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Total phenolic content and free radical scavenging activities of methanolic extract powders of tropical fruit residues

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ABSTRACT

Methanolic extract powders of acerola, passion fruit and pineapple industrial residues, including pulp, seeds and peel, altogether (except for acerola) devoid of seeds, were screened for antioxidant capacity. The total phenolic contents (TPCs) of the extract powders were compared with their radical-scavenging activities (RSA) against both DPPH$^{\bullet}$ and superoxide anion ($O_2^{\bullet-}$) radicals, and their protective effect against liposome peroxidation, triggered by peroxyl radical. Lipid peroxidation was followed by the fluorescence decay of the probe, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid (C_{11}-BODIPY$^{581/591}$). The TPCs of acerola, passion fruit and pineapple extract powders were (94.6 ± 7.4); (41.2 ± 4.2) and (9.1 ± 1.3) mg of gallic acid equivalents g^{-1} of dry extract, respectively. Acerola showed the best RSA-DPPH$^{\bullet}$ scores, whereas passion fruit was more protective on the RSA-$O_2^{\bullet-}$ system. Together with the protective effects against lipid peroxidation (rate of BODIPY decay), which were similar for acerola and passion fruit extracts, these data suggest that the methanolic extracts of acerola and passion fruit residues may be useful as antioxidant supplements, particularly the acerola extract, due to its high phenolic content.

1. Introduction

Imbalanced production and consumption of reactive oxygen species, leading to oxidative stress, is implicated in the pathophysiology of a plethora of genetic and acquired disorders, such as cancer, arteriosclerosis, malaria and rheumatoid arthritis, as well as neurodegenerative diseases and ageing processes (Halliwell & Gutteridge, 2007; Vasconcelos et al., 2007). Epidemiology shows an inverse association between the daily consumption of fruits and vegetables and the risk of degenerative and chronic diseases (John, Ziebland, Yudkin, Roe, & Neil, 2002; Zibadi et al., 2007). The protective effects of fruits and vegetables have long been attributed to their antioxidant compounds, such as polyphenols, carotenoids, and vitamins C and E. Antioxidants act in various ways, which include complexation of redox-catalytic metal ions, scavenging of free radicals, and decomposition of peroxides. Often, several mechanisms and mechanistic synergisms are involved. Especially in food-related systems (e.g. extracts), antioxidant activity studies (using multiple experimental approaches) allow a complete screening of the putative chain-breaking capacity (Mello & Kubota, 2007). Growing knowledge about the health-promoting impact of antioxidants in everyday foods, combined with the assumption that a number of common synthetic preservatives may have hazardous effects (Krishnakumar & Gordon, 1996), has led to increased investigations in the field of natural antioxidants (Moure et al., 2001).

Efficient, inexpensive and environmentally friendly use of agri-food industry waste is highly cost-effective and minimises environmental impact. One of the most effective options is the recovery of bioactive plant food constituents, which could be used in the pharmaceutical, cosmetics and food industry (Makris, Boskou, & Andrikopoulos, 2007). In addition, economically advantageous alternatives for exploiting the antioxidant content of tropical fruit residues, from juice processing industries, can provide the local food industries and impoverished population with low-cost nutritional supplements. In the food industry, synthetic antioxidants, such as ascorbic acid and butylated hydroxytoluene (BHT), have

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long been widely used as antioxidant additives to preserve and stabilize the freshness, nutritive value, flavour and colour of foods and animal feed products. However, at least one study has revealed that BHT could be toxic, especially at high doses (Moure et al., 2001). It is thus important to consider the potential health risks associated with long-term dietary intake of BHT. For safety purposes, the US Food and Drug Administration (FDA) limits the use of BHT to 0.02% or 200 ppm of food lipid content (Shui & Leong, 2006). Alternative, natural and (probably) safer, sources of food antioxidants have become increasingly more attractive (Williams, Iatropoulos, & Whysner, 1999; Bonilla, Mayan, Merida, & Medina, 1999).

The number of studies on residual sources of antioxidants has increased considerably in recent years (Moure et al., 2001). Shui and Leong (2006) found that antioxidants obtained from star fruit residues slowed the rancidity process of oil to a greater extent than did BHT. They stressed the high potential of this residue for preventing oil rancidity. The antioxidant compounds from agri-waste may not only increase the stability of foods, by preventing lipid peroxidation, but in humans or animals may also protect biomolecules and supramolecular structures e.g. membranes, ribosomes from oxidative damage.

