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Improved loquat (*Eriobotrya japonica* Lindl.) cultivars: Variation of phenolics and antioxidative potential

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ABSTRACT

Leaves and fruits (peel and flesh) of six improved cultivars ('Mizuho', 'Néctar de Cristal', 'Mizauto', 'Mizumo', 'Centenária' and 'NE-3') of loquat (*Eriobotrya japonica* Lindl.) were studied for their phenolic composition and antioxidant capacity. The analysis by HPLC-DAD-ESI-MS/MS allowed the identification of 18 compounds (8 hydroxycinnamic acid derivatives and 10 flavonoid glycosides). The quantification of the identified compounds revealed distinct profiles amongst the three analysed materials. Loquat leaves exhibited the lowest amounts of phenolics. 3- And 5-caffeoylquinic, and 5-feruloylquinic acids were the major compounds. Generally, 'Mizauto' cultivar presented the highest phenolic content. All loquat materials exhibited DDPH scavenging capacity, in a concentration-dependent manner, the leaves being the most active one. This effect seems to be related to the flavonoid content.

1. Introduction

Loquat (*Eriobotrya japonica* Lindl.) belongs to the Rosaceae family, but is one of the few subtropical representatives. It probably had its origin in south-eastern China and has been cultivated in China and Japan since ancient times. Now it is also cultivated in other regions, namely in the Mediterranean area, Australia, South Africa, South America, California and India. The plant is an evergreen shrub or small tree, with narrow leaves, dark green on the upper surface, and a lighter woolly under surface. Its white flowers give rise to pale-yellow or deep-orange pomes (Vaughan & Geissler, 1997). The principal objective of the economic exploration of loquat is the production of its fruits, which can be eaten fresh or processed into jam and jelly, although its leaves have been used for treatment of skin diseases and diabetes (De Tommasi, Aquino, De Simona, & Pizza, 1992), chronic bronchitis, coughs, phlegm, ulcers and cancer (Ito et al., 2000).

Phenolic compounds are secondary metabolites, ubiquitous in the plant kingdom and impacting on human health, decreasing the risk of several diseases by reducing oxidative phenomena (Peluso, 2006; Pulido, Bravo, & Saura-Calixto, 2000). They are important in fruits and vegetables, to which they contribute colour and flavour, also being involved in astringent and bitter tastes. It is known that, amongst other factors like maturity stage or light exposure, phenolic composition varies with cultivar (Macheix, Fleuriet, & Billot, 1990). In addition, the phenolic profile has already been revealed to be a useful parameter for the discrimination of the distinct fruit parts (Silva et al., 2002, 2005).

Previous works with loquat allowed the identification of some phenolics in its leaves (Ito et al., 2000; Jung, Park, Chung, Kim, & Choi, 1999; Kawahara, Satake, & Goda, 2002; Louati, Simmonds, Grayer, Kite, & Damak, 2003), but, as far as we know, no quantification was performed. Phenolics of loquat fruits have been characterised in few works, describing the occurrence of both hydroxycinnamic and benzoic acids derivatives and cyanidine glycoside, with the first being the main ones (Ding, Chachin, Ueda, Imahori, & Wang, 2001; Koba, Matsuoka, Osada, & Huang, 2007). Furthermore, the antioxidant capacity of loquat extracts containing those compounds has been observed (Jung et al., 1999; Koba et al., 2007), although fruits and leaves' potential have not been compared.

The morphology of loquat fruits is conditioned by their origin and cultivar. Fruits can be spherical to pear-shape in form,

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10–80 g mass/unit, with pale-yellow to deep-orange flesh. The pulp, succulent and with a soft and agreeable flavour, can be firm and fleshy in some cultivars and more fusing in others, presenting a white to salmon-orange colour. Loquat has been the subject of horticultural improvement regarding its production capacity and the organoleptic characteristics of the fruits, namely to increase their size and quality. In the American continent, Brazil is a prominent producer of loquat, São Paulo state being the leader in national production. Recently research of different loquat cultivars and their economic viability to further large scale production was developed by "Instituto Agronómico" (IAC, São Paulo, Brazil) (Pio et al., 2007): 'Mizuho' cultivar comes from 'Kusunoki' x 'Tanaka' cross, and 'Néctar de Cristal' results from the free pollination of 'Togoshi' cultivar seed; 'Mizauto' cultivar is the product of 'Mizuho' autofecundation, while 'Mizumo' arises from 'Mizuho' x 'Mogui' cross; 'Centenária' cultivar also proceeds from 'Mizuho' x 'Mogui' cross, but exhibits fruits with tender pulp, better palatability and less prone to the appearance of violaceous spots than the previous cultivar. Seeds of fruits obtained from open pollinated plants of 'Mizuho' were used to produce 'NE-3' cultivar that is still being started-up by IAC.

As far as we are aware, no previous work involved the above mentioned cultivars. This study intended to define the phenolic profile of the different parts of *E. japonica* plant and carry out further comparison of six improved cultivars ('Mizuho', 'Néctar de Cristal', 'Mizauto', 'Mizumo', 'Centenária' and 'NE-3'), and assess the antioxidant potential of the distinct vegetal materials. To achieve these aims phenolic compounds were determined by HPLC-DAD-ESI-MS/MS and antioxidative properties were checked against DPPH radical.

