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## Antioxidant, anti-acetylcholinesterase and cytotoxic activities of ethanol extracts of peel, pulp and seeds of exotic Brazilian fruits

### Antioxidant, anti-acetylcholinesterase and cytotoxic activities in fruits

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## ABSTRACT

Ethanol extracts of powdered *genipap* (*Genipa americana* L.), *umbu* (*Spondia tuberosa* A.) and *siriguela* (*Spondia purpurea* L.) prepared from separate pulp, seeds and peel were investigated for their (i) antioxidant capacity, which was evaluated by various known methods; (ii) acetylcholinesterase (AChE) inhibitory activity; and (iii) cytotoxic effect on corneal epithelial cells of sheep. The highest values of total phenolic content were obtained with peel and seed extracts. Siriguela and umbu (seeds and peel) extracts displayed the highest antioxidant activities. Lipid peroxidation assays using mimetic biomembranes and mouse liver homogenates indicated that genipap pulp is a promising antioxidant. The investigation of phenols and organic acid contents revealed the presence of quercetin, citric and quinic acids, chlorogenic acid derivatives, among others, in several extracts, with the highest amount found in siriguela seeds. Genipap pulp and siriguela seed ethanol extracts presented an AChE inhibition zone similar to that of the positive control, carbachol. AChE inhibition assay with chlorogenic acid, one of the main constituents of siriguela seeds, revealed that this acid showed activity similar to that of the control physostigmine. These data suggest that these extracts are potentially important antioxidant supplements for the everyday human diet, pharmaceutical and cosmetic industries.

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**Abbreviations:** AA, ascorbic acid; AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; ABTS, 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) radical; AChE, Acetylcholinesterase; AD, Alzheimer's disease; ATCI, Acetylthiocholine iodide; BSA, Bovine serum albumin; C<sub>11</sub>-BODIPY<sup>581/591</sup>, 4,4'-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-5-indacene-3-undecanoic acid; CUPRAC, Copper reducing antioxidant capacity; DCFI, 2,6-dichlorophenol-indophenol; DPPH, α,α-diphenyl-β-picrylhydrazyl radical; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); EDTA, Ethylenediamine tetraacetic acid; FC, Folin-Ciocalteu; FCS, Fetal calf serum; FRAP, Ferric reducing antioxidant power; GA, Gallic acid; MEM, Eagle's minimum essential medium; RSA-DPPH<sup>•</sup>, Radical scavenging activity of α,α-diphenyl-β-picrylhydrazyl radical; SD, Standard deviation; TBARS, Thiobarbituric acid reactive substances; TLC, Thin layer chromatography; TPC, Total phenolic content; TPTZ, 2,4,6-Tripyridyl-s-triazine.

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## 1. Introduction

Various phytochemicals found in vegetables, fruits, and medicinal plants have received increasing attention for their potential role in the prevention and treatment of human diseases (Oliveira, Valentim, Goulart, et al., 2009; Youdim & Joseph, 2001). They have long been associated with good health due to the presence of polyphenols, carotenoids, tocopherols, tocotrienols, ascorbic acid and several thiols (Oliveira, Valentim, Goulart, et al., 2009). The health benefits could be attributed to their antioxidant capabilities. Antioxidants can protect biological systems against the potentially harmful effects of processes or reactions that can cause excessive oxidation (Arnao, Cano, & Acosta, 2001). Once the balance between the production of reactive oxygen species and the defense systems is disrupted, oxidative stress sets in,

promoting many diseases such as cancer, atherosclerosis and other cardiovascular disorders, diabetes, accelerated aging (Valko et al., 2007) and Alzheimer's disease (AD) (Ingkaninan, Temkithawon, Chuenchom, Yuyaem, & Thongnoi, 2003). They also function as inducers of cell signals, leading to changes in gene expression, which result in the activation of enzymes that eliminate ROS and/or toxins, including those involved in initial cancer events, through redox regulation of thiols, and their influence on digestive and metabolizing enzymes, among other factors (Finley et al., 2011; Nzaramba, Reddivari, Bamberg, & Miller, 2009). However, the traditional message that oxidative stress, which involves the production of reactive oxygen species (ROS), is the basis for chronic diseases and aging is being reexamined. Accumulating evidence suggests that ROS exert essential metabolic functions and that removal of too many ROS can upset cell signaling pathways and actually increase the risk of chronic disease (Finley et al., 2011).

As stated, among the diseases, AD, a progressive degenerative neurological disorder characterized by impairment of memory and behavior, is especially relevant for phytotherapy through the use of natural antioxidants to minimize oxidative imbalance, since AD is known to result from oxidative damage to the central nervous system (Barnham, Masters, & Bush, 2004; Zaini, Anwar, Hamid, & Saari, 2011). Additionally, one of the most promising approaches for treating this disease is to enhance the acetylcholine level in the brain using acetylcholinesterase (AChE) inhibitors (Ingkaninan et al., 2003). Actually most promising drugs for symptomatic treatment of AD are acetylcholinesterase inhibitors. Natural products with AChE inhibitory activity have been recognized as new multipotent drugs for AD treatment (Matthew Joyner & Cichewicz, 2011; Uriarte-Pueyo & Calvo, 2011; Williams, Sorribas, & Howes, 2011). To screen the almost inexhaustible sources of natural products, an effective and fast assay was developed (Rhee, Meent, Ingkaninan, & Verpoorte, 2001).

Brazil has a plethora of tropical fruits with distinctive exotic flavors. In the Northeast of Brazil there are many areas where the climate and soil are especially favorable for the production of tropical fruits. Fruit production and processing represent important economic activities not only due to relevant regional commercialization but also to growing national and international markets (Tiburski, Rosenthal, Deliza, Godoy, & Pacheco, 2011). Developing countries continue to account for 98% of global production (Clerici and Carvalho-Silva (2011).

In view of the above, the purpose of this study was to evaluate the anti-acetylcholinesterase and antioxidant activities of peel, pulp and seed ethanol extracts of three exotic and commercially important Brazilian fruits: genipap, siriguela, and umbu. To this end, an analysis was made of acetylcholinesterase inhibition of the extracts and of individual selected chemicals, allied to the widely accepted antioxidant capacity assays (total phenolic content, DPPH, ABTS, FRAP and CUPRAC), as recommended, to provide a reliable picture of the total antioxidant capacity (Contreras-Calderon, Calderon-Jaimes, Gerra-Hernandez, & Garcia-Villanova, 2010; Huang, Ou, & Prior, 2005; Rufino et al., 2010). Biomimetic liposomes were prepared to mimic a real situation in biological membranes, and the  $C_{11}$ -BODIPY<sup>581/591</sup> method was also used. TBARS assays were also performed with mouse liver homogenates. A preliminary investigation of phenols and organic acid contents was performed.

*Genipa americana* L. (Rubiaceae) (genipap), which is edible and a popular source of beverages, including liquors, (Ono et al., 2007), is widely distributed in tropical Central and South America. *Spondia purpurea* L. (Anacardiaceae) (siriguela), originally from Central America, is distributed from Mexico through Northern Peru and Brazil, and is endemic to semi-arid regions (Teixeira et al., 2007). The fruit is small and reddish fruits have an aromatic pulp of bittersweet taste (Augusto, Valente, Tada, & Rivellino, 2000). *Spondia tuberosa* A. (Anacardiaceae) (umbu) is a species distributed in Brazil's semi-arid tropical regions. Umbu is a source of vitamins and minerals and has a tannin content of 126.27 mg/100 g of fruit. This high tannin content and the presence of natural antioxidants give umbu its functional appeal (Narain, Bora,

Holschuh, & Vasconcelos, 1992). Its fruit, which has a distinctive aroma, is suitable for raw consumption and for the processing industry (Santos, 1999). To the best of our knowledge, few studies (Rufino, Fernandes, Alves, & Brito, 2009; Rufino et al., 2010) have focused on the antioxidant potential of these fruits, and so far no reports about genipap have been published.