In Brazil, many edible tropical fruits are processed into natural and concentrated juices, jellies, pulp and extracts. In these processes, seeds, peels and other parts are routinely discarded as useless, causing environmental problems.

Scarce information on the antioxidant capacity of residues from acerola, passion fruit and pineapple is available in the literature. This led us to investigate the antioxidant properties of these residues produced by a juice-processing industry located in the state of Alagoas, northeastern Brazil, aiming to fully exploit the nutrient content of these fruit residues, prevent waste and eventually provide the local food industries and impoverished population with low-cost nutritional supplements. The total phenolic content (TPC) and radical-scavenging activity (RSA), against both the $DPPH^{\bullet}$ and the superoxide anion ($O_2^{\bullet -}$) radical, of acerola, passion fruit and pineapple extracts were determined. In parallel, their protective effect against peroxyl radical-initiated membrane lipid peroxidation was evaluated.

2. Materials and methods

2.1. Standards and reagents

Folin-Ciocalteau reagent, BHT (butylated hydroxytoluene), soy phosphatidylcholine, $DPPH^{\bullet}$ (2,2′-diphenyl-2-picrylhydrazyl radical), and AAPH [2,2′-azobis(2-amidinopropane) dihydrochloride] were purchased from Sigma–Aldrich (Steinheim, Germany). EDTA (ethylenediamine tetraacetic acid), NBT (nitro blue tetrazolium), PMS (phenazine methosulfate), and β-NADH (β-nicotinamide adenine dinucleotide) were obtained from Acros Organics (New Jersey, USA). Sodium bicarbonate and gallic acid were from Vetec Química Fina Ltda (Rio de Janeiro, Brazil), Trolox® from Merck (Düsseldorf, Germany), and the fluorescent fatty acid-analogue, 4,4′-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza–*S*-indacene-3-undecanoic acid (C_{11}-$BODIPY^{581/591}$), from Molecular Probes (Ontario, Canada). All the reagents were of analytical grade and the stock solutions and buffers prepared with milliQ purified water.

2.2. Sample preparation and extraction

Pulp, seeds and peels (herein named residues) of pineapple (*Ananas comosus)* and passion fruit (*Passiflora alata*), along with pulp and peels of acerola (*Malpighia emarginata)*, were provided by a local juice factory, located in Pindorama, Alagoas State, Brazil. Fresh fruits were selected and, after 24 h, thoroughly washed with sodium hypochlorite solution (0.05%) and rinsed with water before being processed. Their juices are of commercial value, and therefore, were not used in the present study. The juicing processes were as follows: for passion fruit, after washing and excess water elimination in a perforated stainless steel sieve (holes with 1 cm diameter), the fruits were cut in halves. The endocarpus and seeds were triturated in a mixer, and the solid residues were separated from the liquid portion using sieves with 0.50 cm orifices. For pineapple, after washing and drying, the fruit extremities (crown and bottom) were cut off. The heart of the fruit was cut into four pieces and added to the mixer. Filtration allowed separation of juice and solid parts. In the case of acerola, the seeds are bitter, and hence must be separated before juice manufacture. The whole fruits were added to a special mixer that is used only to break the peels of the fruits. The seeds were removed by filtration.

The fruit residues were kept for 24 h in a −20 °C freezer before the drying process. After juicing, the solid residual portion, pulp, seeds and peel, altogether (except for acerola) were then spread on sieves and washed with distilled water, dried in a ventilated oven at 60 °C for 48 h and ground to a fine powder, using a laboratory-size mill. Extractions of 20 g of either acerola, pineapple and passion fruit residues were carried out by reflux, initially with *n*-hexane, to remove non-polar compounds and then with methanol. Extracts were prepared with 150 ml of each solvent, in a Soxhlet apparatus heated at 60 °C. The residues were extracted with *n*-hexane for 4 h and then by methanol for a further 4 h, heated at 60 °C. The solvents were eliminated using a rotary evaporator (Buchi rotavapor R-114) at 50 + 10 °C and the extract residues were kept under nitrogen in a refrigerator for 1 h. The *n*-hexane extract did not exhibit antioxidant activity, and therefore was discarded. The final amounts of dry powder obtained from the methanol extracts of pineapple, passion fruit, and acerola residues (20 g) were 3.14 g (15.7%), 0.12 g (2.5%), and 1.43 g (7.1%), respectively (Table 1).