2. Materials and methods

2.1. Standards and reagents

5-Caffeoylquinic and ferulic acids, kaempferol-3-*O*-glucoside and quercetin-3-*O*-galactoside were from Extrasynthése (Genay, France). *p*-Coumaric acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co. (St. Louis, USA). Acetonitrile, methanol, sodium hydroxide and acetic, hydrochloric and formic acids were purchased from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

2.2. SPE columns

The Isolute C18 non-endcapped (NEC) columns (50 μm particle size, 60 Å porosity; 10 g sorbent mass/70 ml reservoir volume) were obtained from International Sorbent Technology Ltd. (Mid Glamorgan, UK).

2.3. Plant material and sampling

Samples of six loquat (*E. japonica* Lindl.) cultivars were analysed: 'Mizuho', 'Néctar de Cristal', 'Mizauto', 'Mizumo', 'Centenária' and 'NE-3' cultivars. The fruits were harvested from plants aged 12 years, located on the Germoplasm Bank of the loquat tree from "Centro Avançado de Pesquisa Tecnológica do Agronegócio das Frutas" of "Instituto Agronómico" (IAC), in Jundiaí, São Paulo State (23°8′S; 46°55′W, 700 m altitude), Brazil. The plants were annually fertilized, according to previous soil analysis, and pulverised with fungicides and insecticides when necessary.

Fruits and leaves (about 40 leaves from the medium portion of the tree-top) were harvested in July 2007, when the fruits were completely ripe. Afterwards, the fruits were separated into flesh and peel, and each part was freeze-dried. The leaves were dehydrated in a ventilated stove at 60 °C, for 2 days. Each sample was powdered using mortar and pestle (910 μm).

2.4. Phenolic compound extraction

For the screening of the phenolic compounds in leaves, peel, and flesh from *E. japonica* two extractive procedures were performed: one with methanol:water (1:1) for phenolics in general, and another with methanol:acetic acid:water (25:1:24) to check for possible anthocyanins. In both cases, about 0.2 g of each powdered material was thoroughly mixed with 1.5 ml of extractive solvent. The mixtures were then ultra-sonicated (1 h), macerated for 15 h and ultra-sonicated again (1 h). The resulting extracts were centrifuged (12,000 rpm, 5 min), and the supernatant was collected and filtrated through a 0.45 μm size pore membrane.

For phenolic quantification and antioxidant assay each sample (ca. 2 g for leaves, ca. 1 g for flesh and peel) was thoroughly mixed with methanol until complete extraction of the phenolic compounds (negative reaction to 20% NaOH). The extract was concentrated to dryness under reduced pressure (30 °C) and redissolved in water acidified to pH 2 with HCl. The obtained solution was applied to a Isolute C18 (NEC) column, previously conditioned with 30 ml of methanol and 70 ml of acidified water. Polar compounds were removed with the aqueous solvent and the retained phenolic compounds were then eluted with 50 ml methanol. The extract was concentrated to dryness under reduced pressure (30 °C) and redissolved in methanol (1 ml).

2.5. HPLC-DAD-MS/MS-ESI qualitative analysis

Chromatographic separations were carried out on a 250 × 4 mm, 5 μm, RP-18 LiChroCART column (Merck, Darmstadt, Germany) protected with a 4 × 4 mm LiChroCART guard column, with 1% acetic acid (A) and acetonitrile (B) as solvents, starting with 10% B and using a gradient to obtain 20% B at 40 min, and 40% B at 50 min. The flow rate was 1 ml/min and the injection volume 20 L. The HPLC system was equipped with an Agilent 1100 Series diode array and a mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser and a G1315B photodiode array detector, controlled by ChemStation software (Agilent, v. 08.03). Spectroscopic data from all peaks were accumulated in the range 240–400 nm, and chromatograms were recorded at 285 and 340 nm. For the study of possible anthocyanins spectroscopic data were accumulated between 240 and 600 nm and chromatograms were registered at 545 nm. The mass detector was a G2445A Ion-Trap Mass Spectrometer equipped with an electrospray ionisation (ESI) system and controlled by LCMSD software (Agilent, v. 4.1.). Nitrogen was used as nebulising gas at a pressure of 65 psi and the flow was adjusted to 11 L/min. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. The full scan mass covered the range from *m/z* 100 to 1500. Collision-induced fragmentation experiments were performed in the ion-trap using helium as collision gas, with voltage ramping cycles from 0.3 up to 2 V. MS data were acquired in the negative ionisation mode, and in positive mode for the study of anthocyanins.

2.6. HPLC-DAD quantitative analysis

Twenty microlitres of each extract were analysed using a HPLC unit (Gilson) and a 250 × 4.6 mm i.d., 5 μm Spherisorb ODS2 column (Waters, Milford, USA). The solvent system was a mixture of formic acid 5% in water (A) and methanol (B), with a flow rate of 0.9 ml/min, and the gradient was as follows: 0 min – 5% B; 3 min – 15% B; 13 min – 25% B; 25 min – 30% B; 35 min – 35% B;

39 min – 45% B; 42 min – 45% B; 44 min – 50% B; 47 min – 70% B; 50 min – 70% B; 56 min – 75% B; 61 min – 80% B. Detection was achieved with a Gilson diode array detector. Spectroscopic data from all peaks were accumulated in the range of 200–400 nm, and chromatograms were recorded at 340 nm. The data were processed on Unipoint system Software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

