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Antioxidant capacity of four polyphenol-rich Amazonian plant extracts: A correlation study using chemical and biological *in vitro* assays

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Abstract

Many plants used in Amazonian folk medicine present a high antioxidant activity. In this study, the antioxidant activities of four largely used plants, namely *Byrsonima crassifolia, Davilla kunthii*, *Davilla rugosa* and *Inga edulis*, were evaluated, using methanolic extracts of their leaves, fruits and bark and several different *in vitro* tests, based either on the capacity to scavenge free radicals (ORAC, TRAP) or on the ability to protect biological structures (LDLs, erythrocytes). The total phenolics (TP), flavanoids (TFA) and flavonols (TFO) were also measured. Almost all extracts performed well in all assays of antioxidative capacity, with best activities found in leaves (compared to fruits and bark). Most antioxidative performance indicators (ORAC, TRAP, LDL protection) correlated well with the TP and TFA content of the extracts. Conversely, correlation was lower between TFO and these indicators, reflecting a lower involvement of these compounds in antioxidant processes. Erythrocyte protection against oxidant-triggered haemolysis showed no correlation with any of the phenolic content indicators, suggesting that most of these compounds have a low ability to protect lipid targets in the erythrocyte membrane. On the other hand, protection of erythrocytes against haemolysis correlated positively with LDL protection. The extract of *I. edulis* leaves contained average amounts of polyphenols but ranked first in the majority of the tests, indicating the occurrence of particularly efficient compounds with very important antioxidant properties, which could be used for medicinal and other applications.

Keywords: Antioxidant properties; *In vitro* tests; Phenolics; Flavonoids; Amazonian plants

1. Introduction

The antioxidant activity of fruits and vegetables is mainly correlated with their contents of polyphenols, carotenoids and vitamins C and E (Benzie, 2003; Cadenas & Packer, 2002). Among these antioxidants, polyphenols constitute a large and complex category of compounds. They include flavonoids, the largest and most-studied group of polyphenols. These low molecular weight polyphenols are built upon a C_6–C_3–C_6 flavone skeleton in which the three-carbon bridge between the phenyl groups is commonly cyclized with oxygen. Several classes are differentiated according to the degrees of unsaturation and oxidation of this three-carbon segment (Rice-Evans, Miller, & Paganga, 1996; Robards & Antolovich, 1997).

Interest in flavonoid antioxidants has increased considerably because of their elevated capacity for scavenging free radicals associated with various diseases. This property has been evidenced by a large number of *in vitro* tests (Cao

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& Prior, 1998; Nijveldt et al., 2001). Notably, some studies have reported that flavonoids inhibit lipid peroxidation and LDL oxidation (Sanchez-Moreno, Jimenez-Escrig, & Saura-Calixto, 2000; Sang et al., 2003). Moreover, *in vitro* experimental data also suggest that flavonoids possess anti-inflammatory, anti-allergic, anti-viral and anti-carcinogenic properties (Nijveldt et al., 2001). Epidemiological studies also indicate a protective role of dietary flavonoids against cardiovascular diseases. In fact, some association between flavonoid intake and a reduced mortality by coronary heart disease has been demonstrated (Hertog et al., 1995; Knekt, Jarvinen, Reunanen, & Maatela, 1996). These biological properties are thought to be linked to the antioxidant activity of these compounds (Cadenas & Packer, 2002).

The extraction of phenolic compounds in plant material is influenced by their chemical nature, the employed extraction method, sample particle size, storage time and conditions, as well as the presence of interfering substances (Prior & Cao, 1999). Phenolic extracts of plant materials are always a mixture of different classes of phenolics that are soluble in the used solvent system. The use of a hydroalcoholic solution appears to offer satisfactory results for this process (Chirinos, Rogez, Campos, Pedreschi, & Larondelle, 2007; Perva-Uzunalic et al., 2006; Silva, Rogez, & Larondelle, 2007a).