## 2. Materials and methods

### 2.1. Standards and reagents

Folin-Ciocalteu reagent, soy phosphatidylcholine, vitamin C, resveratrol, ethanol, methanol, DPPH<sup>•</sup> ( $\alpha,\alpha$ -diphenyl- $\beta$ -picrylhydrazyl radical), ABTS (2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt), potassium persulfate, AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride), TPTZ (2,4,6-tripyridyl-s-triazine), tris(hydroxymethyl)aminomethane, copper chloride II, neocuproine, ammonium acetate, bovine serum albumin (BSA), NaCl, MgCl<sub>2</sub>·6H<sub>2</sub>O, ascorbic acid (AA), chlorogenic acid, acetylcholinesterase (AChE) from *Electrophorus electricus* (electric eel) – Type VI-S, acetylthiocholine iodide (ATCI), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), carbachol, physostigmine, Eagle's minimum essential medium (MEM), fetal calf serum (FCS), streptomycin, penicillin, amphotericin B, trypsin, EDTA (ethylenediaminetetraacetic acid), and trypan blue were purchased from Sigma Aldrich (Steinheim, Germany). Methanol, ethanol, hydrochloric acid, 2,6-dichlorophenol-indophenol (DCFI), sodium carbonate and gallic acid (GA) were supplied by Vetec Química Fina Ltda (Rio de Janeiro, Brazil); DC-Aluminofolien, Silica gel 60 F<sub>254</sub>, 0.2-mm thickness, and Trolox® were supplied by Merck (Düsseldorf, Germany), and the fluorescent fatty acid analogue 4,4'-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-5-indacene-3-undecanoic acid ( $C_{11}$ -BODIPY<sup>581/591</sup>) was purchased from Molecular Probes (Ontario, Canada). All the reagents were of analytical grade and the stock solutions and buffers were prepared with Milli-Q purified water.

### 2.2. Fruit sampling

Genipap (*G. americana* L., Rubiaceae), siriguela (*S. purpurea* L., Anacardiaceae) and umbu (*S. tuberosa*, Anacardiaceae) fruits, intact and in a suitable state of ripeness for harvesting, were purchased at the municipal market of Maceió, Alagoas, in the morning, during the months of March and April, 2008. These fruits came from small noncommercial farms in the state of Alagoas. The fruit selection criteria were the absence of lesions on the peels and the fruits' state of ripeness suitable for harvesting and consumption. Although the fruits were not labeled as organically grown products, they were cultivated without agrochemicals.

The fruits were stored at room temperature for about two days post-harvest. Immediately after they were purchased, the samples were washed with tap water and the parts to be analyzed were separated and dried for subsequent ethanol extraction. Siriguela and umbu seeds were dried in an air circulation oven at 40 °C for 24 h, while their peels were oven-dried at 35 °C. Their pulps were freeze-dried.

### 2.3. Sample preparation and extraction

The crude extracts were prepared from the peels, pulp and seeds of the fruits. Table 1 lists the weight of fruit parts used to prepare the extracts (column 2). Each part was oven-dried at 35 °C or 40 °C, crushed and then extracted three times with 95% ethanol. The solvents were eliminated in a rotary evaporator (Buchi rotavapor R-114) at 40 °C and the residues were dried and stored at 27 °C, wrapped in aluminum foil, to avoid incidence of light. Table 1 lists the extraction yields (column 3), obtained after evaporation of the solvent (ethanol).

**Table 1**  
Initial dry weights (g) and ethanol extraction yields (%) from samples.

Samples		Initial dry weight (g)	Extraction yield (%)
Genipap	Peel	259.9	36.3
	Pulp	50.0	45.4
	Seeds	451.1	25.3
Siriguela	Peel	149.8	5.9
	Pulp	50.0	32.3
	Seeds	285.7	1.4
Umbu	Peel	88.5	11.3
	Pulp	50.0	17.5
	Seeds	149.0	5.6

#### 2.4. Phytochemical screening of extracts

The presence of anthocyanidins, anthocyanins, anthraquinones, anthrone, catechins, coumarins, flavonols, flavonoids, flavanones, leucoanthocyanidins, phenols, proanthocyanidins, saponins, steroids, triterpenoids, tannins and xanthenes in the extracts was verified, qualitatively, according to the methods described by Souza et al. (2003), based on chemical reactions and colorimetric changes. In summary, flavonoids were verified by Shinoda reaction with  $\text{AlCl}_3$  and  $\text{H}_3\text{BO}_3$ , using  $\text{NaOH}$   $1 \text{ mol L}^{-1}$ ; tannins with lead acetate; coumarins by reaction with  $\text{KOH}$   $2 \text{ mol L}^{-1}$ , triterpenoids and steroids by Kedde, Liebermann-Burchard and Baljet reactions; saponins by the foam index test, and alkaloids by Dragendorff and Mayer reactions.

#### 2.5. Thin layer chromatography (TLC) bioassay for the detection of AChE inhibition

Inhibition of the enzyme acetylcholinesterase (AChE) was evaluated by TLC following the methodology described by Ellman, which was later adapted by Rhee et al. (2001). The peel, pulp and seed extracts were dissolved individually in ethanol. Each sample was diluted to a concentration of  $2 \text{ mg mL}^{-1}$  and spotted on an alumina chromatography plate of (1.5–2.5  $\mu\text{L}$ ). The plate was then sprayed with DTNB [5,5'-dithiobis(2-nitrobenzoic acid)]/ATCI [acetylthiocholine iodide] reagent [ $1 \text{ mmol L}^{-1}$  DTNB (Ellman's reagent) and  $1 \text{ mmol L}^{-1}$  ATCI in Tris-HCl buffer, pH 8.0] until the silica was saturated with the solvent. The TLC plates were allowed to dry for 3 min, after which they were sprayed with  $3 \text{ U mL}^{-1}$  of AChE enzyme solution. After 10 min, a yellow background with white spots revealing compounds with enzyme inhibitory activity became visible. Carbachol ( $0.01 \text{ mol L}^{-1}$ ) was used as a positive control and ethanol and methanol as negative controls. The same procedure was employed for pure compounds, e.g., chlorogenic acid, and physostigmine was also used as a positive control.

#### 2.6. Cytotoxicity assay

The possible cytotoxic effect of the tested plant extracts was examined using sheep corneal epithelial cells. The cells were cultured in MEM supplemented with 10% fetal bovine serum (FBS),  $0.10 \text{ mg mL}^{-1}$  of streptomycin, 100 UI of penicillin G and  $0.25 \mu\text{g}$  of amphotericin B in an incubator with 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ . The cells were harvested using trypsin 0.05% and 0.02% EDTA solution. The cells were seeded in 96-well plates ( $1 \times 10^4$  cells per well) and incubated for 24 h with 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ . The culture medium was then substituted for 200 mL of fresh medium (control) or medium containing an extract concentration of  $100 \mu\text{g mL}^{-1}$ . After 48 h, the cells were dispersed and evaluated by the Trypan Blue Exclusion assay (Weisenthal, Marsden, Dill, & Macaluso, 1983). All the tests were performed in triplicate.

#### 2.7. Determination of ascorbic acid (AA)

The determination of ascorbic acid (AA) was conducted according to AOAC (1997) adapted by Oliveira, Godoy, and Prado (2010). Five hundred mg of the extracts were homogenized in 50 mL of aqueous solution of metaphosphoric acid (1%) and submitted to titration using 2,6-dichlorophenol-indophenol (DCFI). The titration end point was evidenced by coincidence of color between the titration solution and the extract. For the determination of AA, the following equation was used:

$$C = [(p \times c \times 50)/(V \times m)],$$

where  $C$  is the quantity of AA (mg) per gram of dry extract;  $p$  is the volume (mL) of standard AA solution utilized, whose concentration is  $c$  ( $\text{mg mL}^{-1}$ ) in the standardization of DCFI;  $V$  is the volume (mL) of the titrant solution used, and  $m$  is the quantity of the sample.

#### 2.8. Determination of total phenolic content

The total phenolic content (TPC) of the ethanol extracts obtained from the powdered fruits was determined using Folin-Ciocalteu (FC) reagent, as described by Cicco, Lanorte, Paraggio, Viggiano, and Lattanzio (2009), with the following modifications. Solutions of dried extracts in methanol were prepared at a concentration of  $50 \mu\text{g mL}^{-1}$ . Aliquots (120  $\mu\text{L}$ ) of these solutions were placed in test tubes and 180  $\mu\text{L}$  of water was added to obtain 4% methanol in the final solution. Then, 300  $\mu\text{L}$  of FC reagent was added to each tube. After 2 min, 2.4 mL of a 5% (w/v) sodium carbonate solution was added. The mixture was shaken and heated at  $40^\circ\text{C}$  in a water bath for 20 min. The tubes were then cooled rapidly and the developed color was read at 767 nm in a MutiSpec-1501 UV-Vis spectrophotometer (Shimadzu, Japan). The concentration of phenolic compounds was estimated using a calibration curve traced with gallic acid (GA) in methanol ( $0.10 \times 10^{-4}$ – $17.5 \times 10^{-4} \text{ mol L}^{-1}$ ) as a polyphenol reference ( $n = 3$ ). The results are expressed as mg of GA equivalents/g extract ( $\text{mg GAE g}^{-1}$ ). The same procedure was performed using 120  $\mu\text{L}$  of methanol as a negative control.