2.3. Determination of total phenolic content

Total phenolic content (TPC) of the methanolic extracts obtained from the fruit dry powders was determined using the Folin-Ciocalteau reagent, as described by Singleton, Orthofer, and Lamuela-Raventós (1999), with minor modifications. The extracts were dissolved in milliQ purified water (0.50 mg ml^{-1}) and 0.50 ml aliquots or deionized water (control) were mixed by manual shaking, for 10–15 s, with 0.50 ml of Folin-Ciocalteau reagent. After 3 min, 0.50 ml of saturated sodium carbonate solution was added and the solution diluted to 5 ml with deionized water. The reaction mixture was kept in the dark for 2 h and its absorbance

Table 1
Methanol extraction yields (EY) and total phenolic contents (TPC) measured in methanolic extract powders of fruit residues (FR) and calculated for 100 g of dry fruit residues (FR).

Methanolic extracts	EY (%)	TPC (dry extract weight) (mg GAE g^{-1})	TPC (dry FR weight) (mg GAE 100 g^{-1})
Acerola (AC)	7.1	94.6 ± 7.4	681 ± 53.5
Pineapple (PA)	30.2	9.1 ± 1.3	275 ± 38.0
Passion fruit (PF)	2.5	41.2 ± 4.2	103 ± 10.4

measured at 760 nm against water in a UV–vis spectrophotometer (MutiSpec–1501, Shimadzu, Japan). The concentration of phenolic compounds was estimated using a calibration curve traced with gallic acid (0.01–0.4 mol l^{-1}) as a polyphenol reference ($n = 3$). The results were expressed as mean ± standard deviations of mg of gallic acid equivalents g^{-1} extract) and mg of gallic acid equivalent 100 g^{-1} fruit residue), considering the extraction yields. A study of the interference of vitamin C on TPC was performed. UV–vis spectra were recorded, in triplicate, after adding enough and sequential amounts of vitamin C solution in gallic acid solution in order to get ratios of 0.1:1; 0.5:1; 1:1; 2:1. The absorbance was measured in the absence and presence of vitamin C.

2.4. Radical-scavenging activity of 2,2′-diphenyl-β-picrylhydrazyl radical (*RSA*-DPPH•)

The antioxidant capacity of fruit samples and, for comparison, the antioxidant activities of gallic acid and BHT were measured in terms of their radical-scavenging ability (RSA), using the DPPH• method (Sânchez-Moreno, Larrauri, & Saura-Calixto, 1999). Thus, 0.30 ml of powder extract dissolved in methanol (0.25, 0.5 and 1.0 mg ml^{-1}) were mixed with 2.7 ml of DPPH• radical solution (40 μg ml^{-1} in methanol) in a 3 ml-quartz cuvette. The mixture was homogenised using the micropipette pointer (pushing and pulling liquids) and kept in the dark prior to analysis. The DPPH• absorption values at 516 nm were obtained every 5 min during 50 min. The percentage of remaining DPPH• ($DPPH^{\bullet}_R$) was calculated as follows:

$$\%DPPH^{\bullet}_R = 100 \times [(DPPH^{\bullet})_t/(DPPH^{\bullet})_{t=0}]$$

where:

$DPPH^{\bullet}_t$ was the concentration of DPPH• at any time t;

$DPPH^{\bullet}_{t=0}$ was the concentration of DPPH• at time zero.

The results are expressed as $DPPH^{\bullet}_R$ disappearance (%) as a function of time. All determinations were performed in triplicate. The percentage of DPPH radical-scavenging activity (RSA- {DPPH•) of each extract was calculated as below:

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

where:

A_c is the absorbance of the solution when the extract was added at a particular concentration;

A_D is the absorbance of the DPPH• solution.

The results were expressed as RSA%-DPPH• against total extract concentration in the cuvette.

2.5. Superoxide anion radical ($O_2^{\bullet -}$)-scavenging activity (*RSA*- $O_2^{\bullet -}$)

Antioxidant capacity of the dried methanolic extracts against $O_2^{\bullet -}$, radicals was determined by the method of Ewing and Janero (1995) with minor modifications. The final reaction system (250 μl in each reaction-well) consisted of 50 mM phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 50 μM NBT, 78 μM NADH, and 3.3 μM PMS (final concentrations). The absorbance at 560 nm, after a 10 μl sample addition (0.5 mg methanolic extract ml^{-1}), was continuously monitored for 5 min as an index of NBT reduction (formazan production) using a SpectraMax 250 microplate reader (Molecular Devices).