Brazil, and specifically the Amazonian Region has a great variety of exotic food and medicinal plants with antioxidant activity, whose characterisation could present some potential for sustainable development in Amazonia. The aim of this study was to evaluate the *in vitro* antioxidant properties of four Amazonian plants (*Byrsonima crassifolia* H. B. & K., *Davilla kunthii* St. Hil., *Davilla rugosa* Poir. and *Inga edulis* Mart) used in folk medicine, and to link these properties with their polyphenol and flavonoid contents. *B. crassifolia* is a tropical tree of the Malpighiaceae family distributed in tropical America. The leaves and barks are used in folk medicine to treat coughs, skin fungal diseases, diarrhoea, indigestion and snake bites (Bejar, Amarquaye, Che, Malone, & Fong, 1995; Bejar & Malone, 1993; Martinez-Vazquez, Gonzalez-Esquinca, Luna, Gutierrez, & Garcia-Argaez, 1999). *D. kunthii* and *D. rugosa* are members of a predominantly woody plant family present in all tropical regions, traditionally prescribed in cases of inflammation and ulcer (Silva, Souza, Rogez, Rees, & Larondelle, 2007b). Hydroalcoholic extracts of *D. rugosa* are known to display an anti-ulcer activity in rats (Guaraldo, Sertie, & Bacchi, 2001). *I. edulis* is a tree native to the tropical rain forest of America. Its leaf extracts have been used in folk medicine as an anti-inflammatory and anti-diarrheic product (Silva et al., 2007b). In a previous study, Silva et al. (2007b) observed that extracts of leaves and barks of these four plants, among a wide variety of other Amazonian plants, present particularly high antioxidant activities and phenolic contents. The present work further investigates their antioxidant activity through the use of several *in vitro* assay systems.

2. Materials and methods

2.1. Plant materials and chemicals

B. crassifolia (Malpighiaceae) (leaf, bark and fruit), *D. kunthii* (Dilleniaceae) (leaf and bark), *D. rugosa* (Dilleniaceae) (leaf and bark) and *I. edulis* (Leguminosae) (leaf, bark and fruit) were taxonomically identified by a pharmacognosy expert of the Botanical Museum Emilio Goeldi (Belém, Brazil) through comparison with witness specimens deposed in this herbarium (MG 130939, MG 122249, MG 103777 and 0153192, respectively). The parts of the plants to be studied (leaf, bark and fruit) were collected in two different villages (Igarapé-açu and Bonito) of the State of Pará (Brazil). The plant samples were carried to the laboratory within a maximum of 12 h after harvest. The plants were cut into small pieces, lyophilized for 48 h, and stored at −20 °C under a N_2 atmosphere.

Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO).

2.2. Extraction of the phenolic compounds

The extraction procedure for phenolic compounds was based on Perva-Uzunalic et al. (2006) and Silva et al. (2007a). Lyophilised and triturated samples (1 g) were diluted in 20 ml of methanol–water solution (80:20 v/v). The solution was agitated with a vortex and left for 3 h in the dark at room temperature. The suspensions were centrifuged at 27,000*g* for 15 min at 4 °C and the supernatants were removed and concentrated in a rotary evaporator under vacuum. Ultrapure water (1 ml) was added to the crude extracts and the solutions were conserved in dark glass bottles at −20 °C and under a N_2 atmosphere. These samples were shipped in dry ice to the Université catholique de Louvain (Belgium) and stored at −20 °C prior to analysis.

2.3. Preparation of human erythrocytes

EDTA-treated tubes (3 ml) were used to collect human blood in the morning from 15 fasting patients of the Saint-Pierre Hospital (Ottignies, Belgium). The tubes were centrifuged at 1000*g* for 10 min in order to separate erythrocytes from plasma. The buffy coats were washed three times with phosphate buffered saline (PBS) at pH 7.4, centrifuged at 1000*g* for 5 min at 4 °C and resuspended in 1 ml PBS solution containing 10 mM glucose (Zhang et al., 1997). The suspensions were then pooled, kept at 4 °C and used within 48 h for *in vitro* studies of anti-haemolysis activity.

2.4. Isolation of human LDLs

Human serum (~200 ml/isolation) was provided by the André Vésale Hospital (Montigny-le-Tilleul, Belgium). LDLs were isolated by the discontinuous density gradient centrifugation procedure adapted from Redgrave, Roberts,

and West (1975). The density of the serum was adjusted at 1.25 g/ml with KBr before centrifugation at 200,000*g* for 16 h at 7 °C in quick-seal tubes. The isolated lipoprotein fraction was adjusted to a density of 1.27 g/ml with KBr and centrifuged at 200,000*g* for 3 h at 7 °C. The isolated LDL fraction was then dialyzed against a buffered solution (8.77 g/l NaCl and 2.21 g/l tris[hydroxymethyl]aminomethane in ultrapure water, pH 7.2–7.4) with sodium EDTA (0.1 g/l). EDTA protected LDLs against oxidation and was removed by a 150 min dialysis against EDTA-free PBS before the oxidation experiments. The LDL solution can be rather unstable at this stage, and therefore the time between desalting and the final oxidation experiment did not exceed 6 h. The protein concentration in the LDL fraction was determined by the BCA protein assay, using bovine serum albumin (BSA) as a standard (Smith et al., 1985).