#### 2.9. Radical scavenging activity of $\alpha, \alpha$ -diphenyl- $\beta$ -picrylhydrazyl radical (RSA-DPPH')

The antioxidant capacity of fruit samples and, for purposes of comparison, the antioxidant activity of Trolox were measured as their radical scavenging ability (RSA), using the DPPH' method (Sánchez-Moreno, Larrauri, & Saura-Calixto, 1999). Thus, 0.30 mL of powder extract solution ( $50 \mu\text{g mL}^{-1}$ ) was mixed with 2.7 mL of DPPH' radical solution ( $40 \mu\text{g mL}^{-1}$  in methanol) in a 3 mL quartz cuvette. The mixture was homogenized and stored in the dark prior to analysis. The DPPH' absorbance values at 516 nm were recorded at 5 min intervals for 50 min.

The percentage of DPPH' radical-scavenging activity (RSA% – DPPH') of each extract was calculated as follows:

$$\% \text{ RSA} = (1 - A_C/A_D) \times 100,$$

where  $A_C$  is the absorbance of the solution when extract was added at a particular concentration in 30 min, and  $A_D$  is the absorbance of the DPPH' solution. All the determinations were performed in triplicate.

The  $\text{IC}_{50}$  (half maximal inhibitory concentration) was calculated graphically using a calibration curve in the linear range by plotting the extract concentration vs. the corresponding scavenging effect. The  $\text{IC}_{50}$  or I% (percentage of inhibition) was calculated according to Scherer and Godoy (2009), using the following equation:

$$I\% = [(Abs_0 - Abs_1)/Abs_0] \times 100,$$



where  $Abs_0$  is the absorbance of the control and  $Abs_1$  the absorbance in the presence of the test compound.

### 2.10. ABTS assay

The method described by Arnao et al. (2001) was used with minor modifications as follows. Stock solutions (50 mL) were prepared, one containing 7 mmol L<sup>-1</sup> ABTS<sup>•+</sup> and the other 140 mmol L<sup>-1</sup> potassium persulfate. The reagent was then prepared by mixing the two stock solutions in equal volumes and allowing them to react for 16 h at room temperature in the dark. The final solution was then diluted by mixing 5 mL of freshly prepared ABTS<sup>•+</sup> solution (7 mmol L<sup>-1</sup>) with 90  $\mu$ L methanol to obtain an absorbance of  $0.70 \pm 0.50$  at 734 nm. Extract solutions were prepared in methanol at a concentration of 50  $\mu$ g mL<sup>-1</sup>. Fruit extracts (aliquot of 30  $\mu$ L) were allowed to react with 3.0 mL of the ABTS<sup>•+</sup> solution for 6 min in the dark. The absorbance was then measured at 734 nm. The standard curve was linear between 100 and 2000  $\mu$ mol L<sup>-1</sup> of Trolox®. The results are expressed in mmol Trolox® equivalent (TE)/g fresh mass.

### 2.11. FRAP assay

The assay was performed according to the method described by Rufino et al. (2006), which is based on the reduction of a ferric tripyridyl triazine complex to its dark blue ferrous form, in the absence and presence of antioxidants. Briefly, the FRAP reagent is prepared by mixing 2.5 mL of a solution of 10 mmol L<sup>-1</sup> TPTZ in 40 mmol L<sup>-1</sup> HCl, and adding 2.5 mL of 20 mmol L<sup>-1</sup> FeCl<sub>3</sub> and 25 mL of 0.30 mol L<sup>-1</sup> acetate buffer (pH = 3.6), after which the reagent is heated to 37 °C.

Sample aliquots (90  $\mu$ L) were mixed with 270  $\mu$ L of distilled water and 2.7 mL of FRAP reagent and incubated at 37 °C for 30 min. The absorbance of the reaction mixture was measured at 595 nm and a calibration curve was prepared with Trolox® (5–1000  $\mu$ mol L<sup>-1</sup>). The results are expressed as TEAC<sub>FRAP</sub>, i.e., Trolox Equivalent Antioxidant Capacities calculated with respect to the original FRAP in mmol of Trolox g<sup>-1</sup>.

### 2.12. CUPRAC (copper reducing antioxidant capacity)

These assays are based on the reduction of Cu(II) to Cu(I) by the combined action of all the antioxidants (reducing agents) in a sample. One milliliter each of CuCl<sub>2</sub> ( $1 \times 10^{-2}$  mol L<sup>-1</sup>), neocuproine ( $7.5 \times 10^{-3}$  mol L<sup>-1</sup>) and NH<sub>4</sub>Ac buffer solutions (1.0 mol L<sup>-1</sup>, pH = 7.0) were placed in a test tube. Then, 0.5 mL of the extract solution and 0.6 mL of H<sub>2</sub>O were added to the initial mixture to reach a final volume of 4.1 mL. The tubes were stoppered, and after 1 h, the absorbance at 450 nm was recorded against a blank reagent. The extract solutions were prepared in methanol at a concentration of 50  $\mu$ g mL<sup>-1</sup>. The calibration curve was prepared with Trolox (5–600  $\mu$ mol L<sup>-1</sup>) (Apak, Güçlü, Özyürek, & Karademir, 2004). The results are expressed as the total antioxidant capacity in mmol Trolox g<sup>-1</sup> of extract, which was calculated as follows:

$$\text{Total Antioxidant Capacity (in mmol Trolox/g)} = \frac{AV_f rV_i}{\epsilon_{\text{Trolox}} V_s m} / 1000$$

where  $A$  is the absorbance;  $\epsilon_{\text{Trolox}}$  is the molar absorptivity of Trolox;  $V_f$  is the final volume (after the addition of reagents);  $V_s$  is the volume of sample taken for analysis from the diluted extract;  $r$  is the time prior to analysis;  $V_i$  is the volume used to prepare the initial solution (diluted extract); and  $m$  is the weight in grams used in the preparation of the initial solution (diluted extract).

## 2.13. Antioxidant capacity in a biomimetic membrane system

### 2.13.1. Preparation of unilamellar vesicles

Unilamellar vesicles of soy phosphatidylcholine (1 mM) were prepared by extrusion in 10 mL of phosphate buffer (50 mmol L<sup>-1</sup>, pH 7.4), as previously described (MacDonald et al., 1991), with the additional incorporation of  $10^{-7}$  mol L<sup>-1</sup> of the peroxy-sensitive fluorescent probe C<sub>11</sub>-BODIPY<sup>581/591</sup> (Drummen, Gadella, Post, & Brouwers, 2002). The multilamellar dispersion obtained in this step was transferred to a membrane extrusion system with 100 nm pore diameter operating at 25 °C. The multilamellar dispersion was put through the extruder 15 times to produce BODIPY-labeled unilamellar vesicles with an average diameter of 120 nm.

### 2.13.2. Lipid peroxidation measurements

Fluorescence measurements were carried out at 37 °C in a Spex Fluorolog 1681® fluorometer by mixing, in a 1 mL-quartz cuvette, 800  $\mu$ L of unilamellar vesicle suspension, 50  $\mu$ L of 50 mmol L<sup>-1</sup> phosphate buffer pH 7.4, and 50  $\mu$ L of sample (0.5 mg mL<sup>-1</sup> dried extract solution) or Trolox (1, 5 and 10  $\mu$ mol L<sup>-1</sup>), vitamin C and resveratrol (100  $\mu$ mol L<sup>-1</sup>) as positive controls. The solvents (methanol and buffer) were used as negative controls. The reaction was initiated with the addition of 100  $\mu$ L of AAPH (100 mM). The fluorescence decay ( $\lambda_{\text{exc}} = 580$  nm,  $\lambda_{\text{em}} = 600$  nm) was monitored continuously for 30 min.