2.6. Antioxidant capacity in a membrane biomimetic system

2.6.1. Preparation of unilamellar vesicles

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

2.6.2. Lipid peroxidation measurements

Fluorescence measurements were carried out at 37 °C using a Spex Fluorolog – 1681® fluorometer by mixing, in a 1 ml-quartz cuvette, 800 μl of unilamellar vesicle suspension, 50 μl of 50 mM phosphate buffer, pH 7.4, and 50 μl of sample (0.5 mg ml^{-1} dried extract solution) or trolox (positive control, 1, 3 and 5 μM). The reaction was initiated by adding 100 μl of AAPH (100 mM). The fluorescence decay ($\lambda_{excitation} = 580$ nm, $\lambda_{emission} = 600$ nm) was continuously monitored for 30 min.

2.7. Statistical analysis

All data were expressed as means ± standard deviation. A significant difference was considered at the level of $p < 0.05$. The results were analysed using ANOVA, unpaired Student's t-test and qui-square, using Microsoft Excel statistical functions and Microcal Origin 7.0.

3. Results and discussion

3.1. Extract yields

Table 1 lists the methanol extraction (EY) and dry powder (g) yields and the most effective extraction, from 20 g of fruit residues was, in decreasing order of yield: pineapple (30.2%) > acerola (7.1%) > passion fruit (2.5%). The much higher yield of pineapple dry powder can be attributed to the higher content of pineapple fibres compared to the other fruits.

3.2. Total phenolic content (TPC)

Polyphenolic compounds are very important fruit constituents, by virtue of their antioxidant activity by chelating redox-active metal ions, inactivating lipid free radical chains and preventing hydroperoxide conversion into reactive oxyradicals. Table 1 summarises the contents of total phenolics, expressed as gallic acid equivalents (GAE), either as mg GAE g^{-1} or mg GAE 100 g^{-1}, found in the methanolic extracts and calculated for corresponding fruit residues. These values were obtained from the absorbance of the extracts treated with Folin-Ciocalteau reagent.

The TPC of acerola methanolic extract was approximately 2-fold and 10-fold higher than those of the passion fruit and pineapple methanolic extracts, respectively ($p < 0.05$). These data indicate substantial differences in the TPCs of the tested fruit extracts, which could strongly account for the distinct antioxidant activities of the samples. One cannot exclude the presence of additional antioxidant components in the residues that could contribute to these differences because some non-phenolic compounds, such as ascorbate and reducing carbohydrates, can also reduce the Folin reagent (Stratil, Klejdus, & Kubán, 2007). However, phenolic compounds react with Folin-Ciocalteau reagent only under basic conditions (pH ~ 10) due to a much higher reactivity of the phenolate anion with the molybdenum-based compounds present in the reagent (Huang, Ou, & Prior, 2005). Duplicate experiments performed by adding ascorbic acid to gallic acid in different proportions (0.1:1.0; 0.5:1.0; 1.0:1.0; 2.0:1.0) corroborated results reported elsewhere (Stratil et al., 2007), revealing interference of 20.0 + 2.89% but only when the ratio ascorbate/gallate ⩾ one. In

Table 2
Reported total phenolic contents (TPC) in methanolic extracts of fruit residues.

Residues, entire fruits and pulps	TPC	References
Residues of apple	52.2 ± 4.80[a]	Peschel et al. (2006)
Residues of strawberry	59.8 ± 4.24[a]	Peschel et al. (2006)
Residues of pear	18.4 ± 2.12[a]	Peschel et al. (2006)
Residues of star fruit	32.2 ± 3.6[a]	Shui and Leong (2006)
Residues of winery	2.77 ± 0.23[a]	Lafka, Sinanoglou, and Lazos (2007)
Pineapple (total fruit)	2.58 ± 0.05[b]	Gorinstein et al. (1999)
Pineapple (total fruit)	40.4 ± 1.0[b]	Sun, Chu, Wu, and Liu (2002)
Pineapple pulp	21.7 ± 45[b]	Kuskoski, Asuero, Morales, and Fett (2006)
Acerola	896–1888[b]	Lima et al., 2005
Acerola pulp	580 ± 4.6[b]	Kuskoski, Asuero, Morales, and Fett (2006)
Passion fruit pulp	20.0 ± 2.6[b]	Kuskoski, Asuero, Morales, and Fett (2006)

[a] mg GAE g^{-1} dry extract.
[b] mg GAE 100 g^{-1} fresh dry total fruit.

addition, evaluation of ascorbate concentration in the fruit powder extracts is not reliable because it is well documented that it is both thermally unstable and easily oxidised under manipulation and storage.