2.5. Determination of total phenolics, total flavanoids and total flavonols

The total phenolic content (TP) was determined by the Folin–Ciocalteu colorimetric method (Singleton, Orthofer, & Lamuela-Raventos, 1999). Results were expressed as mg of gallic acid equivalents (GAE) per g of dry matter (DM).

The total flavanoids (TFA) were estimated using the chromogen, *p*-dimethylaminocinnamaldehyde (DMACA), following the protocol proposed by Delcour and Devarebeke (1985). The results were expressed as mg of catechin equivalents (CE) per g of DM.

The quantification of flavonols (TFO) was done by means of the aluminium chloride (Merck, Darmstadt, Germany) colorimetric method (Chang, Yang, Wen, & Chern, 2002). Results were expressed as mg of quercetin equivalents (QE) per g of DM.

2.6. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed as described by Silva et al. (2007b). It was conducted on microplates using fluorescein as the fluorescent probe. All extracts were set to a standardized concentration of 20 μM on a GAE basis. The antioxidant activity by ORAC was expressed as μmol of trolox equivalents (TE) per g of DM or as TE per mg of GAE.

2.7. Total radical-trapping antioxidant potential (TRAP) assay

The method was adapted from Krasowska et al. (2001) and Leontowicz et al. (2002). In this protocol, the peroxyl radicals produced at a constant rate by thermal decomposition of AAPH (2,2′-azobis(2-methylpropionamidine) dihydrochloride) induce the chemiluminescence of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione). The presence of antioxidants competing for the peroxyl radicals delays the onset of the luminescence. The AAPH solution (133 mM) was prepared in 100 mM phosphate buffer (pH 7.4). Luminol was solubilised (70.8 g/l) in 1 M NaOH. Before analysis, this stock solution was diluted 60 times in 50 mM borate buffer (pH 9.5). All extracts were set to a standardized concentration of 20 μM on a GAE basis. In microplates, trolox standards or plant extracts (50 μl) were added to 50 μl phosphate buffer (100 mM; pH 7.4) and the mixtures were allowed to incubate at 37 °C for 5 min in the microplate luminometer (EG&G Berthold LB96P, Bad-Wildbach, Germany) before automatic injection of 50 μl luminol and 50 μl AAPH. The chemiluminescence signal was measured every minute over a 180 min period. Tests were made in triplicate and the lag-times induced by the extracts were expressed as μmol of TE per g of DM and TE per mg of GAE.

2.8. Determination of anti-haemolysis activity

A method adapted from Zhang et al. (1997) and Zhu et al. (2002) was used to evaluate the improved resistance conferred by the extracts to erythrocytes against the free radical attacks induced by AAPH. The erythrocyte suspension (100 μl) in PBS ($Abs_{534\,nm} \sim 2$ UAcm^{-1}) was added to 50 μl of extract or standard (quercetin, catechin, trolox) in a transparent 96-well microplate (Costar, Corining, NY). After 5 min of incubation at 37 °C in a thermoregulated microplate spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA), 50 μl AAPH (100 mM in a 100 mM phosphate buffer, pH 7.4) were added to each well and the absorbance of the erythrocyte suspension was followed at 534 nm for 60 min. The protection of erythrocytes by the extracts was deduced from the time required for half-haemolysis (50% reduction of $A_{534\,nm}$) compared to control values (100 mM phosphate buffer, pH 7.4).

2.9. Protection of LDLs against oxidation

2.9.1. Copper-induced oxidation followed by conjugated dienes assay

The method used was adapted from Stulnig et al. (1996) and Proudfoot et al. (1997). EDTA-free LDLs, suspended in PBS (170 μl, pH 7.4) at a concentration of 100 μg protein/ml, were mixed with 5 μl of extracts or standards in a UV-transparent 96-well microplate maintained at 37 °C in a spectrophotometer. Oxidation was initiated by the addition of 25 μl $CuSO_4$ (final concentration 10 μM) and monitored by the production of conjugated dienes at 234 nm over a 5 h time period. Protection of the LDLs was measured by the delay in the onset of the surge in conjugated dienes level. This onset is determined as the intercept of the slope during the propagation phase with the time in abscissa. All extracts were tested at a standardized concentration of 2.5 μM GAE.