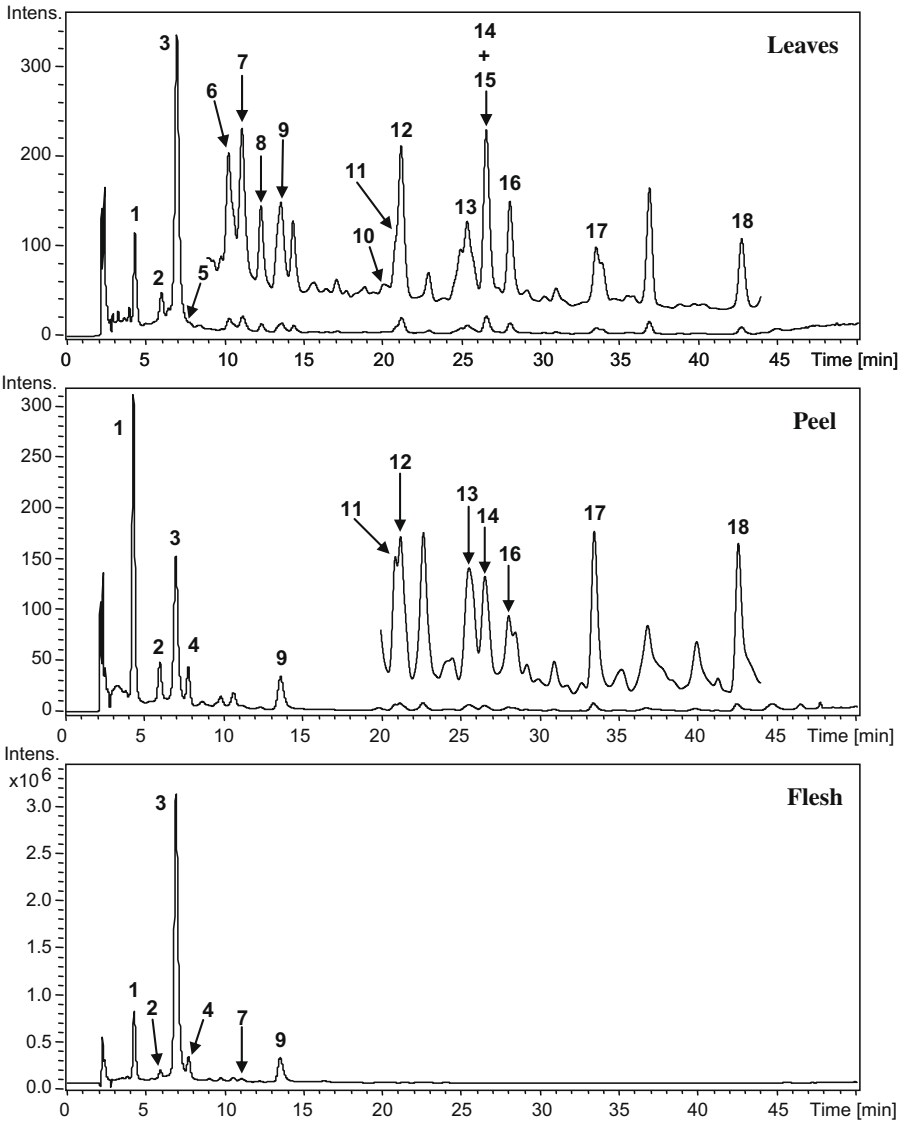

Fig. 1. HPLC-DAD chromatogram of loquat material hydromethanolic extracts. Detection at 340 nm. (**1**) 3-caffeoylquinic acid; (**2**) 3-*p*-coumaroylquinic acid; (**3**) 5-caffeoylquinic acid; (**4**) sinapoyl glucoside; (**5**) naringenin-6,8-di-*C*-glucoside; (**6**) 4-*p*-coumaroylquinic acid; (**7**) 5-*p*-coumaroylquinic acid; (**8**) 4-feruloylquinic acid; (**9**) 5-feruloylquinic acid; (**10**) naringenin-8-*C*-rhamnosyl(1 → 2)glucoside; (**11**) quercetin-3-*O*-neohesperidoside; (**12**) quercetin-3-*O*-sambubioside; (**13**) quercetin-3-*O*-galactoside; (**14**) kaempferol-3-*O*-neohesperidoside; (**15**) quercetin-3-*O*-glucoside; (**16**) kaempferol-3-*O*-sambubioside; (**17**) quercetin-3-*O*-rhamnoside; (**18**) kaempferol-3-*O*-rhamnoside.

Table 1
Rt; UV and −MS: $[M-H]^-$, −MS2$[M-H]^-$ data of hydroxycinnamoyl chlorogenic acids from loquat.[a]

Compounds[b]		*Rt* (min)	UV (nm)	$[M-H]^-$ (*m*/*z*)	−MS2$[M-H]^-$ (*m*/*z*) (%)					
					[ferulic-H]$^-$	[quinic-H]$^-$	[caffeic-H]$^-$	[191-18]$^-$	[*p*-coumaric-H]$^-$	[(cinnamic der-H)-44]$^-$
1	3-CQA	4.2	325, 295sh	353		191(100)	179(45)	173(4)		135(13)
2	3-*p*-CoQA	5.9	311	337		191(4)		173(3)	163(100)	119(13)
3	5-CQA	6.8	325, 295sh	353		191(100)	179(25)	173(50)		135(4)
6	4-*p*-CoQA	10.2	311	337		191(1)		173(100)	163(9)	119(1)
7	5-*p*-CoQA	11.1	311	337		191(100)		173(4)	163(4)	
8	4-FQA	12.2	320, 281sh	367	193(15)			173(100)		
9	5-FQA	13.3	322, 290sh	367	193(4)	191(100)		173(4)		

[a] Main observed fragments. Other ions were found but they have not been included.
[b] CQA: caffeoylquinic acid; *p*-CoQA: *p*-coumaroylquinic acid; FQA: feruloylquinic acid.

