid	2.41 ± 0.035
Gallic acid	195 ± 7.35
Flavanols and procyanidins	
(−)-Epicatechin gallate	3.34 ± 1.05
(+)-Catechin	1.76 ± 0.13
(−)-Epicatechin	2.32 ± 0.92
Procyanidin B1	2.04 ± 0.001
Procyanidin B2	1.02 ± 0.13
Flavonols	
Isoquercitrin	2.97 ± 0.000
Quercetin	0.37 ± 0.001
Rutin	1.94 ± 0.13

4. Discussion

The fruits from the *Fabaceae* family are well known for their protein contribution to the diet. In this study, the pulp of copaiba showed carbohydrates as the main nutrient (Table 1). However, a previous study has shown that its pulp has 26.43% of insoluble fibers and 2.15% of soluble fibers (Esteves et al., 2011). Thus, about 34% of the carbohydrates in copaiba pulp represents a non-digestible fraction, which could be associated with the glycemic- and cholesterol-lowering effects shown in the same work.

Phenolic compounds could also be associated with these effects. They are the major contributor to dietary antioxidant capacity. A study has reported that the consumption of polyphenols from fruits represents 320 mg of GAE daily among North Americans, and contributes to the highest antioxidant value when compared to other vegetables in the diet (Chun et al., 2005). However, phenolics have different structures, which means different interactions, stabilities and targets in biological fluids (Robards et al., 1999).

Concerning total polyphenol contents in copaiba, the results of the present study were higher when compared with previous work (Esteves et al., 2011). Furthermore, in this study the phenolic compounds were identified by HPLC (Fig. 2, Table 2).

Gallic acid was the major polyphenol identified in copaiba (Table 2). This compound is related to antioxidant, neuroprotective (Mansouri et al., 2013), anticarcinogenic, antimutagenic and anti-inflammatory activities (Verma, Singh, & Mishra, 2013), which suggests possible indications for the use of this fruit pulp.

In addition, tannins, such as epicatechin gallate, procyanidins, catechin and epicatechin, identified in copaiba pulp, could also contribute to its antioxidant power. Catechins, for example, have

Table 3
DPPH-IC_{50}, H-ORAC values and linearity of copaiba fruit pulp.

Extracts		Mean ± SD	Conc[a] (g l^{-1})	Slope	Intercept	R^2
EtOH	DPPH IC_{50} (g l^{-1})	0.51 ± 0.002^{c}	0.3–1	66.0	16.7	0.983
60% EtOH		1.17 ± 0.07^{a}	0.5–3	23.7	23.0	0.986
80% MeOH		0.87 ± 0.001^{b}	0.2–1	57.5	0.169	0.988
EtOH	H-ORAC (μmol TE g^{-1})	295 ± 8.85^{b}	0.5–2.5	28.1	0.652	0.995
60% EtOH		301 ± 10.8^{b}	0.1–0.3	201	−0.335	0.997
80% MeOH		219 ± 6.70^{c}	0.05–0.45	128	4.50	0.993
Hydrolyzed		416 ± 46.1^{a}	0.05–0.2	264	4.45	0.997

[a] Concentration ranges in pulp fruit. Different letters in the column indicate statistical differences according to ANOVA and Tukey tests ($P < 0.05$).

Table 4
Frequency of micronucleated polychromatic erythrocytes (MNPCE) of bone marrow cells of experimental groups treated with copaiba fruit pulp.

Groups	Treatments	Number of PCE	MNPCE		% Reduction
			No.	%	
G1	NaCl + CP	12,000	21	0.21	–
G2	NaCl + NaCl	12,000	5	0.06	
G3	30 mg kg^{-1} copaiba + CP	12,000	21	0.18	0.00
G4	30 mg kg^{-1} copaiba + NaCl	10,000	9	0.09	
G5	100 mg kg^{-1} copaiba + CP	10,000	8	0.08	38.0
G6	100 mg kg^{-1} copaiba + NaCl	12,000	11	0.09	
G7	300 mg kg^{-1} copaiba + CP	10,000	21	0.21	0.00
G8	300 mg kg^{-1} copaiba + NaCl	12,000	17	0.14	

MNPCE: chromosomal damage in immature erythrocytes; CP: cyclophosphamide. Copaiba fruit powder was dissolved in water and administered daily by gavage during 15 days.

been associated with anticancer, anti-inflammatory, antidiabetes, antiobesity and neuroprotective effects (Braicu, Ladomery, Chedea, Irimie, & Berindan-Neagoe, 2013; Williams & Spencer, 2012; Zaveri, 2006).