Compared with TPC data reported for several fruits and vegetables, obtained under similar experimental conditions and using specific units, the acerola residues studied here present a value higher than those of pear, strawberry, star fruit, apple and red beet (Table 2). It is noteworthy that apple residues have already been applied in food and cosmetic industrial products (Peschel et al., 2006). In terms of results expressed as 100 g of total fruits, the passion fruit and pineapple residues have TPC values (Table 1) higher than those of the respective pulp (Kuskoski, Asuero, Morales, & Fett, 2006) and total fruit (Gorinstein et al., 1999; Sun, Chu, Wu, & Liu, 2002) (Table 2).

Acerola (total fruit) always had the highest TPC value (Lima et al., 2005), depending on the variety; however, it is clear from the comparison between Tables 1 and 2 (Kuskoski et al., 2006) that the results reported herein from fruit residues still contain a significant amount of "phenols".

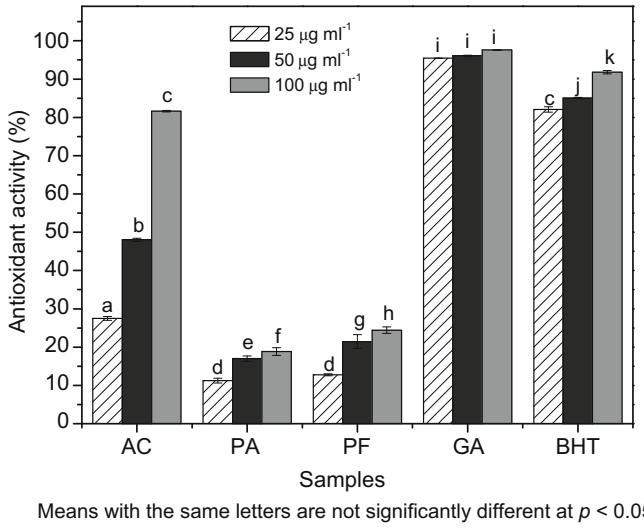

Fig. 1. Antioxidant capacities (%) of acerola (AC), pineapple (PA), and passion fruit (PF) powdered methanolic extracts, compared to gallic acid (GA) and butylated hydroxytoluene (BHT) solutions in terms of % DPPH• decomposition for 30 min. Hatched column: 25 μg l^{-1}; dark grey: 50 μg l^{-1} and grey: 100 μg l^{-1}. All experiments were performed in triplicate. Means with the same letters are not significantly different at $p < 0.05$.

3.3. Radical-scavenging activity towards DPPH• (*RSA- DPPH•*)

This assay allows comparison of the reactivities of powerful antioxidants such as BHT and gallic acid with those present in fruit methanolic extracts against DPPH•, a relatively stable radical species (actually one of the few stable and commercially available organic nitrogen-centred radicals). In addition, DPPH• has the advantage of being unaffected by certain side reactions of polyphenols, such as metal ion chelation and enzyme inhibition. A freshly prepared DPPH• solution displays a deep purple colour (λ_{max} = 516 nm) that gradually vanishes in the presence of a good hydrogen donor, i.e., a potent antioxidant. The DPPH•-scavenging activities (%) of acerola, pineapple and passion fruit extracts (at concentrations *c.a.* 25, 50 and 100 μg l^{-1}) were compared to those of gallic acid and BHT (Fig. 1).

Acerola extract clearly displays a dose-dependent antioxidant activity against DPPH• (Fig. 1), an antioxidant threshold value recorded at around 20–25% when higher doses (50 and 100 μg l^{-1}) of both pineapple and passion fruit were analysed. Gallic acid and BHT, as expected, were highly effective at all concentrations tested. Samples of acerola extracts presented higher antioxidant activity when compared with samples of pineapple and passion fruit, especially at higher concentrations. The acerola extract presented more promising results as an antioxidant, at least when evaluated for RSA-DPPH• and TPC, indicating that there is a possible correlation between these results.