Phenolic compound quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Caffeoylquinic, *p*-coumaric and ferulic acid derivatives and quercetin and kaempferol glycosides were quantified as 5-caffeoylquinic, *p*-coumaric and ferulic acids, quercetin-3-*O*-galactoside and kaempferol-3-*O*-glucoside, respectively. Kaempferol-3-*O*-neohe-

Table 2
Rt; UV, and −MS: $[M-H]^-$ and −MS2$[M-H]^-$ data of flavonol-3-*O*-glycosides from loquat.[a]

Compounds[b]		*Rt* (min)	UV (nm)	$[M-H]^-$ (*m/z*)	−MS2$[M-H]^-$ (*m/z*) (%)				
					Flavonol-3-*O*-diglycosides				
					−132	−146	−150	−164	$[Aglc-H/2H]^-$
11	Q-3-Nhp	20.8	–[c,d]	609		445(11)		445(5)	300(100)
12	Q-3-Sbb	21.1	–[c,d]	595	463(14)		445(9)		300(100)
14	K-3-Nhp	26.2	265, 301sh, 350	593		447(2)		429(15)	284(100)
16	K-3-Sbb	28.0	265, 295sh, 349	579	447(14)		429(30)		284(100)
					Flavonol-3-*O*-monoglycosides				
13	Q-3-Gal	25.4	255, 266sh, 299sh, 350	463					301(100)
15	Q-3-Glc	26.4	–[c]	463					301(100)
17	Q-3-Rhmn	33.3	255, 265sh, 302sh, 349	447					301(100)
18	K-3-Rhmn	42.6	265, 299sh, 349	431					285(100)

[a] Main observed fragments. Other ions were found but they have not been included.
[b] Q: quercetin. K: kaempferol. Nhp: neohesperidoside (rhamnosyl(1 → 2)glucoside). Sbb: sambubioside (xylosyl(1 → 2)glucoside). Gal: galactoside. Glc: glucoside. Rhmn: rhamnoside.
[c] Compounds hidden by others. Their UV spectra have not been properly observed.
[d] UV of **11** + **12**: 257, 265sh, 297sh, 352 nm.

Table 3
Phenolic composition of loquat leaves, peel and flesh samples (mg/kg, dry basis).[a]

Samples[b]	Compound[c]																Σ
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11 + 12**	**13**	**14 + 15**	**16**	**17**	**18**	
Leaves																	
A	3.1 (0.5)	2.0 (0.1)	20.1 (5.7)	–	nq	5.6 (0.0)	4.6 (0.9)	1.0 (0.0)	11.2 (4.0)	nq	0.1 (0.0)	4.9 (0.0)	7.0 (0.0)	0.5 (0.1)	4.3 (0.1)	7.6 (0.0)	75.6
B	1.0 (0.1)	0.7 (0.0)	4.8 (0.9)	–	nq	0.8 (0.1)	0.6 (0.1)	0.3 (0.0)	5.9 (0.3)	nq	0.5 (0.0)	1.7 (0.0)	0.9 (0.0)	0.8 (0.0)	0.8 (0.0)	2.7 (0.1)	21.9
C	2.6 (0.2)	0.8 (0.1)	9.2 (0.9)	–	nq	2.4 (0.1)	0.9 (0.1)	0.8 (0.0)	18.8 (0.4)	nq	2.6 (0.0)	2.1 (0.1)	3.4 (0.0)	2.0 (0.0)	2.1 (0.1)	2.4 (0.2)	51.8
D	2.4 (0.6)	1.3 (0.1)	21.7 (7.6)	–	nq	1.9 (0.0)	1.3 (0.3)	0.5 (0.0)	24.4 (6.0)	nq	3.9 (0.1)	3.9 (0.0)	11.9 (0.4)	2.0 (0.0)	4.8 (0.1)	5.8 (0.0)	91.7
E	3.6 (0.6)	1.1 (0.2)	17.0 (3.3)	–	nq	1.8 (0.0)	1.5 (0.3)	0.5 (0.1)	11.3 (2.3)	nq	2.2 (0.0)	3.7 (0.0)	4.4 (0.0)	1.8 (0.1)	2.2 (0.0)	3.2 (0.1)	56.7
F	5.9 (0.2)	1.3 (0.1)	34.7 (6.6)	–	nq	2.1 (0.1)	1.9 (0.1)	0.4 (0.0)	12.3 (3.1)	nq	2.6 (0.0)	4.8 (0.0)	7.5 (0.0)	1.7 (0.1)	2.7 (0.1)	4.9 (0.0)	86.5
Peel																	
A	1121.1 (165.1)	171.8 (37.8)	580.7 (95.8)	nq	–	–	–	–	330.1 (79.7)	–	12.6 (0.5)	2.6 (0.1)	18.0 (0.2)[d]	2.8 (0.0)	36.5 (0.2)	10.5 (0.4)	2286.8
B	473.4 (36.7)	62.6 (5.7)	237.6 (11.7)	nq	–	–	–	–	51.9 (9.9)	–	11.9 (0.5)	2.6 (0.1)	15.8 (0.5)[d]	3.1 (0.0)	15.8 (0.9)	7.8 (0.1)	882.4
C	13.1 (2.8)	20.0 (0.5)	13.0 (3.4)	nq	–	–	–	–	282.6 (0.8)	–	1.1 (0.2)	3.1 (0.2)	3.2 (0.7)[d]	1.4 (0.6)	3.9 (0.5)	3.2 (0.4)	344.8
D	930.5 (8.7)	122.3 (2.0)	577.6 (0.3)	nq	–	–	–	–	22.1 (7.5)	–	17.9 (0.9)	1.4 (0.1)	33.8 (0.5)[d]	4.3 (0.7)	57.4 (3.7)	13.3 (0.3)	1780.5
E	21.2 (6.1)	2.5 (0.5)	21.7 (8.0)	nq	–	–	–	–	60.0 (3.9)	–	7.2 (0.2)	0.2 (0.0)	3.5 (0.2)[d]	1.5 (0.1)	5.9 (0.2)	1.8 (0.1)	125.7
F	1349.8 (144.1)	159.7 (24.4)	640.2 (75.0)	nq	–	–	–	–	267.9 (40.8)	–	26.6 (2.4)	4.3 (0.7)	82.1 (1.5)[d]	8.4 (0.5)	46.2 (3.9)	18.2 (1.0)	2603.3
Flesh																	
A	644.4 (9.3)	nq	nq	nq	–	–	nq	–	537.0 (34.7)	–	–	–	–	–	–	–	1181.4
B	516.6 (34.9)	40.9 (7.7)	1130.3 (17.0)	nq	–	–	103.3 (6.2)	–	490.8 (83.4)	–	–	–	–	–	–	–	2281.9
C	72.5 (1.8)	4.3 (0.2)	539.4 (55.0)	nq	–	–	6.9 (0.6)	–	661.5 (41.0)	–	–	–	–	–	–	–	1284.6
D	209.5 (11.4)	15.9 (0.6)	772.3 (48.9)	nq	–	–	18.4 (1.3)	–	1295.5 (30.7)	–	–	–	–	–	–	–	2311.7
E	295.3 (13.7)	14.8 (1.3)	1087.8 (57.6)	nq	–	–	51.9 (1.8)	–	273.1 (28.1)	–	–	–	–	–	–	–	1722.9
F	3.3 (0.3)	12.8 (0.1)	22.1 (1.2)	nq	–	–	161.9 (1.0)	–	1027.2 (25.6)	–	–	–	–	–	–	–	1227.4