## 2.14. Antioxidant capacity in a biological system

### 2.14.1. Preparation of mouse hepatic homogenates

Five 24 to 26-week-old male C56Bl/6 mice were kept in standard experimental conditions. Mice were euthanized with a mixture of ketamine and xylazine (100 mg kg<sup>-1</sup>, and 10 mg kg<sup>-1</sup>, respectively; IP), and immediately thereafter sacrificed by cardiac puncture. The liver was then removed, washed in PBS solution, dried with paper, and stored in a biofreezer at -80 °C. Approximately 100 mg of hepatic tissue from each animal was pulverized in liquid nitrogen, using a mortar and pestle. Samples were resuspended in lysis buffer (pH 7.5) containing 50 mmol L<sup>-1</sup> Tris-HCl, 0.1 mmol L<sup>-1</sup> EDTA, 0.1 mmol L<sup>-1</sup> EGTA, 0.1% SDS, 0.1% deoxycholate, 1% IGEPAL, and a 1000-fold dilution of a mammalian protease inhibitor cocktail (all from Sigma Chemical Co). The samples were then centrifuged twice for 30 min at 4000 rpm, at 4 °C, and the resulting pellets were discarded, keeping the supernatant for biological assays. The protein content of the supernatant (hepatic homogenate) was measured according to the Bradford method (1976). The estimation of TBARS levels was performed in quadruplicate.

### 2.14.2. Estimation of lipid peroxidation

As an index of inhibition of lipid peroxidation, TBARS levels were measured using mouse liver homogenates prepared as described previously. Synthetic butylhydroxytoluene (BHT) ([BHT]<sub>final</sub> = 1 mM) was used as a positive control. The protein concentration in the homogenate was adjusted to 2 mg mL<sup>-1</sup> with PBS. The supernatant (100  $\mu$ L) was preincubated with the vegetal extracts (10  $\mu$ L, in concentrations of 25, 50 and 100  $\mu$ g mL<sup>-1</sup>) at 37 °C for 45 min. Peroxidation was then initiated by adding 10  $\mu$ L of freshly prepared FeSO<sub>4</sub>·7H<sub>2</sub>O to a final concentration of 10  $\mu$ M, and the samples were incubated at 37 °C for another 90 min. The TBARS assay was performed as described by Wallin, Rosengren, Shertzer, and Camejo (1993). Briefly, 10  $\mu$ L of BHT ([BHT]<sub>final</sub> = 5 mM), SDS (10%, 100  $\mu$ L) as a precipitant, and 1000  $\mu$ L of TBA solution at 1.3% were added to the reaction mixture, which was then heated to 95 °C in a boiling water bath for 60 min. After cooling on ice for 10 min, the analytes were centrifuged at 4000 rpm for 8 min at 10 °C. The absorbance was read at 532 nm in a Thermo Scientific® Multiskan GO microplate spectrophotometer.

Results are expressed as percent inhibition after 60 min, calculated as the inhibition ratio (IR) in comparison with the positive control BHT in the different extract concentrations (Dominguez et al., 2005).

$$IR(\%) = [(C-E)/C] \times 100$$

where C) is the absorbance of the control and E) is that of the test sample.

### 2.15. UPLC-MS analysis of phenolic compounds and some organic acids

In order to characterize the plant material, UPLC-MS profiles of the extracts were obtained. Chromatographic separation (3  $\mu$ L injection) was performed in an Acquity UPLC system (Waters, Milford, MA, USA) using a UPLC BEH column (2.1  $\times$  50 mm, 1.7  $\mu$ m particle size) at a temperature of 30 °C. A gradient of (A) Milli-Q purified water with 0.1% formic acid and (B) acetonitrile (Tedia, Brazil) was used, starting with 5% B and ramping up to 100% B at 9 min, holding for 9.50 min, and then returning to the initial conditions. Detection in the negative ion mode was performed in an Acquity TQD mass spectrometer (Micromass Waters, Milford, MA, USA) operating in the following conditions: capillary – 3000 V, cone – 30 V, source temperature 150 °C; in the range of 110 to 1000 m/z. Compounds were identified and quantified in comparison to standards.

### 2.16. Statistical analysis

The experimental design was completely randomized with 9 treatments and 3 replicates. The data were analyzed by analysis of variance (ANOVA), followed by Tukey's test to detect significant differences between treatments. The results were expressed as mean  $\pm$  SD (standard deviation). Statistical analysis was performed using SAEG 9.1 (System for Statistical Analysis, MG, Brazil). Spearman's correlation was used to determine if the bioactive compounds contribute to the antioxidant capacity. Data related to Table 5 were normalized and analyzed by analysis of variance (ANOVA), followed by Tukey's test and Spearman's correlation. A probability value of  $p < 0.05$ ,  $p < 0.001$  was considered statistically significant.

## 3. Results and discussion

### 3.1. Phytochemical screening and UPLC/MS analyses of extracts

Phytochemical screening was performed to identify the classes of compounds in the extracts before using them. Sterols, terpenoids, oils, flavonoids, alkaloids and other phenolic compounds have been shown to possess anti-acetylcholinesterase and antioxidant activities (Ji & Zhang, 2008; Ross & Kasum, 2002).

The interest in polyphenols is due to their great abundance in our diet, and their probable role in the prevention of various diseases

associated with oxidative stress. The present phytochemical screening revealed the presence of phenols and tannins in siriguela and umbu (peel and seeds); anthocyanins, proanthocyanidins and flavonoids in siriguela peel; leucoanthocyanidins, catechins and flavanones in genipap peel and in genipap, siriguela and umbu seeds; anthraquinones, anthrone and coumarins in genipap, siriguela and umbu (peel and seeds); triterpenoids and steroids in all the samples; and saponins in siriguela seeds. The pulps were not analyzed due to the formation of precipitates during the tests (Table 2).

Albeit not comprehensive in terms of total phytochemical content, UPLC/MS analyses revealed that quercetin predominated in most of the analyzed extracts: genipap peel ( $48.38 \pm 0.97 \mu\text{g g}^{-1}$ ); genipap seeds ( $34.73 \pm 0.69 \mu\text{g g}^{-1}$ ); umbu peel ( $32.65 \pm 0.65 \mu\text{g g}^{-1}$ ); siriguela peel ( $31.44 \pm 0.63 \mu\text{g g}^{-1}$ ); genipap pulp ( $9.84 \pm 0.20 \mu\text{g g}^{-1}$ ) and siriguela pulp ( $3.88 \pm 0.08 \mu\text{g g}^{-1}$ ).

The phytochemical composition of siriguela seeds contained a noticeable amount of chlorogenic acid (3-caffeoyl-D-quinic acid; CGA) ( $356.93 \pm 7.13 \mu\text{g g}^{-1}$ ). This acid (3-caffeoyl-D-quinic acid; CGA), an ester formed between caffeic and quinic acids, is one of the major polyphenol compounds found in numerous plant species (Kono et al., 1997). It has two vicinal hydroxyl groups on an aromatic residue and, in vitro, exhibits cardioprotective effects, antimutagenic, carcinogenic, and antioxidant activities, along with lipid peroxidation inhibitory properties (Kwon et al., 2010; Mullen et al., 2011; Niggeweg, Michael, & Martin, 2004; Sato et al., 2011).

When our study focused on analyzing organic acids, we found citric acid (umbu peel –  $3.78 \pm 0.07 \mu\text{g g}^{-1}$ ; umbu seeds –  $3.11 \pm 0.06 \mu\text{g g}^{-1}$ ; umbu pulp –  $2.12 \pm 0.04 \mu\text{g g}^{-1}$ ; siriguela seeds –  $1.50 \pm 0.03 \mu\text{g g}^{-1}$ ; siriguela peel –  $0.88 \pm 0.01 \mu\text{g g}^{-1}$ ; siriguela pulp –  $0.52 \pm 0.01 \mu\text{g g}^{-1}$  and genipap pulp –  $0.43 \pm 0.00 \mu\text{g g}^{-1}$ ) and quinic acid (genipap peel –  $43.28 \pm 0.86 \mu\text{g g}^{-1}$  and umbu pulp –  $41.88 \pm 0.84 \mu\text{g g}^{-1}$ ). Organic acids are primary metabolites that can be found in large amounts in all plants, especially in fruits (Silva et al., 2011). Citric acid is an important organic acid for plant growth and is related with heavy metal stress tolerance (Sun & Hong, 2011).

Apart from the above mentioned compounds, Costa, Ballus, Teixeira-Filho, and Godoy (2010) have reported total phytosterol content of genipap pulp and seeds of 216 and 233 mg 100 g<sup>-1</sup>, respectively. They observed the presence of phytosterols such as campesterol, stigmasterol,  $\beta$ -sitosterol + sitostanol,  $\Delta^5$ -avenasterol +  $\Delta^7$ -stigmasterol and  $\Delta^7$ -avenasterol.