Isoquercitrin was highlighted among the flavonols (Table 2). This compound is more water-soluble than the aglycone-quercetin, and is related to functional effects, such as antioxidant, antitumoral, anti-inflammatory, and is used also against diabetes and cardiovascular disorders (Valentová, Vrba, Bancírová, Ulrichová, & Kren, 2014).

In general, the different extractions used to obtain the polyphenols and the antioxidant capacity of copaiba showed that EtOH was more efficient than 60% EtOH, probably due to lability to higher temperatures of some compounds, such as catechin (Fig. 1). EtOH was also more efficient than 80% MeOH; however, the MeOH-hydrolysis improved the amount of the polyphenols and antioxidant capacity (Fig. 1, Table 3), most likely because of the release of glycoside compounds and hydrolysis of complex tannins (Batista et al., 2014). Moreover, the differences found among the extracts could be explained by the effects of dielectric constants of different solvents used in the extractions (Haminiuk, Plata-Oviedo, Mattos, Carpes, & Branco, 2014), and also by the peculiarity of each antioxidant method used.

Despite the wide use of FRAP and DPPH methods for determination of antioxidant power of vegetables (Rufino et al., 2010), their assays are based on the single electron donation and other parameters weakly correlated with physiological events (Ou, Hampsch-Woodill, & Prior, 2001). The ORAC method is based on hydrogen atom transference, approaching the capacity of quenching a physiological radical (peroxyl) and reproducing other biological conditions, such as body temperature, and physiological pH (Prior et al., 2003). Additionally, the linearity of copaiba antioxidant power, at the concentrations used in the ORAC assay, did not demonstrate any interference from other material components (Ou et al., 2001).

The antioxidant value of copaiba in this study, measured by *in vitro* methods, was correlated with the gallic acid content. Moreover, the dietary antioxidant compounds in plant-based diets are well associated with the minimization of oxidative stress *in vivo* (Batista et al., 2014; Braicu et al., 2013). Thus, associated with the growing interest in the use of natural compounds to prevent oxidative stress, cell damage, and several metabolic and degenerative diseases (Braicu et al., 2013; Vincent, Innes, & Vincent, 2007), the addition of copaiba fruit to diet, or use of its extracts, could improve the supply of daily bioactive compounds.

Admittedly, the presence of non-polar compounds in copaiba, such as some vitamins and carotenoids, could also play a role in its antioxidant capacity (Cardoso, Bedetti, Ribeiro, Esteves, & Pinheiro-Sant'ana, 2013; Pereira et al., 2012). The L-ORAC value of copaiba supports this idea, showing that the non-polar compounds present in the dichloromethane extract exhibited greater antioxidant power, than extracts from freeze-dried pine-nuts and strawberry (Prior et al., 2003).

Antioxidant capacity of fruits is also linked to the prevention of genetic alterations (Malta et al., 2012). This is the first study that explores the antioxidant potential of copaiba pulp fruit in cyclophosphamide-induced mutagenicity.

Cyclophosphamide is an alkylating cytotoxic agent used in the treatment of immunosuppressant malignancies. Tumors lead to compounds that can bind to DNA generating chromosomal aberrations, micronuclei (MN) and sister chromatid changes in rats, mice and hamsters. Therefore, cyclophosphamide has been used as a positive control in studies on the protective effect of compounds (Leite-Legatti et al., 2012). The micronuclei (MN) are the results of chromosome fragments that fail to incorporate into any of the nuclei of the mitotic cells during telophase. Thus, the frequency of MN in polychromatic erythrocytes (PCE) of mouse bone marrow is a very sensitive index of DNA damage (Vilar, Ferreira, Ferri, Guillo, & Chen Chen, 2008).

The dose of 100 mg kg^{-1} copaiba powder showed great reduction of micronuclei, suggesting that this dose could be used in further experiments (Table 4). Studies with species from the same family (*Fabaceae*) as copaiba corroborate the results found. Treatment with a diet containing black bean (*Phaseolus vulgaris*) showed a similar reduction in mutagenic damage (Azevedo et al., 2003). The ethanol fraction of a wild poinsettia extract (*Pterogyne nitens*) showed antimutagenic activity at concentrations 0.115 and 0.230 mg ml^{-1} (Ferreira et al., 2009). *Stryphnodendron adstringens* extract showed antiproliferative activity at concentrations of 100 and 200 mg kg^{-1} (Silva-De-Andrade, Barros-De-Castro, & Chen-Chen, 2006).