[a] Values are expressed as mean (standard deviation) of three determinations for each sample.
[b] A, 'Nectar de Cristal'; B, 'Centenária'; C, 'NE-3'; D, 'Mizauto'; E, 'Mizuho'; F, 'Mizumo'.
[c] Identity of compounds as in Fig. 1.
[d] Values correspond only to compound 14. Abbreviations: nq, not quantified; Σ, sum of the determined compounds.

speridoside and quercetin-3-*O*-glucoside were quantified together, as kaempferol-3-*O*-glucoside.

2.7. DPPH scavenging assay

The antiradical activity was determined spectrophotometrically in a Multiskan Ascent microplate reader (Thermo Lab Systems) by monitoring the disappearance of DPPH at 515 nm, following a described procedure (Silva et al., 2004). The reaction mixtures in the sample wells consisted of aqueous extract (five different concentrations) and DPPH 150 μM methanolic solution. The reaction was conducted at room temperature for 30 min. Three experiments were performed in triplicate.

2.8. Statistical analysis

The evaluation of statistical significance was determined by ANOVA, followed by Newman–Keuls testing. The level of significance was set at $p < 0.05$.

3. Results and discussion

3.1. Phenolic compound characterisation

The HPLC-DAD-ESI-MS/MS screening of the hydromethanolic extracts of loquat leaf, peel and flesh (Fig. 1) revealed the presence of several phenolic compounds, namely hydroxycinnamic acid derivatives (**1**–**4**, **6**–**9**) and flavonoid glycosides (**5**, **10**–**18**).

3.1.1. Hydroxycinnamic acid derivatives

Compounds **1**–**4** and **6**–**9** presented an UV spectra characteristic of hydroxycinnamic acid derivatives. Compound **4** (*R*t 7.7 min; UV 301 nm) exhibited a deprotonated molecular ion at *m/z* 385 u and in its MS2 the loss of 162 u was observed to originate and ion at *m/z* 223 u ([sinapic acid-H]$^-$), which indicates that this compound is sinapoyl glucoside. The other compounds are esters of *trans* cinnamic acids (caffeic, *p*-coumaric and ferulic acids) and quinic acid. According to Clifford, Johnston, Knight, and Kuhnert (2003), the relative abundance of the ions obtained from their MS2 fragmenta-