### 3.2. Thin layer chromatography (TLC) bioassay for the detection of AChE inhibition

Partial inhibition of AChE activity in the brain has been shown to be therapeutically beneficial. AChE inhibitors that penetrate the blood-brain barrier elevate the levels of endogenous acetylcholine and are useful in the symptomatic treatment of Alzheimer's disease (Rosenberry, Sonoda, Dekat, Cusack, & Johnson, 2008). The ethanol

**Table 2**  
Phytochemical screening of extracts of parts of the fruits.

Ethanol extracts	Phenols and tannins	Anthocyanins, proanthocyanidins and flavonoids	Leucoanthocyanidins, catechins and flavanones	Anthraquinones, anthrones and coumarins	Flavonols, flavanones and xanthenes	Triterpenoids, sterols	Saponins
Genipap	Peel	—	+	+	—	+	—
	Seed	—	+	+	—	+	—
	Pulp	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
Siriguela	Peel	+	—	+	—	+	—
	Seed	+	+	+	—	+	+
	Pulp	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
Umbu	Peel	+	—	+	—	+	—
	Seed	+	+	+	—	+	—
	Pulp	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>

<sup>a</sup> Could not be performed due to the formation of precipitates.

extracts of genipap pulp and of umbu and siriguela seeds present anti-acetylcholinesterase activity. The carbamate carbachol was used for comparison because it is an isosteric analogue of the physiological substrate acetylcholine (Rosenberry et al., 2008). Genipap pulp ( $0.8 \pm 0.01$  cm) and siriguela seed ( $0.9 \pm 0.01$  cm) presented an AChE inhibition zone similar to that of the positive control ( $1.0 \pm 0.01$  cm), a finding that is significant and hitherto unreported. Umbu seeds presented reasonable activity ( $0.5 \pm 0.01$  cm) and the other extracts were inactive. In view of the aforementioned significant presence of chlorogenic acid in siriguela extracts and the knowledge about its acetylcholinesterase inhibitory activity *ex vivo* reported by Kwon et al. (2010), and dose-dependently *in vitro* with  $IC_{50} = 98.17 \mu\text{g mL}^{-1}$ , as well as its free radical scavenging activity ( $IC_{50} = 3.09 \mu\text{g mL}^{-1}$ ), the inhibitory assay was performed as described, using physostigmine as the positive control. The chlorogenic acid inhibition halo ( $0.63 \pm 0.05$  cm) is very similar to that of the positive control ( $0.67 \pm 0.05$  cm), confirming that it is partially responsible for this important activity.

### 3.3. Cytotoxicity of plant extracts

Some natural products from plants are reportedly harmful to health. Therefore, cytotoxic studies are necessary and can indicate the toxic profile of plant extracts (Oke & Aslim, 2010). The extracts were evaluated on sheep corneal epithelial cells in order to evaluate their cytotoxic effects on normal cells. The medium [Minimum Essential Medium (MEM) + 10% Fetal Calf Serum (FCS)] and 1% formaldehyde were used as negative and positive controls, respectively. In this work, the ethanol extracts of parts of the fruits, at concentrations of 100 ppm, did not show cytotoxicity (Table 3). None of the extracts led to the reduction of cell viability below 90%. The fruit parts (seed and peel) evaluated here are not commonly eaten and there are no reports about the cytotoxicity of their pulp.

Based on these results, the most promising extracts were those obtained from genipap pulp and siriguela seeds.

### 3.4. Total phenolic content (TPC), antioxidant assays and ascorbic acid determination

There are several methods for determining antioxidant capacity. The methods used here were DPPH, ABTS, FRAP, CUPRAC and lipid peroxidation in a biomimetic membrane system and in a mouse liver homogenate representative of direct antioxidant activity.

Table 4 lists the TPC, ABTS, FRAP, CUPRAC and ascorbic acid content results obtained from the ethanol extracts of fruits. A significant difference ( $p < 0.05$ ) among all the analyzed extracts was obtained, with the best general performance shown by siriguela seeds.

Ascorbic acid content was determined (Table 4, column 8), since it was shown to interfere with some of the measurements of antioxidant capacity. The AA content in siriguela seeds ( $3.32 \text{ mg g}^{-1}$ ) and umbu

pulp ( $1.74 \text{ mg g}^{-1}$ ) was higher than the amounts shown in Brasil (2002). The present results indicate that AA does not influence TPC. The literature reports different values for the whole fruits, e.g.,  $18.4 \text{ mg } 100 \text{ g}^{-1}$  for umbu (Rufino et al., 2010), unlike Genovese, Pinto, De Souza, and Lajolo (2008), who obtained  $2.5 \text{ mg } 100 \text{ g}^{-1}$ . Almeida et al. (2011) reported different values for siriguela ( $29.6 \text{ mg } 100 \text{ g}^{-1}$ ) and umbu ( $12.1 \text{ mg } 100 \text{ g}^{-1}$ ), while Narain et al. (1992) showed that AA content in umbu fruit decreases as the fruit ripens.

The intragroup comparison of the results was performed considering the highest activity as 100% in each assay (each individual column), in order to normalize the data (Fig. 1). The ratios are shown as percentage of each extract. An intergroup observation indicated that the siriguela seeds and peels showed better and more significant performance ( $p < 0.05$ ), in most cases, than the other samples.

Fig. 1 and Table 5 show the results of Spearman correlations between two assays at a time. All the assayed showed a good correlation, particularly those with coefficients higher than 0.7000, showing a statistical significance of 0.01.

With regard to TPC, siriguela and umbu seeds showed the highest values; genipap pulp showed an intermediate value, and siriguela and umbu pulp showed the lowest values (Table 4, Fig. 1). Evaluating only the fresh pulp of *S. purpurea* (siriguela) and *S. tuberosa* (umbu), Almeida et al. (2011) found moderate values of 55.0 and 44.6 mg of GAE/100 g, respectively. In an investigation of the yellow mombin (pulp + peel) (*Spondia mombin* L.), which belongs to the Anacardiaceae family, Tiburski et al. (2011) found a total phenolic content of 260.21 mg GAE/100 g. Assessing the intact fruit (peel, pulp and seed) of *Spondia pinnata* K., Satpathy, Tyagi, and Gupta (2011) reported the highest value of 210 mg GAE for TPC in 100 g of the extract.

Non-edible parts of exotic fruits have shown important antioxidant activities. In general, phenolic compounds are located preferentially in peel and seeds and to a lesser extent in the pulp. In their review, Ayala-Zavala et al. (2011) showed that the seed, peel and pulp of avocado (*Persea americana*) have TPC of 5160, 1260 and 490 mg GAE  $100 \text{ g}^{-1}$ , while those of mango (*Mangifera indica* L.) have 11,700.0, 7000.0 and 240.0 mg GAE  $100 \text{ g}^{-1}$ . The seed of jackfruit (*Artocarpus heterophyllus*) and longan (*Dimocarpus longan* Lour.) have TPC of 2770 and 6260 mg GAE  $100 \text{ g}^{-1}$ , and their pulp contains 160 mg GAE  $100 \text{ g}^{-1}$ , respectively. Banana (*Musa cavendish*) and guava (*Psidium guajava* L.) have TPC of 928 and 5870 mg GAE  $100 \text{ g}^{-1}$  in their peel and 232 and 159 mg GAE  $100 \text{ g}^{-1}$  in their pulp, respectively.

The TPC of siriguela and umbu appeared in a decreasing order of seed > peel > pulp, which is consistent with most of the findings reported in the literature. In contrast, genipap peel presented a higher phenolic content than its seeds or pulp. Similar results were found for jujube (*Ziziphus jujuba* Mill) fruit, with a higher TPC of around 46.23 mg GAE  $\text{g}^{-1}$  dry weight in the peel (Wang, Liu, Zheng, Fan, & Wei Cao, 2011). In addition, Silva et al. (2004) showed that quince (*Cydonia oblonga* Miller) peel extract had an approximately five-fold higher content of phenolics (7087.1 mg of phenolic compound  $\text{kg}^{-1}$  dry matter) than that of the pulp. Prasad et al. (2010) investigated the application of different solvents (ethyl acetate, butanol, and water) to extract antioxidant compounds of peel, pulp, and seeds of dabai (*Canarium odontophyllum* Miq.) fruit. They reported that the peel contains higher phenolic content than seed or pulp and showed that the ethyl acetate (68 mg GAE  $100 \text{ g}^{-1}$ ) fraction contained higher TPC than the butanol (35 mg GAE  $100 \text{ g}^{-1}$ ) and water (18 mg GAE  $100 \text{ g}^{-1}$ ) fractions.