These studies indicate that copaiba could also exhibit antiproliferative activity, probably generated by polyphenolic compounds present in the fruit able to inhibit the mutagenic/carcinogenic compound, eliminate electrophilic metabolite, interact directly and non-enzymatically with the mutagenic agent, or by reducing the bioavailability of the agent, forming a complex with it (de Mejia, Castano-Tostado, & Loarca-Pina, 1999). In addition, more studies are needed, especially those concerning antiproliferative activity, and also bioavailability and bioactivity of polyphenol compounds from copaiba that may elucidate their mechanisms in living organisms. The minimum and maximum doses for such foods should also be investigated, since an excess of phenolic compounds, e.g. catechins and gallic acid, may also have a pro-oxidant effect (Braicu et al., 2013; Niho et al., 2001).

5. Conclusions

The high content of polyphenol compounds of copaiba pulp fruit contributes to its antioxidant capacity, which was confirmed by different methods of extraction and analyses. Among the polyphenol compounds found, gallic acid was the most abundant. However, the lowest and highest doses did not induce mutagenic or antimutagenic effects, but the medium dose was able to reduce the deleterious effect of cyclophosphamide.

Acknowledgments

The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, #300533/2013-6), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and, Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support. We cordially thank Aline Camarão Telles Biasoto for kind cooperation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.11.093.

References

Azevedo, L., Gomes, J. C., Stringheta, P. C., Gontijo, A. M., Padovani, C. R., Ribeiro, L. R., & Salvadori, D. M. (2003). Black bean (*Phaseolus vulgaris* L.) as a protective agent against DNA damage in mice. *Food and Chemical Toxicology, 41*(12), 1671–1676.

Batista, A. G., Lenquiste, S. A., Cazarin, C. B. B., da Silva, J. K., Luiz-Ferreira, A., Bogusz, S., ... Marostica, M. R. (2014). Intake of jaboticaba peel attenuates oxidative stress in tissues and reduces circulating saturated lipids of rats with high-fat diet-induced obesity. *Journal of Functional Foods, 6*, 450–461.

Braicu, C., Ladomery, M. R., Chedea, V. S., Irimie, A., & Berindan-Neagoe, I. (2013). The relationship between the structure and biological actions of green tea catechins. *Food Chemistry, 141*(3), 3282–3289.

Cardoso, L. D., Bedetti, S. D., Ribeiro, S. M. R., Esteves, E. A., & Pinheiro-Sant'ana, H. M. (2013). 'Jatoba do cerrado' (*Hymenaea stigonocarpa*): Chemical composition, carotenoids and vitamins in an exotic fruit from the Brazilian Savannah. *Fruits, 68*(2), 95–107.

Carvalho-Silva, L. B., Oliveira, M. d. V., Gontijo, V. S., Oliveira, W. F., Derogis, P. B. M. C., Stringheta, P. C., ... dos Santos, M. H. (2012). Antioxidant, cytotoxic and antimutagenic activities of 7-epi-clusianone obtained from pericarp of *Garcinia brasiliensis*. *Food Research International, 48*(1), 180–186.

Chun, O. K., Kim, D. O., Smith, N., Schroeder, D., Han, J. T., & Lee, C. Y. (2005). Daily consumption of phenolics and total antioxidant capacity from fruit and vegetables in the American diet. *Journal of the Science of Food and Agriculture, 85*(10), 1715–1724.

Costa-Machado, A. R. M., Bastos, J. K., & de Freitas, L. A. P. (2013). Dynamic maceration of *Copaifera langsdorffii* leaves: A technological study using fractional factorial design. *Revista Brasileira De Farmacognosia-Brazilian Journal of Pharmacognosy, 23*(1), 79–85.

Davalos, A., Gomez-Cordoves, C., & Bartolome, B. (2004). Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. *Journal of Agricultural and Food Chemistry, 52*(1), 48–54.

de Mejia, E. G., Castano-Tostado, E., & Loarca-Pina, G. (1999). Antimutagenic effects of natural phenolic compounds in beans. *Mutation Research, 441*(1), 1–9.

dos Santos, H. M., Oliveira, D. F., de Carvalho, D. A., Pinto, J. M. A., Campos, V. A. C., Mourao, A. R. B., ... Costa-Lotufo, L. V. (2010). Evaluation of native and exotic Brazilian plants for anticancer activity. *Journal of Natural Medicines, 64*(2), 231–238.