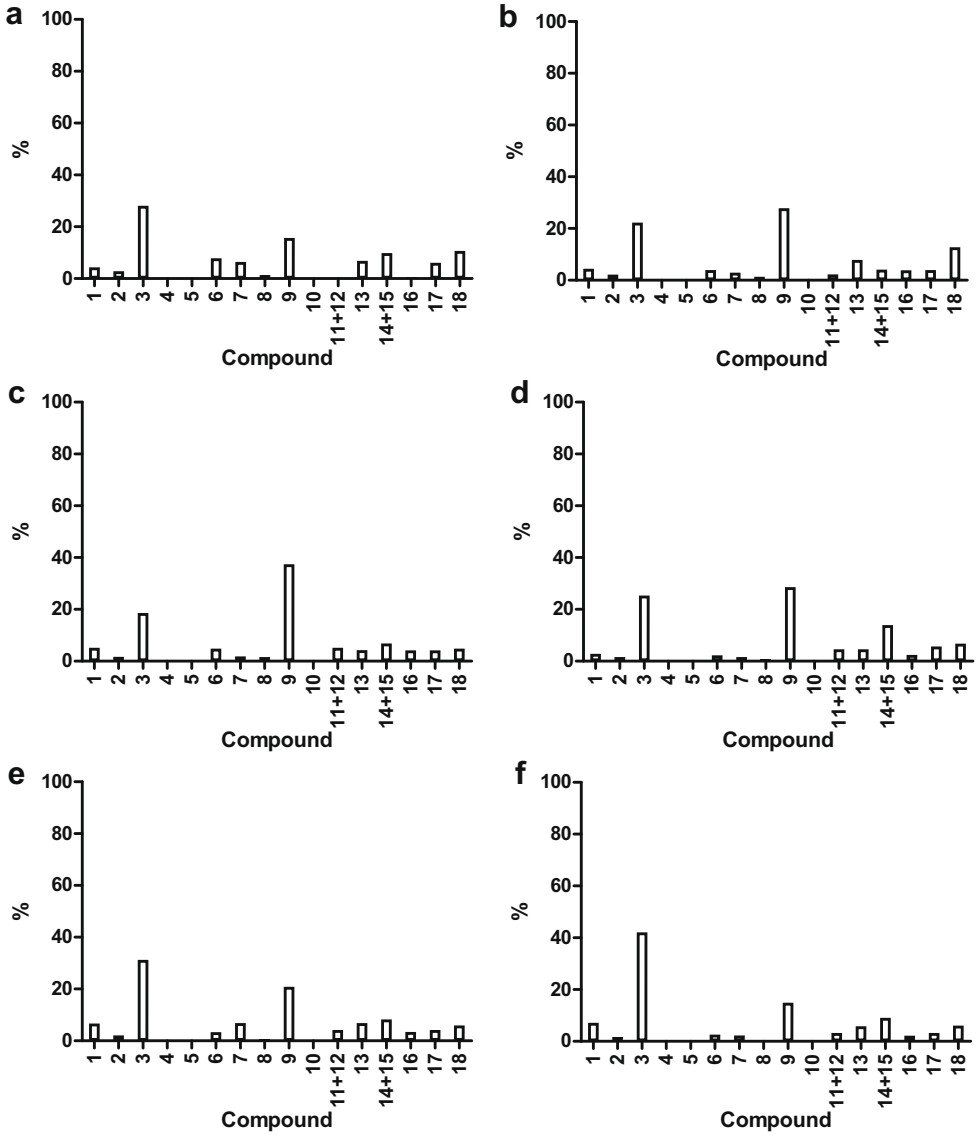

Fig. 2. Phenolic profiles of loquat leaves: (a) 'Néctar de Cristal', (b) 'Centenária', (c) 'NE-3', (d) 'Mizauto', (e) 'Mizuho' and (f) 'Mizumo' cultivars. Identity of compounds as in Fig. 1.

tions allows the differentiation of the distinct isomers present (Table 1). Thus, were identified two caffeoylquinic acid isomers (3-caffeoylquinic (3-CQA) (**1**) and 5-caffeoylquinic acids (5-CQA) (**3**)), three *p*-coumaroylquinic acid derivatives (3-*p*-coumaroylquinic (3-*p*-CoQA) (**2**), 4-*p*-coumaroylquinic (4-*p*-CoQA) (**6**) and 5-*p*-coumaroylquinic acids (5-*p*-CoQA) (**7**)) and two of feruloylquinic acid (4-feruloylquinic (4-FQA) (**8**) and 5-feruloylquinic acids (5-FQA) (**9**)).

Leaves are the loquat material containing the highest number of acylated derivatives, with the flesh exhibiting the lowest one. Compounds **1**, **2**, **3**, and **9** are common to leaves, peel and flesh (Fig. 1), chlorogenic acid (5-CQA) being the most abundant in leaves and flesh, while neochlorogenic acid (3-CQA) is predominant in the peel. Sinapoyl glucoside (**4**) was not detected in the leaves. Previous studies have reported the occurrence in loquat leaves of several hydroxycinnamic acid derivatives, with structures similar to those found in the analysed samples, but only 3-caffeoylquinic, 5-caffeoylquinic and 4-*p*-coumaroylquinic acids were already described (Ito et al., 2000).

3.1.2. Flavonoid glycosides

The study of the UV and MS2 fragmentation of compounds **11–18** (Table 2) indicates that they are quercetin-3-*O*-glycosides (**11–13, 15** and **17**) and kaempferol-3-*O*-glycosides (**14**, **16** and **18**). This was achieved by their UV spectra (Mabry, Markham, & Thomas, 1970) and by the deprotonated aglycone ion ([Aglc-2H/H]$^-$: 300/301 quercetin, 284/285 kaempferol), which is the base peak in every case, from which it can be deduced that, in the diglycosides (**11**, **12**, **14** and **16**), the glycosilation is in one single phenolic hydroxyl (Cuyckens, Ma, Pocsfalvi, & Claeys, 2000). On the other hand, the presence of ions deriving from the interglycosidic fragmentation ([(M−H)−146]$^-$ and [(M−H)−(146+18)]$^-$ for compounds **11** and **14**, and [(M−H)−132]$^-$ and [(M−H)−(132+18)]$^-$ for compounds **12** and **16**), indicates their (1 → 2) interglycosidic union (Cuyckens, Rozenberg, Hoffmann, & Claeys, 2001; Ferreres, Llorach, & Gil-Izquierdo, 2004): rhamnosyl(1 → 2)hexoside (**11** and **14**) and pentosyl(1 → 2)hexoside (**12** and **16**).