Orhan and Aslan (2009) studied anti-acetylcholinesterase and antioxidant activities in ethanol extracts of *Salvia triloba*, *Teucrium polium* and *Melissa officinalis* and found values of 10.9, 107.8 and 68.7 mg GAE  $\text{g}^{-1}$  extract, respectively. In the Anacardiaceae family, Moyo, Ndhkala, Finnie, and Staden (2010) evaluated leaf, stem bark, young stem and opercula of *Sclerocarya birrea* subsp. *caffra* and *Harpephyllum caffrum* and obtained significant anti-acetylcholinesterase activity, with TPC values varying from 2.46 to 14.15 mg GAE  $\text{g}^{-1}$  extract.

**Table 3**

Effects of ethanol extracts of fruits on sheep corneal epithelial cell viability.

Ethanol extracts (100 ppm)		Cell viability (%)
Genipap	Peel	98.5 <sup>b,c</sup> ± 0.53
	Pulp	100.0 <sup>a</sup> ± 0.00
	Seeds	99.1 <sup>a,b,c</sup> ± 0.18
Siriguela	Peel	90.4 <sup>c</sup> ± 0.39
	Pulp	99.6 <sup>a,b</sup> ± 0.26
	Seeds	92.8 <sup>d</sup> ± 0.61
Umbu	Peel	98.9 <sup>a,b,c</sup> ± 0.30
	Pulp	98.8 <sup>a,b,c</sup> ± 0.53
	Seeds	92.0 <sup>e</sup> ± 0.79
Negative control 1: MEM + 10% FCS		98.0 <sup>e</sup> ± 0.25
Positive control 2: formaldehyde 1% (w/v)		16.2 <sup>f</sup> ± 0.68

Within each column, averages denoted with the same letter were not significantly different in this test ( $p < 0.05$ ).



**Table 4**  
TPC, DPPH, ABTS, FRAP, CUPRAC and ascorbic acid content of ethanol extracts of fruits.

Ethanol extracts		TPC	DPPH	ABTS	FRAP	CUPRAC	Ascorbic acid content
		mg GAE <sup>b</sup> g <sup>-1</sup> dry extract	RSA% – DPPH <sup>i</sup>	TEAC <sup>j</sup>	TEAC <sub>FRAP</sub> (mmol Trolox. g <sup>-1</sup> )	TEAC <sub>CUPRAC</sub> (mmol Trolox. g <sup>-1</sup> )	mg g <sup>-1</sup>
Genipap	Peel	187.7 ± 11.3 <sup>b</sup>	15.43 ± 0.70 <sup>d</sup>	0.75 ± 0.09 <sup>d,e</sup>	0.71 ± 0.06 <sup>d</sup>	1.32 ± 0.05 <sup>c,d</sup>	0.0 ± 0.00 <sup>h</sup>
	Seeds	122.7 ± 18.9 <sup>c</sup>	11.61 ± 0.47 <sup>e</sup>	1.09 ± 0.35 <sup>d</sup>	1.0 ± 0.07 <sup>d</sup>	0.81 ± 0.30 <sup>f</sup>	1.01 ± 0.05 <sup>g</sup>
	Pulp	61.6 ± 3.6 <sup>d</sup>	10.17 ± 0.36 <sup>e</sup>	0.40 ± 0.00 <sup>e,f,g</sup>	1.02 ± 0.11 <sup>d</sup>	1.04 ± 0.13 <sup>d,e</sup>	0.0 ± 0.00 <sup>h</sup>
Siriguela	Peel	112.2 ± 13.2 <sup>c</sup>	31.89 ± 0.92 <sup>b</sup>	5.06 ± 0.15 <sup>a</sup>	16.20 ± 1.05 <sup>a</sup>	5.06 ± 0.15 <sup>b</sup>	1.15 ± 0.01 <sup>f</sup>
	Seeds	254.7 ± 42.1 <sup>a</sup>	37.64 ± 1.26 <sup>a</sup>	3.06 ± 0.22 <sup>b</sup>	13.73 ± 0.53 <sup>b</sup>	8.87 ± 0.23 <sup>a</sup>	3.32 ± 0.09 <sup>a</sup>
	Pulp	13.5 ± 1.3 <sup>e</sup>	6.40 ± 0.35 <sup>f</sup>	0.03 ± 0.01 <sup>g</sup>	0.28 ± 0.09 <sup>d</sup>	0.37 ± 0.01 <sup>f</sup>	1.30 ± 0.05 <sup>e</sup>
Umbu	Peel	52.5 ± 5.9 <sup>d</sup>	19.71 ± 1.47 <sup>c</sup>	0.53 ± 0.01 <sup>e,f</sup>	4.98 ± 0.21 <sup>c</sup>	1.46 ± 0.07 <sup>c</sup>	1.52 ± 0.05 <sup>d</sup>
	Seeds	202.2 ± 6.9 <sup>b</sup>	20.78 ± 0.34 <sup>c</sup>	2.18 ± 0.35 <sup>c</sup>	5.46 ± 0.08 <sup>c</sup>	5.17 ± 0.13 <sup>b</sup>	1.60 ± 0.06 <sup>d</sup>
	Pulp	40.4 ± 8.7 <sup>d</sup>	16.10 ± 0.88 <sup>d</sup>	0.22 ± 0.05 <sup>g</sup>	1.19 ± 0.15 <sup>d</sup>	0.80 ± 0.04 <sup>e</sup>	1.74 ± 0.04 <sup>c</sup>

<sup>b</sup>Gallic acid equivalents. <sup>i</sup>Percentage of DPPH radical-scavenging activity in 30 min.

<sup>j</sup>TEAC: Trolox Equivalent Antioxidant Capacity (mmol TE/g dry extract). Mean values followed by the same letters in each column do not differ significantly at  $p < 0.05$  by a Tukey test.

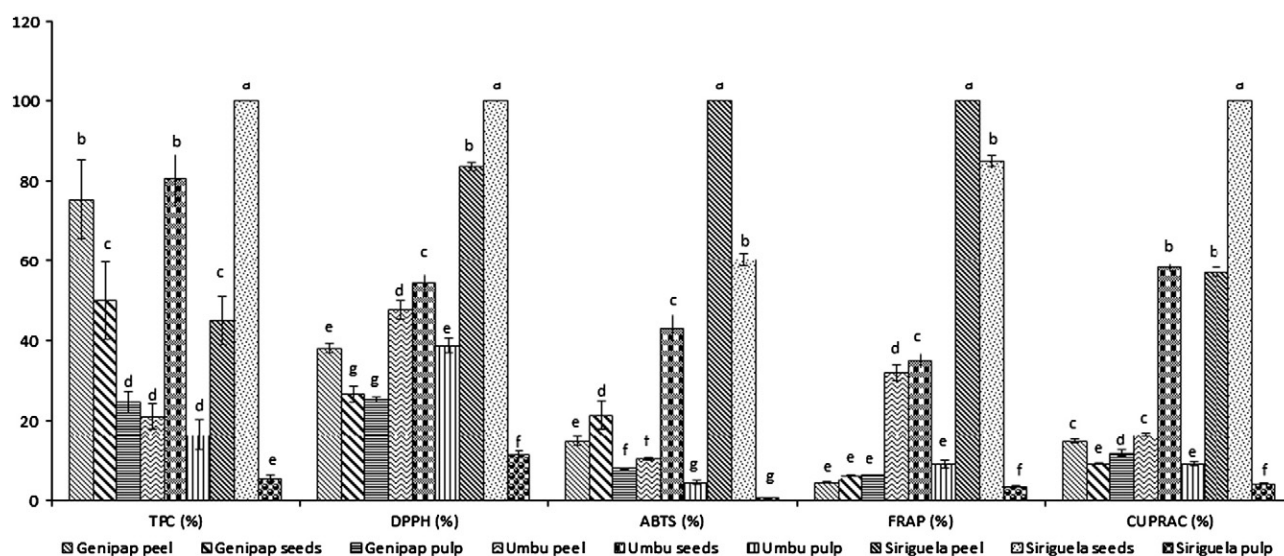
All the extracts of genipap, siriguela and umbu parts in our study presented higher values than those obtained by Orhan and Aslan (2009) and Moyo et al. (2010). It is noteworthy that Folin–Ciocalteu reagent, which is used to determine TPC, is not specific for phenolic compounds because it can also be reduced by many non-phenolic compounds, including ascorbic acid (Karadag, Ozcelik, & Sanner, 2009).