Fig. 2 depicts the kinetics of DPPH• annihilation by samples of the fruit extracts and, for comparison, those of gallic acid and

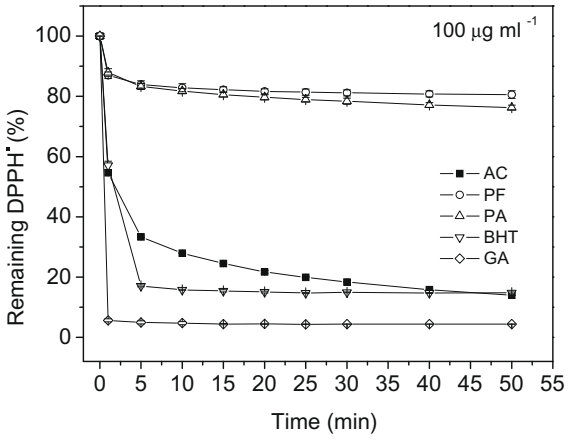

Fig. 2. RSA-DPPH• kinetic behaviour of methanolic extracts prepared from powdered tropical fruits residues (100 μg ml^{-1}). AC, acerola; PA, pineapple; PF, passion fruit; BHT, butylated hydroxytoluene; GA, gallic acid.

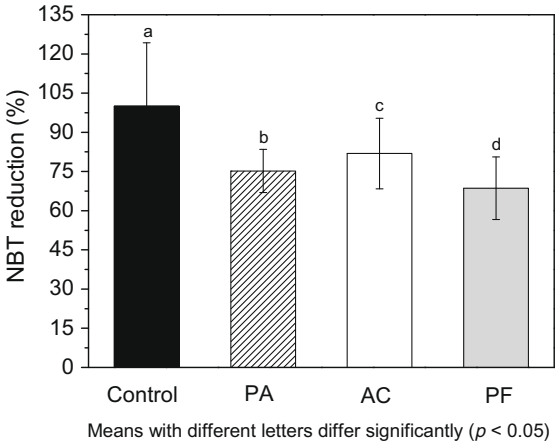

Fig. 3. Extent of NBT reduction (%) by the methanolic extracts of powdered tropical fruits residues, added to the superoxide-generated NADH/PMS/dioxygen system. AC, acerola; PA, pineapple and PF, passion fruit. $^{*}p < 0.05$.

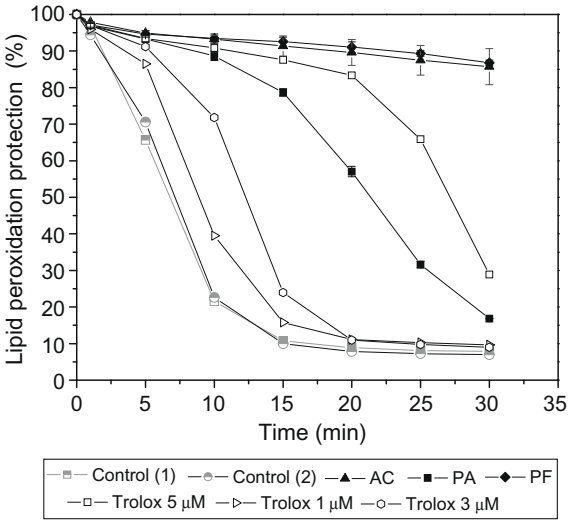

Fig. 4. Lipid peroxidation protection (%) afforded by AC, PA and PF extracts and a positive control (trolox) in several concentrations. (1, 3 and 5 μM). Negative controls (1 and 2) = PB (phosphate buffer) ± methanol. PB (phosphate buffer), liposome plus C_{11}-BODIPY$^{581/591}$ were added in all cases.

BHT controls (100 μg ml^{-1}). Clearly, the highest rate of DPPH$^{\bullet}$ decay occurs within the first 5 min of reaction (e.g., kinetics of gallic acid and BHT); albeit, fruit extract solutions maintained their antioxidant effect until the end of the experiment (50 min). Again, the efficiency of radical decomposition by acerola extract was markedly higher than those obtained with other fruits (pineapple and passion fruit), and closer to that observed for gallic acid and BHT.