2.9.2. AAPH-induced oxidation followed by thiobarbituric acid reactive substances (TBARS) assay

The peroxidation of LDLs was induced by AAPH and assessed by measuring the levels of TBARS, which mainly

correspond to malondialdehyde (MDA), produced by the peroxidation process. The isolated LDLs were suspended in PBS (pH 7.4) at a concentration of 200 μ g protein/ml. All extracts were set to a standardized concentration of 2.5 μM of GAE. The method described by Yagi (1984) was modified for microplate measurements. Briefly, 20 μl of LDLs was incubated with 5 μl of extracts and 5 μl of AAPH (final concentration 20 mM) in wells of a white 96-well microplate. The microplate was incubated at 37 °C for 30 min before adding 24 μl trichloroacetic acid (15%) and 48 μl thiobarbituric acid (0.67%). The microplate was covered with a Teflon lid and the solution was heated at 95 °C for 30 min. After cooling on ice, 100 μl butanol were added and the plate centrifuged at 200*g* for 5 min (4 °C). The fluorescence of the supernatant was measured at 515 nm ($\lambda_{excitation}$) and 555 nm ($\lambda_{emission}$) on a Fluoroskan Ascent (Labsystems, Helsinki, Finland). MDA, submitted to the same conditions, was used as a standard. The results were expressed as nmol MDA per mg of LDL protein.

2.10. Statistical analysis

Variables were expressed as means ± SD. Statistical significances were determined using an independent samples *t*-test. Results were considered as significant for *p*-values lower than 0.05. Correlation analyses were performed using linear regression and the Pearson's correlation coefficient (*r*). These analyses were conducted using the Excel 2000 SR-1 program (Microsoft, Troy, NY) and Statistica Kernel Release 7.1 (StartSoft Inc., Tulsa, OK) for Windows.

3. Results and discussion

3.1. Phenolic, flavanoid and flavonol contents

Table 1 presents the TP, TFA and TFO contents and polymerization degree of the different parts of the four plants that have been investigated. The leaves and bark had high TP (26–120 mg GAE/g DM) and TFA (4–16 mg CE/g DM) contents, confirming the previous report (Silva et al., 2007b). Data on the polyphenolic profile of these plants are scarce in the literature. Only *B. crassifolia* has so far been investigated (Bejar et al., 1995). Six out of 22 compounds in methanolic extracts were identified as flavonoids (catechin, epicatechin, guaijaverin, hyperin, quercetin and its 3-*O*-[6″-galloyl]galactoside). TFO were not detected in bark and fruits of *I. edulis*. As expected from their roles as UV-B filters protecting the plant organs (Harborne & Williams, 2000), TP were found in higher concentrations in leaves and barks than in fruits. The TP content of fruits in *I. edulis* (2.9 ± 0.2 mg GAE/g DM) and *B. crassifolia* (2.4 ± 0.7 mg GAE/g DM) appear low if compared with other fruits, such as *Rubus* sp. (13.5 mg GAE/g DM) and *Fragaria ananassa* (10.3 mg/g DM) (Wang & Lin, 2000). TP contents of leaves (44–63 mg GAE/g DM) are in the same range as those of *Rubus* sp. (82.3 mg GAE/g DM) and *F. ananassa* (55.2 mg GAE/g DM) (Wang & Lin, 2000), while the level of TP in barks of the four species are rather different (26–120 mg GAE/g DM).

As shown in Table 1, TFA and TFO represented only 18.3% (±7.2) and 5.4% (±4.9) of the TP, respectively, suggesting either that the extracts are very complex, and contain many other polyphenols, such as flavanones, isoflavones, phenolic acids or tannins, or that the degree of polymerization of the polyphenols present in the samples is high. This polymerization degree can be estimated by the ratio between the TP and TFA contents (Table 1). In general, the barks present higher values than do the leaves and the fruits, probably because barks are normally richer in tannins and procyanidins.

3.2. Antioxidant activity of the extracts

The results of the determination of the antioxidant capacity of an extract depend greatly on the methodology

Table 1
Concentrations of total phenolics (TP), flavanoids (TFA) and flavonols (TFO) in methanolic extracts of leaves, bark and fruits of four Amazonian plants[A]