The highest consumption of DPPH<sup>•</sup> (Table 4, column 3) was observed in siriguela (seed and peel) and umbu seed extracts, with a positive association between anti-acetylcholinesterase activity and TPC (Table 4, column 2), which showed the same tendency, i.e., higher values for siriguela seed. The consumption of DPPH<sup>•</sup> was found to stabilize at about 27 min, which indicates the time of action of possible antioxidants present in the samples. Moyo et al. (2010), in *S. birrea* subsp. *caffra* and *H. caffrum*, found percent RSA values varying from 2.5 up to 27.2. Some exotic fruits have high RSA (Kong et al., 2011), such as indigenous kembayau (*Dacryodes rostrata* (Blume)) fruit, with seeds (92.18–92.19%), pulp (91.47–92.15%) and peels (70.39–86.51%), with the seeds showing the highest amount of total flavonoids and phenolics. Ribeiro, Barbosa, Queiroz, Knödler, and Schieber (2008) showed that the DPPH radical-scavenging capacity of peel and seed kernel extracts of the mango variety Ubá was concentration-dependent (1000–5000 ppm). At concentrations of >2000 ppm, the peel extract (53.3–89.1%) showed significantly higher activity (minimum 1.3 times) than the seed extract. The higher activity of the peel extract can be attributed to a more elevated concentration of xanthenes and a 23.5-fold higher

flavonol content than in the kernel. The radical-scavenging activity of the extracts was higher than that of the commercial synthetic antioxidant BHA at all the tested concentrations.

In our work, the IC<sub>50</sub> values for most promising extracts were determined as 36.37 µg mL<sup>-1</sup>, 173.37 µg mL<sup>-1</sup>, 1069.13 µg mL<sup>-1</sup>; for siriguela seeds, umbu seeds and genipap pulp, respectively. Gacche and Dhole (2011) evaluating the ethanolic extract of *Morinda centrifolia* L., from the same family as genipap, exhibited IC<sub>50</sub> of 0.620 mg mL<sup>-1</sup>, a lower value than genipap pulp. The IC<sub>50</sub> values of *S. birrea* and *H. caffrum* extracts (Anacardiaceae) – leaf, young stem and opercula from 4.26 to 6.92 µg mL<sup>-1</sup>, below the values found for siriguela and umbu (Moyo et al., 2010).

Thitilertdecha and Rakariyatham (2011) demonstrated that free radical scavenging properties in the peel, seed and pulp of rambutan (*Nephelium lappaceum* L.) of the Rongrien and See-Chompoo cultivars varied during fruit maturation. In both cultivars, the IC<sub>50</sub> values of the peels remained low throughout fruit development (1.42–4.75 µg mL<sup>-1</sup>), but the values of the seed and pulps were low at first, increasing markedly as the fruit developed until harvest (4.87 to >1000 µg mL<sup>-1</sup>). On the other hand, Santos et al. (2011) investigated different grape parts, peel, pulp, and seeds of *Vitis vinifera* (Brazil and Benitaka) and *Vitis labrusca* (Niagara and Isabel) and showed that the lowest concentrations necessary for 50% inhibition of DPPH ranged from 9.26 to 126.91 µg mL<sup>-1</sup> in seeds in different solvents. No antioxidant activity was found in the pulp, and the activity in the peel



**Fig. 1.** Normalized activities of the extracts in relation to each antioxidant test. The highest activity was considered 100% and the other values correspond to a relative percent of activity. Within each column, averages denoted with the same letter were not significantly different by this test ( $p < 0.05$ ).

**Table 5**

Spearman correlation among the variables TPC, ABTS, FRAP, CUPRAC and DPPH.

	TPC	DPPH	ABTS	FRAP	CUPRAC
TPC	1	0.5894*	0.8036**	0.4614*	0.7105**
DPPH	0.5894*	1	0.7430**	0.8267**	0.8673**
ABTS	0.8036**	0.7430**	1	0.7625**	0.7234**
FRAP	0.4614*	0.8267**	0.7625**	1	0.7230**
CUPRAC	0.7105**	0.8673**	0.7234**	0.7230**	1

Statistical significance (*p* value) reported such as: \* for  $p < 0.05$ , \*\* for  $p < 0.01$ .

varied from 50.92 to  $>400 \mu\text{g mL}^{-1}$ . Resveratrol was detected in the peels and seeds of the four varieties, but not in the pulp.

The ABTS method is based on the ability of antioxidant molecules to quench the long-lived  $\text{ABTS}^{\bullet+}$  radical (Arnao et al., 2001). The highest ABTS values were observed in siriguela peel and in the seed extracts of all the fruits, showing the same trend already observed for siriguela (seed and peel) and umbu seed (Table 4). The best results shown by seeds are probably attributable to their chemical compositions rich in phenols and the presence of substances required for the germination process, as described by Martins, Vasconcelos, Rossetto, and Carvalho (2010). Almeida et al. (2011) found values of 6.25 and  $1.07 \mu\text{M}$  of Trolox equivalents/g fresh mass of siriguela and umbu, respectively. For *S. pinnata* K., Satpathy et al. (2011) reported ABTS (TEAC) values of 0.68 to 0.83 in 100 mg extract.

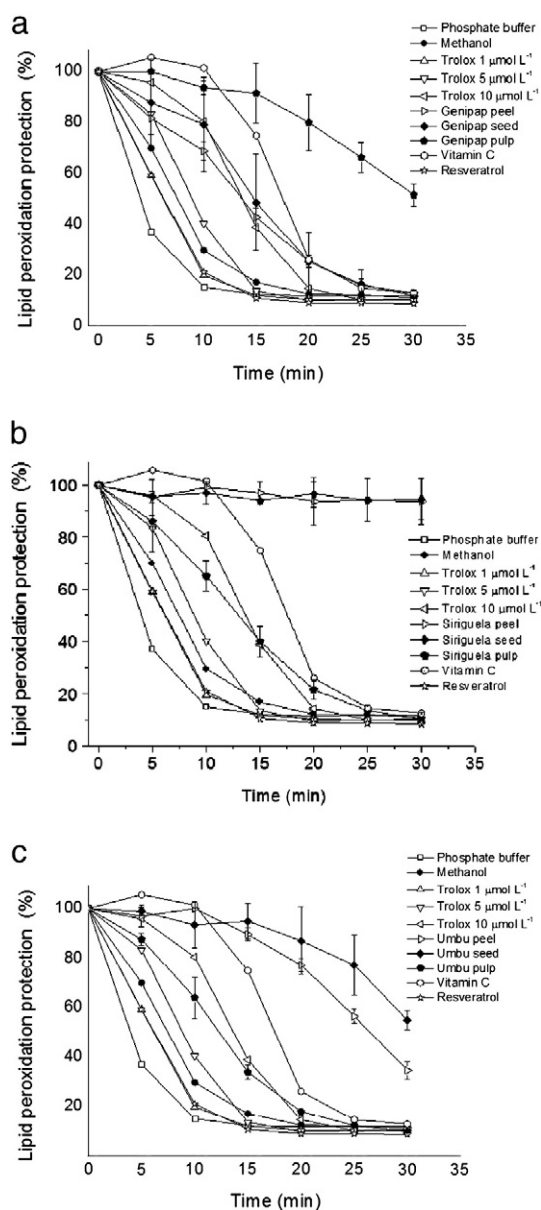
Among the metal reduction-based antioxidant methods, the FRAP method reduces the  $\text{Fe(III)-TPTZ}$  complex to  $\text{Fe(II)-TPTZ}$  at low pH (Rufino et al., 2006). Another method employed here was CUPRAC, which uses  $\text{Cu(II)-neocuproine}$  [ $\text{Cu(II)-Nc}$ ] as the chromogenic oxidizing agent. This method offers advantages over the FRAP method because the redox chemistry of  $\text{Cu(II)}$  involves faster kinetics than that of ferric ions (Apak et al., 2004). According to the metal reduction potential in the Iron-FRAP and Copper-CUPRAC complexes, the latter method has shown higher selectivity due to its lesser ability to receive electrons. Satpathy et al. (2011) report FRAP values of 5.97 to  $7.93 \text{ mg TE}/100 \text{ mg}^{-1}$  extract from *S. pinnata* K.

FRAP and CUPRAC methods showed higher values for siriguela and umbu (peels and seeds). Genipap pulp showed the worst profile, following the same tendency as that of the other aforementioned methods (Table 4). Contreras-Calderon et al. (2010) tested seed samples of 12 families: Sapotaceae; Arecaceae; Caesalpinaceae; Myrtaceae; Passifloraceae; Rubiaceae; Anacardiaceae; Cucurbitaceae; Sterculiaceae; Proteaceae; Caricaceae and Icacinaceae for antioxidant activity and TPC. Their findings showed that the seeds present a wide range of FRAP (1.40 to  $1690 \text{ FRAP } \mu\text{mol TE}/\text{g}$  of fresh weight), ABTS (4.92 to  $1700 \text{ ABTS } \mu\text{mol TE}/\text{g}$  of fresh weight) and TPC (20.4 to  $4851 \text{ TP mg}$  of GAEs/100 g of fresh weight) values.