3.4. Superoxide anion ($O_2^{\bullet-}$) radical-scavenging activity (RSA-$O_2^{\bullet-}$)

Overproduction of superoxide anion radical ($O_2^{\bullet-}$) has long been known as the starting point of ROS/RNS accumulation in cells, contributing to redox imbalance and associated harmful physiological consequences (Pervaiz & Clement, 2007). One simple, rapid and low-cost method for evaluating $O_2^{\bullet-}$-scavenging activity of extracts is based on the triad NADH/reduced phenazine methosulfate (PMS)/dioxygen as a source of $O_2^{\bullet-}$ radical. Reduction of nitro blue tetrazolium (NBT), as a $O_2^{\bullet-}$ scavenging compound, results in a stable formazan. Fig. 3 shows the extent of NBT reduction by the negative control (blank) and fruit extract samples added to the reaction mixture. The blank shows total NBT reduction due to absence of antioxidants in the solution. All extracts revealed modest scavenging activity of $O_2^{\bullet-}$ ($p < 0.05$), monitored as a significant inhibition of the NBT reduction index. In fact, the results of all residues might not be substantially different. It is well known that superoxide radical itself is not a "super" redox agent, but is a key upstream source of highly oxidising derivatives, such as hydroxyl radicals and reactive nitrogen species (Radi, Peluffo, Alvarez, Naviliat, & Cayota, 2001). Passion fruit extracts prevented, in a more effective manner, the reduction of NBT to formazan, whilst acerola was not significantly active (conflicting with results of TPC and RSA- DPPH$^{\bullet}$, already discussed). Perhaps the passion fruit antioxidant activity is afforded, not only by phenolic compounds, but also has important contributions from other $O_2^{\bullet-}$ radical scavengers, such as essential oils, carotenoids and vitamins (Moure et al., 2001).

3.5. Antioxidant activity of fruit extracts evaluated with a membrane biomimetic system

The antioxidant capacity of fruit residue extracts was evaluated in a peroxyl radical-mediated lipid peroxidation membrane model (soy lecithin unilamellar liposomes) loaded with the peroxyl radical-sensitive fluorescent probe C_{11}-BODIPY$^{581/591}$. Fig. 4 shows lipid peroxidation protection (%) *versus* time, using two negative controls: (1) liposome plus C_{11}-BODIPY$^{581/591}$ and AAPH (generator of peroxyl radical) in PBS, and (2) liposome plus C_{11}-BODIPY$^{581/591}$ and AAPH in phosphate buffer containing methanol. A positive control, namely trolox (a more water-soluble α-tocopherol derivative) was used at three concentrations (1, 3 and 5 μM) for comparison with the extracts obtained from acerola, passion fruit and pineapple.

Total AAPH-induced liposomal lipid peroxidation is observed in the absence of antioxidants (controls (1) and (2); Fig. 4), in both the presence and absence of methanol, attesting to innocuous interference of methanol in the process. A dose-dependent effect of trolox on the membrane oxidation confirms its well-known antioxidant ability (positive control). A significant retarding of lipid peroxidation – as indicated by the slight decrease in the fluorescence intensity of membrane-bound C_{11}-BODIPY$^{581/591}$ – was evident for both acerola and passion fruit extracts. In contrast, pineapple extracts exhibited a marked inhibition of liposome peroxidation over the same range (3 and 5 μM) as the trolox concentrations tested in this assay.

In order to infer a major contributing effect of the total phenolic content (TPC) to the total antioxidant activities of methanolic extracts of acerola, pineapple and passion fruit, linear correlation studies were performed with the three antioxidant assays (RSA-$O_2^{\bullet-}$, RSA-DPPH, and C_{11}-BODIPY$^{581/591}$ test) and total gallic acid equivalent were used in each assay. Such an approach was necessary since the RSA-DPPH assay was sketched with 3 different doses of each fruit methanolic extract (0.25, 0.5 and 1.0 mg ml^{-1}). No linear dependence was observed between RSA-$O_2^{\bullet-}$ scores and TPC, as revealed by a low correlation index ($R^2 = 0.278$; data not shown). As depicted in Fig. 4, the fluorescence decay at 600 nm from C_{11}-BODIPY$^{581/591}$ oxidation by AAPH obeys a sigmoid function. Thus, sigmoid curves were fitted for all C_{11}-BODIPY$^{581/591}$ kinetics and EC_{90} values were calculated. EC_{90} is here defined as the elapsed time necessary for 10% fluorescence decrease (at 600 nm) after AAPH addition in the reaction system. The application of EC_{90} values (rather than the traditional EC_{50}) was recommended since only slight fluorescence decreases at 600 nm were observed when either acerola or passion fruit extracts were added to the lipid system (Fig. 4). Nevertheless, no significant correlation was observed between TPC and the inhibition of lipid oxidation by the methano-