Plant		TP (mg GAE/g DM)	TFA (mg CE/g DM)	TFO (mg QE/g DM)	Polymerization degree[B]
Species	Part				
B. crassifolia	Leaf	58.1 ± 14.9[a]	9.4 ± 3.1[a]	2.1 ± 0.3[a]	6.18
	Bark	121 ± 19.2[b]	16.2 ± 4.3[b]	0.1 ± 0.0[b]	7.44
	Fruit	2.9 ± 0.2[c]	0.2 ± 0.0[c]	0.4 ± 0.0[c]	14.50
D. kunthii	Leaf	63.0 ± 10.5[d,a]	12.3 ± 1.0[b,a]	3.3 ± 1.3[d,a]	5.12
	Bark	26.4 ± 1.8[e]	4.1 ± 0.9[d]	0.5 ± 0.0[e]	6.44
D. rugosa	Leaf	49.5 ± 3.6[a]	12.4 ± 0.3[b,a]	3.7 ± 0.4[d]	3.99
	Bark	55.4 ± 14.5[d,a]	6.4 ± 1.5[a]	0.2 ± 0.0[a]	8.66
I. edulis	Leaf	44.2 ± 18.3[d,a]	10.5 ± 4.7[b,a]	4.5 ± 1.4[d]	4.21
	Bark	48.3 ± 1.8[a]	10.1 ± 1.9[a]	0.0[b]	4.78
	Fruit	2.4 ± 0.1[f]	0.7 ± 0.1[e]	0.0[b]	3.43

[A] Results are means ± SD of four replicates: GAE, CE and QE: gallic acid, cathechin and quercetin equivalents, respectively. Values marked by the same letter within a column are not significantly different ($P > 0.05$).
[B] The estimation of the polymerization degree is calculated by the ratio between TP and TFA.

used, that is the oxidant and the oxidisable substrate used in the measurement. Therefore, it is important to compare different analytical methods varying in their oxidation initiators and targets in order to understand the biological activity of an antioxidant (Cao & Prior, 1998). The present study relied on a variety of *in vitro* tests, based either on the capacity to scavenge free radicals (ORAC, TRAP) or on the ability to protect biological structures (LDLs, erythrocytes).

The ORAC and TRAP assays use the same peroxyl radical generator (AAPH), but different substrates and reaction mechanisms and may thus be considered as complementary. The results obtained for both assays are summarized in Table 2. In order to allow a comparison between the two assays, ORAC and TRAP values were calculated against a standard curve constructed with trolox and expressed both as TE/g DM and TE/mg GAE. The latter expression gives a better idea of the antioxidative capacity of the polyphenols present. The ORAC values differed markedly among the extracts when expressed as a function of DM, ranging from 23.4 to 1502 μmol TE/g DM. With the exception of *B. crassifolia*, leaves presented higher ORAC values than did barks or fruits. However, when ORAC values were expressed relatively to the phenolic contents, the antioxidant activities tended to be similar (~15 μmol TE/mg GAE) among the various species and parts, except for the leaves of *I. edulis* and *D. rugosa*, which presented higher values (~ 30–35 μmol TE/mg GAE). In the literature, ORAC values obtained for fruit extracts were: avocado (52.6 μmol TE/g DM), cashew (12.1 μmol TE/g DM) and strawberry (140 μmol TE/g DM) (Wang & Lin, 2000; Wu et al., 2004). The two fruit extracts analysed in the present study thus compare well with other anthocyanin-free fruits, which are recognized for possessing high ORAC values. On the other hand, the ORAC values determined in the present study for leaf extracts (736–1462 μmol TE/g DM) are higher than those reported for iceberg lettuce (267 μmol TE/g DM) (Wu et al., 2004) and *F. ananassa* (527 μmol TE/g DM) (Wang & Lin, 2000) but lower than that of black tea (2871 μmol TE/g DM) (Senthilmohan, Zhang, & Stanley, 2003). As for the barks, the ORAC values of the present study (381–1145 μmol TE/g DM) are lower than those reported for a proanthocyanidin-rich concentrated extract of *Pinus radiata* (4698 μmol TE/g DM) (Senthilmohan et al., 2003).

Like the ORAC values, TRAP values also varied widely, when expressed on a DM basis, ranging from 4.0 to 1621 μmol TE/g DM. That variability could again be lowered upon expression in terms of μmol TE/mg GAE (3.0–27.3). Leaves tended to present higher TRAP values than did barks, except for the bark of *B. crassifolia*, which reached the highest values of all extracts. As with ORAC, low antioxidant activities were measured in the fruit extracts.

To evaluate the ability of the extracts to prevent oxidative damage to human blood constituents, three *in vitro* methods were utilised. Two of them evaluated the protection conferred by extracts to LDLs subjected to oxidative damage, whereas the third one analysed their ability to delay oxidant-induced haemolysis of erythrocytes. Figs. 1–3 present the results obtained for these tests. The extracts were used at the concentration of 2.5 μM of gallic acid equivalents. To allow a comparison with reference compounds, this concentration was also used for catechin, quercetin and trolox standards.