Esteves, E. A., Oliveira, L. G., Pires, S. T., Batista, A. G., Dessimoni-Pinto, N. A. V., & Santana, R. C. (2011). Nutritional composition of *Copaifera langsdorffii* Desf. aril flour and its effect on serum lipids and glucose in rats. *Food Research International, 44*(7), 2357–2361.

Ferreira, F. G. et al. (2009). Avaliação de mutagenicidade e antimutagenicidade de diferentes frações de Pterogyne nitens (Leguminosae), utilizando ensaio de micronúcleo em Tradescantia pallida. *Brazilian Journal of Pharmacognosy, 19*(1A), 61–67.

Gelmini, F., Beretta, G., Anselmi, C., Centini, M., Magni, P., Ruscica, M., ... Facino, R. M. (2013). GC–MS profiling of the phytochemical constituents of the oleoresin from *Copaifera langsdorffii* Desf. and a preliminary in vivo evaluation of its antipsoriatic effect. *International Journal of Pharmaceutics, 440*, 170–178.

Haminiuk, C. W. I., Plata-Oviedo, M. S. V., Mattos, G., Carpes, S. T., & Branco, I. G. (2014). Extraction and quantification of phenolic acids and flavonols from *Eugenia pyriformis* using different solvents. *Journal of Food Science and Technology, 51*(10), 2862–2866.

IAL (1985). *Normas analíticas. Métodos químicos e físicos para análise de alimentos*. São Paulo-SP: Instituto Adolfo Lutz.

Leite-Legatti, A. V., Batista, A. G., Dragano, N. R. V., Marques, A. C., Malta, L. G., Riccio, M. F., ... Marostica, M. R. (2012). Jaboticaba peel: Antioxidant compounds, antiproliferative and antimutagenic activities. *Food Research International, 49*(1), 596–603.

MacGregor, J. T., Heddle, J. A., Hite, M., Margolin, B. H., Ramel, C., Salamone, M. F., ... Wild, D. (1987). Guidelines for the conduct of micronucleus assays in mammalian bone marrow erythrocytes. *Mutation Research/Genetic Toxicology, 189*(2), 103–112.

Malta, L. G., Ghiraldini, F. G., Reis, R., Oliveira, M. D., Silva, L. B., & Pastore, G. M. (2012). In vivo analysis of antigenotoxic and antimutagenic properties of two Brazilian cerrado fruits and the identification of phenolic phytochemicals. *Food Research International, 49*(1), 604–611.

Mansouri, M. T., Farbood, Y., Sameri, M. J., Sarkaki, A., Naghizadeh, B., & Rafeirad, M. (2013). Neuroprotective effects of oral gallic acid against oxidative stress induced by 6-hydroxydopamine in rats. *Food Chemistry, 138*(2–3), 1028–1033.

Natividade, M. M. P., Correa, L. C., de Souza, S. V. C., Pereira, G. E., & Lima, L. C. D. (2013). Simultaneous analysis of 25 phenolic compounds in grape juice for HPLC: Method validation and characterization of Sao Francisco Valley samples. *Microchemical Journal, 110*, 665–674.

Niho, N., Shibutani, M., Tamura, T., Toyoda, K., Uneyama, C., Takahashi, N., & Hirose, M. (2001). Subchronic toxicity study of gallic acid by oral administration in F344 rats. *Food and Chemical Toxicology, 39*(11), 1063–1070.

Ou, B. X., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry, 49*(10), 4619–4626.

Paiva, L. A. F., Cunha, K. M. D., Santos, F. A., Gramosa, N. V., Silveira, E. R., & Rao, V. S. N. (2002). Investigation on the wound healing activity of oleo-resin from *Copaifera langsdorffii* in rats. *Phytotherapy Research, 16*(8), 737–739.

Paiva, L. A. F., Gurgel, L. A., De Sousa, E. T., Silveira, E. R., Silva, R. M., Santos, F. A., & Rao, V. S. N. (2004). Protective effect of *Copaifera langsdorffii* oleo-resin against acetic acid-induced colitis in rats. *Journal of Ethnopharmacology, 93*(1), 51–56.