Previously, from the leaves of *E. japonica* was isolated and characterised, amongst other phenolics, quercetin-3-*O*-sambubioside

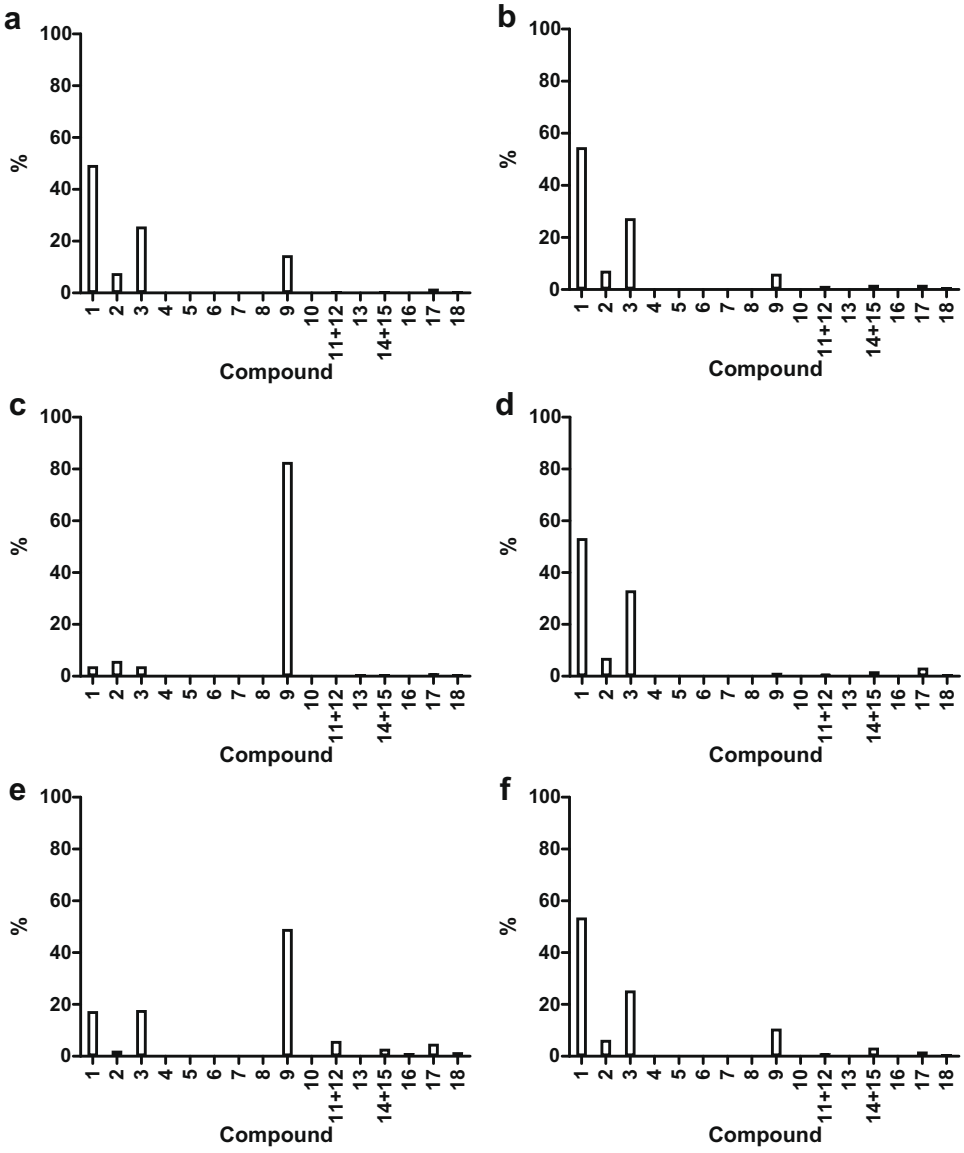

Fig. 3. Phenolic profiles of loquat peel: (a) 'Néctar de Cristal', (b) 'Centenária', (c) 'NE-3', (d) 'Mizauto', (e) 'Mizuho' and (f) 'Mizumo' cultivars. Identity of compounds as in Fig. 1. Compound **15** is not identified in peel.

(quercetin-3-*O*-xylosyl(1 → 2)glucoside) (Jung et al., 1999) and kaempferol-3-*O*-neohesperidoside (kaempferol-3-*O*-rhamnosyl(1 → 2)glucoside) (Ito et al., 2000; Louati et al., 2003). Therefore, the compounds analysed herein must be quercetin-3-*O*-neohesperidoside (**11**), quercetin-3-*O*-sambubioside (**12**), kaempferol-3-*O*-neohesperidoside (**14**) and kaempferol-3-*O*-sambubioside (**16**). Concerning the flavonoids monoglycosides, two quercetin-3-*O*-hexoside isomers were detected (compounds **13** and **15**) that, due to their mobility in RP-HPLC (*R*t) (Tsao, Yang, Young, & Zhu, 2003), must be coincident with quercetin-3-*O*-galactoside (**13**) and quercetin-3-*O*-glucoside (**15**), already detected before in the leaves (Louati et al., 2003). In the same way, quercetin-3-*O*-rhamnoside (**17**) and kaempferol-3-*O*-rhamnoside (**18**) were characterised, already described previously (Jung et al., 1999). For the study of the possible presence of *C*-glycosylflavonoids, already detected in leaves (Ito et al., 2000; Louati et al., 2003), we proceed to the extraction of the ions (Constant Neutral Loss Chromatogram) in which fragmentation it was possible to observe the loss of 60, 90 and/or 120 u, that would indicate *C*-glycosyl derivatives. These together with the presence of the ions $[Aglc+41]^-$/$[Aglc+71]^-$ (mono-*C*-glycosyl) or $[Aglc+83]^-$/$[Aglc+113]^-$ (di-*C*-glycosyl) could confirm these structures (Ferreres, Silva, Andrade, Seabra, & Ferreira, 2003). In this way, two *C*-glycosylflavones (compounds **5** and **10**) were detected, in low quantities, whose presence was best observed at 285 nm (data not shown). Thus, at *R*t 7.7 min an ion at *m/z* 595 u $[M-H]^-$ was noticed, whose MS2 fragmentation gives rise to ions at *m/z* 505 ($[(M-H)-90]^-$, 8%), 475 ($[(M-H)-120]^-$, 20%), 385 ($[272+113]^-$, 86%) and 355 ($[272+83]^-$, 100%) that characterise a trihydroxyflavanone-di-*C*-hexoside, tentatively identified as naringenin-6,8-di-*C*-glucoside (**5**) and not detected until now in *E. japonica*. Additionally, at *R*t 20.0 min an ion at *m/z* 579 u $[M-H]^-$ was found, whose MS2 fragmentation gives rise to ions at *m/z* 459 ($[(M-H)-120]^-$, 70%), 415 ($[(M-H)-(146+18)]^-$, 12%), 343 ($[272+71]^-$, 20%) and 313 ($[272+41]^-$, 100%). The presence of the ion at *m/z* 415, produced by the loss of the rhamnosyl fragment plus water (146+18) from the deprotonated molecular ion, indicates a *O*-glycosylation on the hydroxyl at 2́ of the sugar moiety from *C*-glycosylation (Ferreres, Gil-Izquierdo, Andrade, Valentão, & Tomás-Barberán, 2007). On the other hand, the absence of the ion $[(M-H)-90]^-$ might indicate *C*-glycosylation at eight po-