All extracts induced a significant delay in the onset of the rise in conjugated dienes after exposure of LDLs to copper (Fig. 1). With the exception of *B. crassifolia*, leaf extracts were the most efficient inhibitors of LDL oxidation with latencies increasing from 70 min (control) to 250–300 min. Among reference antioxidants, quercetin presented a shorter lag time than did catechin or trolox. The chain-breaking capacity of extracts was confirmed by the analysis of TBARS on AAPH-induced peroxidation (Fig. 2). Here also, leaf extracts were particularly powerful as TBARS levels were reduced from 39.8 to 9.7–16.9 nmol

Table 2
Oxygen radical absorbance capacity (ORAC) and total radical-trapping antioxidant potential (TRAP) values of methanolic extracts of leaves, bark and fruits of four Amazonian plants[A]

Plant		ORAC		TRAP	
Species	Part	μmol TE/g DM	μmol TE/mg GAE	μmol TE/g DM	μmol TE/mg GAE
B. crassifolia	Leaf	736 ± 99.1^{a}	16.6 ± 3.1^{a}	288 ± 10.8^{a}	6.7 ± 2.4^{a}
	Bark	1145 ± 99.0^{b}	$13.9 \pm 4.9^{b,a}$	1621 ± 70.6^{b}	27.3 ± 0.3^{b}
	Fruit	26.5 ± 3.2^{c}	$13.3 \pm 7.8^{b,a}$	6.4 ± 1.5^{c}	3.6 ± 0.8^{c}
D. kunthii	Leaf	$1007 \pm 166.0^{d,b}$	15.1 ± 3.4^{a}	620 ± 25.9^{d}	$9.2 \pm 0.4^{d,a}$
	Bark	$840 \pm 57.6^{e,d}$	17.0 ± 4.2^{a}	154 ± 25.8^{e}	3.0 ± 0.5^{c}
D. rugosa	Leaf	1502 ± 323.3^{b}	29.9 ± 1.9^{c}	339 ± 1.8^{f}	6.7 ± 0.1^{a}
	Bark	381 ± 116.3^{f}	9.6 ± 2.1^{b}	112 ± 7.14^{g}	3.0 ± 1.0^{c}
I. edulis	Leaf	$1463 \pm 321.9^{b,d}$	34.9 ± 6.2^{c}	652 ± 74.5^{d}	16.2 ± 6.3^{d}
	Bark	462 ± 78.2^{f}	14.7 ± 0.5^{a}	112 ± 9.5^{g}	3.7 ± 0.6^{c}
	Fruit	23.4 ± 1.8^{c}	17.0 ± 3.0^{a}	4.0 ± 0.9^{h}	2.4 ± 0.3^{c}

[A]Results are means ± SD of six replicates. TE, Trolox equivalent; GAE, gallic acid equivalents. Values marked by the same letter within a column are not significantly different ($P > 0.05$).

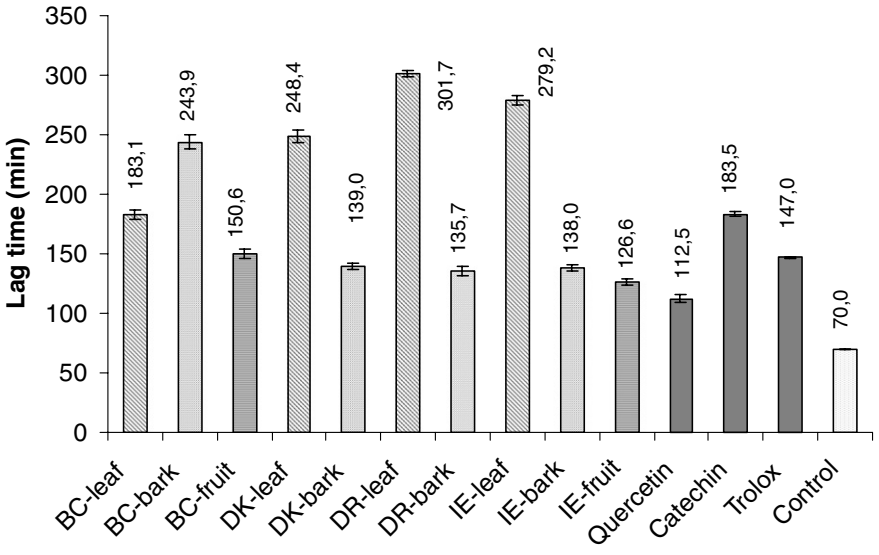

Fig. 1. Protective effect conferred by plant extracts and standards to human LDLs subjected to copper-induced oxidation. The protection was quantified by the increase in the lag time (min) before the rise in conjugated dienes (mean ± SD: $n = 6$). The extracts were tested at 2.5 μM GAE and the standards at 2.5 μM. BC = *B. crassifolia*, DK = *D. kunthii*, DR = *D. rugosa* and IE = *I. edulis*.