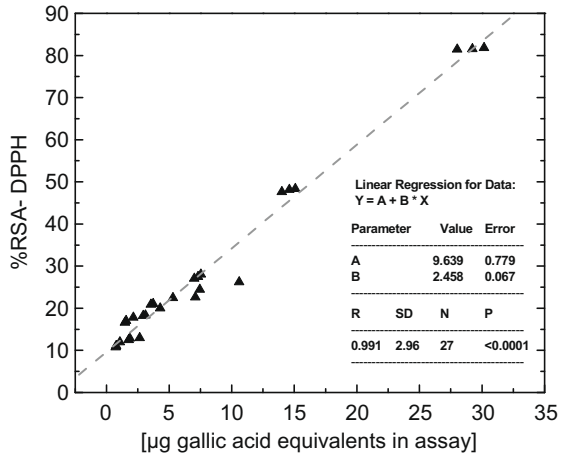

Fig. 5. Linear correlation between the percentage of DPPH radical-scavenging activity (%RSA-DPPH) and total gallic acid equivalent content in each assay of antioxidant activity ($n = 27$). Sources of gallic acid equivalent were not discriminated (acerola, pineapple or passion fruit methanolic extracts).

lic extracts ($R^2 = 0.478$; data not shown). However, a remarkable correlation of $R^2 = 0.991$ was calculated between TPC (expressed as μg of gallic acid equivalent in the assay) and the percentage of DPPH radical-scavenging activity (%RSA- DPPH), as shown in Fig. 5. It is well known that most antioxidant compounds display distinct scavenging ability in aqueous or hydrophobic milieus or even at lipid-water interfaces (Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005). Thus, it is not unusual to observe diverse antioxidant efficiencies for a specific antioxidant when tested in different analytical assays (Kim & Lee, 2004).

Polyphenols are abundant antioxidants present in fruits and vegetables, and attested to as major components of a synergistic-mode system involved in the inhibition of endogenous production of free radicals and progression of oxyradical-mediated degenerative pathologies in humans (Mattei, Barros, Galvão, Bechara, & Carlini, 2001). Synergistic activities observed between only synthetic, only natural and synthetic, and only natural antioxidants have been reported in recent decades (Moure et al., 2001). For example, synergistic increase of antioxidant capacity was observed between vitamin C and vitamin E, which is explained, in this case, as the reduction of tocopheryl radical in the membrane phase by ascorbate in the aqueous phase (Moison, Doerga, & Van Henegouwen, 2002). A similar protective mechanism was described for the pair ascorbate/quercetin acting on liposome-containing cytochrome *c* challenged with peroxides (Bandy & Bechara, 2001). In the present study, a significant and reproducible antioxidant contribution from ascorbate present in the fruits extracts was not expected since it is very unstable under processing and storage. On the other hand, Marinova, Toneva, and Yanishlieva (2008) studied the autoxidation of triacylglycerols of sunflower oil at 100 °C, and found a synergistic protective effect provided by α-tocopherol and myricetin. Conversely, when Heo, Kim, Chung, and Kim (2007) studied the antioxidant capacity of individual phenolics in different combinations contained in fruits and vegetables, such as catechin, quercetin 3-glucoside, chlorogenic acid and others, they found only a modest synergistic effect among the polyphenolics. The most salient finding of these studies is that the design of protocols for the *in vitro* evaluation of antioxidant activities of a single compound or mixture of antioxidants, reliable for interpreting biological data, must take into account their polarity, which will define their distribution in the cell compartments (Niki, 2000). Obviously several other factors affect the efficacy of antioxidants *in vivo*, among them local concentrations of pro- and antioxidants in the tissues, reduction potentials of the pro- and antioxidants involved, and the second order rate constants of the chemical reactions between them.

4. Conclusions

All methanolic extracts obtained from residues of fruit juice industries and studied here – acerola, passion fruit and pineapple - showed antioxidant capacity against different reactive oxygen species, although with different efficiencies. The high content of antioxidant phenolics found in these extracts, with special attention to those of acerola, suggests that powdered residues of these fruits may impart health benefits when used in functional food products. Due to the low cost of fruit residues, which otherwise would be discharged as waste in the environment, they should be regarded as potential nutraceutic resources, capable of offering significant low-cost, nutritional dietary supplements for low-income communities.

More research is required to establish the *in situ* activity, bioavailability and real beneficial effects of these natural antioxidants *in vivo*. Even apparently less efficient than BHT, natural extracts can be advantageous as food antioxidants and should interest food technologists. A detailed economic study, with reliable consideration of the potential toxicity of the present extracts, must be done before any possible application on a large scale in human populations.

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