To summarize, siriguela peel and seed showed the best results in most of the antioxidant assays, while the pulps showed the lowest activity.

Based on the results, a positive correlation was found between acetylcholinesterase inhibition and antioxidant activity in the siriguela and umbu seed extracts, but not in genipap pulp. In the case of genipap, a non-phenolic compound is expected to be an acetylcholinesterase inhibitor.

The antioxidant capacity was also evaluated using a peroxyl radical-mediated lipid peroxidation membrane model (soy lecithin unilamellar liposomes) loaded with the peroxyl radical-sensitive fluorescent probe  $\text{C}_{11}\text{-BODIPY}^{581/591}$  (Oliveira, Valentim, Goulart, et al., 2009; Oliveira, Valentim, Silva, et al., 2009). Figs. 2a, b and c present lipid peroxidation protection (%) as a function of time (total time 30 min) of fruit extracts ( $0.5 \text{ mg mL}^{-1}$ ). Genipap pulp extract showed the best performance, providing more than 95% protection of the membrane for 15 min. Siriguela peel and seed extracts protected the membrane (more than 95%) for more than 30 min, while umbu peel and seed extracts offered more than 95% protection of the membrane for 15 min. These results were considered better than those of the positive



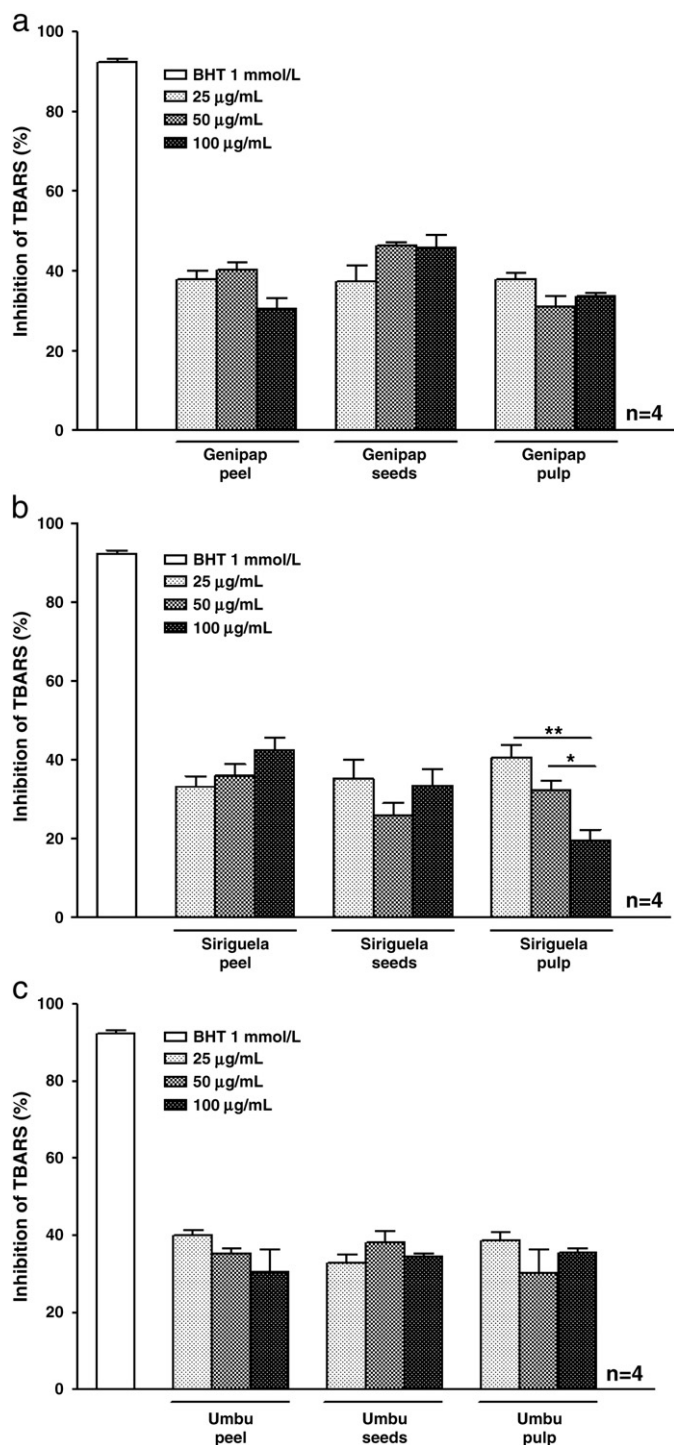
**Fig. 2.** Protection against lipid peroxidation provided by the extracts ( $0.5 \text{ mg mL}^{-1}$ ) of different parts of the fruits of (a) genipap, (b) siriguela and (c) umbu.

controls (Trolox – 1, 5 and  $10 \mu\text{mol L}^{-1}$ ; vitamin C –  $100 \mu\text{mol L}^{-1}$  and resveratrol –  $100 \mu\text{mol L}^{-1}$ ). The extracts that presented acetylcholinesterase inhibitory activity also showed positive performance by this method.

The results indicate that the extracts of genipap pulp, siriguela (peel and seed) and umbu (peel and seed) can work synergistically, since they provided longer protection of the membrane than the controls. It could be meaningless to analyze membrane protection afforded by a single antioxidant present in each extract over time, since some antioxidants play cooperative roles against lipid peroxidation. Such interactions may result in synergistic rather than cumulative antioxidant activity. The most efficient biological interaction seems to be the antioxidant synergism between vitamin E and vitamin C. Vitamin C reduces vitamin E radical to regenerate vitamin E, before the vitamin E radical attacks lipids to induce lipid oxidation (Niki, 2010).

Inhibition of TBARS was also determined (Fig. 3), as an index of inhibition of lipid peroxidation, using mouse hepatic homogenate as a real animal tissue.





**Fig. 3.** Lipid peroxidation protection through TBARS inhibition by the extracts of different parts of the fruits: (a) genipap, (b) siriguela, and (c) umbu. The control is the antioxidant BHT, at 1 mmol/L. Statistical significance ( $p$  value) using one-way analysis of variance (ANOVA) with Tukey test was calculated for intra and intergroup experiments vs. control and is reported as: \* for  $p < 0.05$ , \*\* for  $p < 0.01$  and \*\*\* for  $p < 0.001$ .

Zaini et al. (2011) investigated the in vitro antioxidant activity of Kundur fruit and demonstrated that the seed had higher inhibitory capacity against linoleic acid oxidation and scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals than the peel, pulp and core of the fruit. This may be due to the higher total phenolic content and superoxide dismutase (SOD) activity of the seeds, which is similar to the results observed in the present study for siriguela and umbu seeds. Pande and

Akoh (2010) reported that the seeds of numerous underutilized fruits contain a high polyphenol content, followed by their peels and pulp.

According to Karadag et al. (2009), the antioxidant capacity of complex heterogeneous foods and biological systems is affected by many factors, including the partitioning properties of the antioxidants between lipid and aqueous phases, the conditions of oxidation and the physical state of the oxidizable substrate.

Because of the phytochemical diversity and aqueous and lipid phases in foods, it is reasonable to suggest the existence of various substances that interact for common purposes. Thus, a substance responsible for protecting plants can act as an antioxidant and also as an acetylcholinesterase inhibitor.

As regards the activities found in genipap pulp, it can be assumed that they are not attributable to phenolic compounds due to the low content obtained, while in siriguela and umbu fruits, these compounds may be directly responsible for the activities, as indicated by phytochemical screening.

#### 4. Conclusions

Extracts obtained from siriguela and umbu (seeds and peels) and genipap pulp displayed significant antioxidant properties in most of the tests. The large amount of chlorogenic acid found in siriguela seeds may explain, in part, its better performance in most of the antioxidant assays and its inhibition of acetylcholinesterase activity. With the exception of genipap pulp, a positive correlation was found between acetylcholinesterase inhibitory and antioxidant activities. In view of the significant properties found in this study, including possible beneficial effects on the brain, we recommend that genipap pulp and seed be used in the preparation of foods and that their consumption be encouraged, as well as in the pharmaceutical and cosmetic industries for the development of different products to delay and/or prevent the action of free radicals.

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