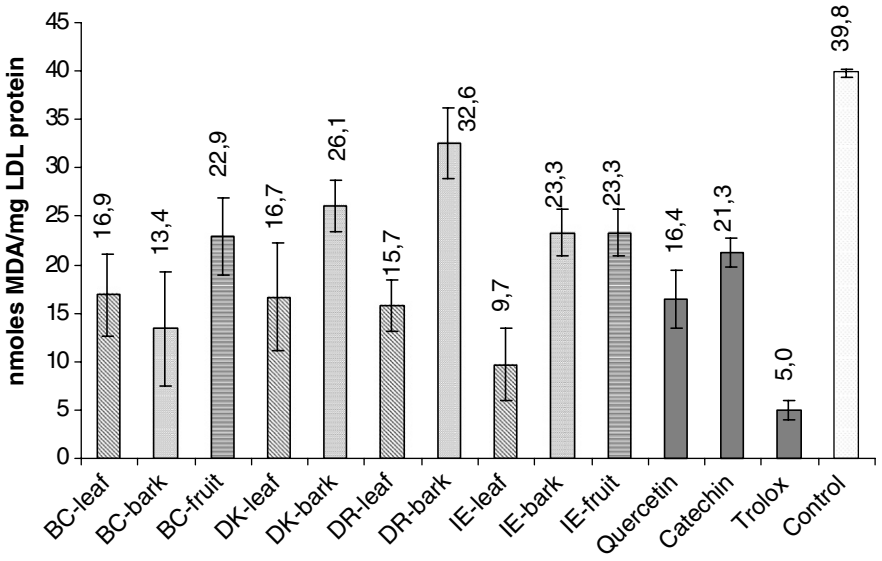

Fig. 2. Protective effect conferred by plant extracts and standards to human LDLs subjected to AAPH-induced oxidation. The protection was quantified by the amount of TBARS produced by the oxidation (mean ± SD: $n = 6$). The extracts were tested at 2.5 μM GAE and the standards at 2.5 μM. BC = *B. crassifolia*, DK = *D. kunthii*, DR = *D. rugosa*, IE = *I. edulis* and MDA = malondialdehyde.

MDA/mg of LDL protein. The most effective extracts were those from *I. edulis* leaves and *B. crassifolia* bark. Interestingly enough, trolox proved superior to all other compounds and extracts, whereas it had not shown a higher efficiency against Cu-induced LDL oxidation. This further stresses the importance of the oxidation inductor in influencing the apparent efficiency of an antioxidant.

As compared to reference polyphenols, the extracts showed a rather limited ability to delay the haemolysis of erythrocytes subjected to AAPH (Fig. 3). The best protection was observed for the *I. edulis* leaf extract, which induced a 32% increase of the lag time, while extracts from *I. edulis* and *B. crassifolia* fruits increased this lag time by 18% and 22%, respectively. Neither the *Davilla* extracts, nor the *B. crassifolia* leaf extract retarded the haemolytic process. Quercetin and catechin were more efficient than all extracts, delaying the haemolysis by 57% and 53%, respectively, whereas trolox did not provide significant protection. These results greatly contrast with those obtained with LDLs. Discrepancies between the protections conferred by the tested material in these assays likely result from differences in their physicochemical properties, which determine their interaction with the different targets used in the different assays. As an example, the liposolubility of