Paiva, L. A. F., Gurgel, L. A., Silva, R. M., Tome, A. R., Gramosa, N. V., Silveira, E. R., ... Rao, V. S. N. (2002). Anti-inflammatory effect of kaurenoic acid, a diterpene from *Copaifera langsdorffii* on acetic acid-induced colitis in rats. *Vascular Pharmacology, 39*(6), 303–307.

Pereira, M. C., Steffens, R. S., Jablonski, A., Hertz, P. F., Rios, A. D., Vizzotto, M., & Flores, S. H. (2012). Characterization and antioxidant potential of Brazilian fruits from the Myrtaceae family. *Journal of Agricultural and Food Chemistry, 60*(12), 3061–3067.

Prior, R. L., Hoang, H., Gu, L. W., Wu, X. L., Bacchiocca, M., Howard, L., ... Jacob, R. (2003). Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC(FL))) of plasma and other biological and food samples. *Journal of Agricultural and Food Chemistry, 51*(11), 3273–3279.

Robards, K., Prenzler, P. D., Tucker, G., Swatsitang, P., & Glover, W. (1999). Phenolic compounds and their role in oxidative processes in fruits. *Food Chemistry, 66*(4), 401–436.

Rufino, M. D. M., Alves, R. E., de Brito, E. S., Perez-Jimenez, J., Saura-Calixto, F., & Mancini, J. (2010). Bioactive compounds and antioxidant capacities of 18 non-traditional tropical fruits from Brazil. *Food Chemistry, 121*(4), 996–1002.

Sebbenn, A. M., Carvalho, A. C. M., Freitas, M. L. M., Moraes, S. M. B., Gaino, A. P. S. C., da Silva, J. M., ... Moraes, M. L. T. (2011). Low levels of realized seed and pollen gene flow and strong spatial genetic structure in a small, isolated and fragmented population of the tropical tree *Copaifera langsdorffii* Desf. *Heredity, 106*(1), 134–145.

Silva-De-Andrade, L., Barros-De-Castro, D., & Chen-Chen, L. (2006). Efeito Modulador do Extrato de Stryphnodendron adstringens Mart. (Barbatimão) Contra Danos Induzidos pela Mitomicina C em Camundongos. *Journal of Brazilian Society of Ecotoxicology, 1*(2), 127–130.

Spagolla, L. C., Santos, M. M., Passos, L. M. L., & Aguiar, C. L. (2009). Extração alcoólica de fenólicos e flavonóides totais de mirtilo "Rabbiteye" (*Vaccinium ashei*) e sua atividade antioxidante. *Revista de Ciências Farmacêuticas Básica e Aplicada, 30*(2), 187–191.

Swain, T., & Hillis, W. E. (1959). The phenolic constituents of *Prunus domestica*. The quantitative analysis of phenolic constituents. *Journal of the Science of Food Agriculture, 10*(27–38).

Valentová, K., Vrba, J., Bancířová, M., Ulrichová, J., & Křen, V. (2014). Isoquercitrin: Pharmacology, toxicology, and metabolism. *Food and Chemical Toxicology, 68*, 267–282.

Verma, S., Singh, A., & Mishra, A. (2013). Gallic acid: Molecular rival of cancer. *Environmental Toxicology and Pharmacology, 35*(3), 473–485.

Vilar, J. B., Ferreira, F. L., Ferri, P. H., Guillo, L. A., & Chen Chen, L. (2008). Assessment of the mutagenic, antimutagenic and cytotoxic activities of ethanolic extract of araticum (*Annona crassiflora* Mart. 1841) by micronucleus test in mice. *Brazilian Journal of Biology, 68*(1), 141–147.

Vincent, H. K., Innes, K. E., & Vincent, K. R. (2007). Oxidative stress and potential interventions to reduce oxidative stress in overweight and obesity. *Diabetes Obesity & Metabolism, 9*(6), 813–839.

Williams, R. J., & Spencer, J. P. E. (2012). Flavonoids, cognition, and dementia: Actions, mechanisms, and potential therapeutic utility for Alzheimer disease. *Free Radical Biology and Medicine, 52*(1), 35–45.

Zaveri, N. T. (2006). Green tea and its polyphenolic catechins: Medicinal uses in cancer and noncancer applications. *Life Sciences, 78*(18), 2073–2080.

Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry, 64*(4), 555–559.