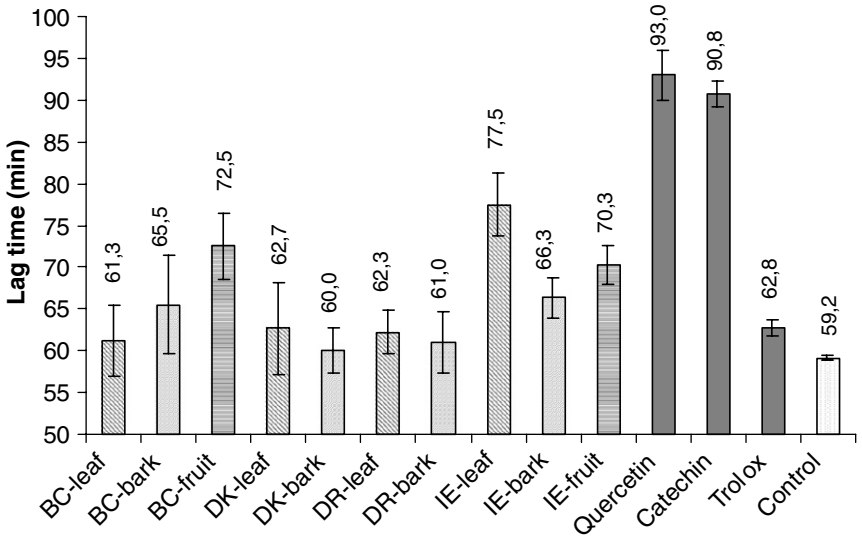

Fig. 3. Protective effect conferred by plant extracts and standards to human erythrocytes subjected to AAPH treatment. The lag-time for the onset of the haemolysis was measured. The protection was quantified by increase in the lag time (min) (mean ± SD; $n = 6$). The extracts were tested at 2.5 μM GAE and the standards at 2.5 μM. BC = *B. crassifolia*, DK = *D. kunthii*, DR = *D. rugosa* and IE = *Inga edulis*.

antioxidants is important in allowing their diffusion into lipoproteins or cell membranes. In that respect, glycosylated flavonoids, which may be abundant in some of the extracts, have been reported to show a quite low diffusibility into erythrocytes (Kitagawa, Sakamoto, & Tano, 2004). On the other hand, the efficiency of the extracts depends on the free radical-scavenging properties of the compounds present (Edenharder & Grunhage, 2003).

3.3. Correlations between antioxidant properties and polyphenols

Correlations were tested to link the antioxidative capacities measured by the different assays used with each other, as well as with the polyphenolic contents of the extracts. Table 3 summarizes the Pearson's correlation coefficients between all analyses carried out on the extracts. ORAC and TRAP values correlated very well with TP and TFA values. This is in agreement with other studies. Wang and Lin (2000) also found a good correlation between ORAC values and the TP contents of *Vaccinium* sp., blackberry and raspberry fruits and leaves. Similarly, Leontowicz et al. (2002) reported a very good correlation between TRAP and TP values in fruit extracts. Although good correlations between ORAC and TRAP values were found, ORAC values positively correlated with TFO whereas TRAP values did not. The protection of LDLs against an oxidation triggered either by AAPH (TBARS) or copper (conjugated dienes) correlated well with TP, TFA and TFO. In the literature, contradictory reports exist on the correlation between flavonoid content and LDL protection. Meyer, Heinonen, and Frankel (1998) observed a correlation between the level of flavonoids in grapes and the inhibition of LDL oxidation, while another study on fruit juices failed to link these parameters (Heinonen, Lehtonen, & Hopia, 1998). Our study confirms that some correlation exists.

Curiously enough, erythrocyte protection did not correlate with any of the phenolic contents, nor with TRAP or ORAC, but did well with the two LDL protection indicators, suggesting that they rely, at least in part, on similar antioxidant effects and impose similar lipid solubility constraints, as already reported by other authors (Simao et al., 2006). ORAC, TRAP and erythrocyte haemolysis

Table 3
Pearson's correlation coefficients (r) between the analysis parameters ($n = 20$)

	TP	TFA	TFO	ORAC	TRAP	LDL-Cu^{2+}	LDL-AAPH
TFA	0.907[b]	1					
TFO	−0.084	0.382	1				
ORAC	0.627[b]	0.787[b]	0.518[a]	1			
TRAP	0.802[b]	0.678[b]	−0.216	0.568[b]	1		
LDL-Cu^{2+}	0.524[b]	0.740[b]	0.734[b]	0.820[b]	0.501[b]	1	
LDL-AAPH	0.406[b]	0.614[b]	0.633[b]	0.762[b]	0.537[b]	0.811[b]	1
Haemolysis	−0.30	0.040	0.295	0.243	0.119	0.382[a]	0.431[a]

[a] Correlation is significant at the 0.05 level (1-tailed).
[b] Correlation is significant at the 0.01 level (1-tailed).

are all induced by AAPH but the mechanism of protection by antioxidants is different in the latter assay. In fact, in the ORAC and TRAP assays, respectively, fluorescein or luminol are water-soluble and are protected by many water-soluble compounds, while the erythrocyte haemolysis is a process involving both water- and lipid-soluble azo compounds.

4. Conclusions

The results of this study show that the four Amazonian plants contain large amounts of polyphenols, and can be considered as good sources of these compounds for medicinal and food applications. Polyphenol contents and antioxidant activities, measured by several methods, suggest that leaves are better sources of polyphenols with antioxidant properties, than are bark and fruits. Among leaves, those of *I. edulis* appear as the most promising source of powerful antioxidants. Although it contains average amounts of polyphenols, the *I. edulis* leaf extract ranked first in all but one of the assays of antioxidative activity. Further studies will attempt to identify the flavonoids responsible for these properties.

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