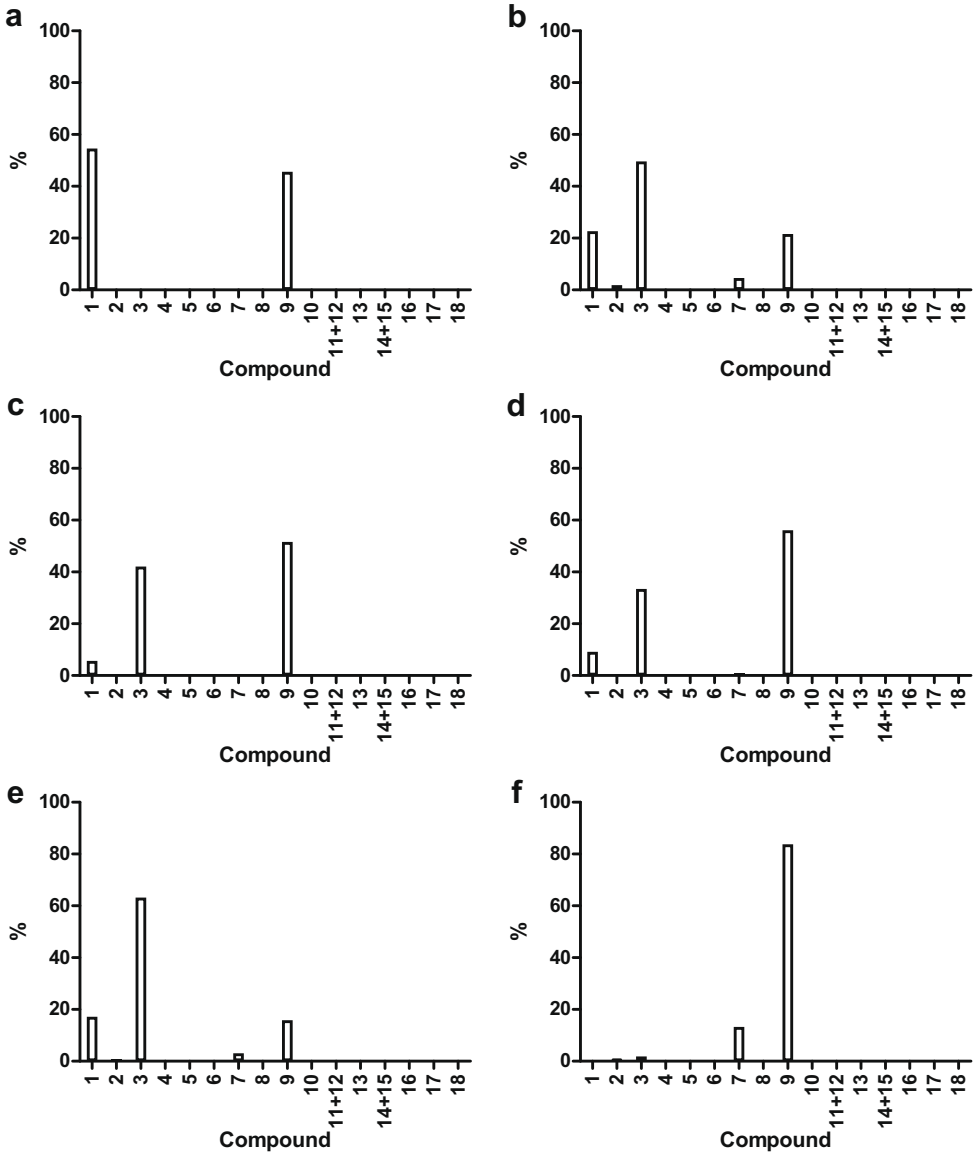

Fig. 4. Phenolic profiles of loquat flesh: (a) 'Néctar de Cristal', (b) 'Centenária', (c) 'NE-3', (d) 'Mizauto', (e) 'Mizuho' and (f) 'Mizumo' cultivars. Identity of compounds as in Fig. 1.

sition. Thus, this compound is coincident with naringenin-8-*C*-rhamnosyl(1 → 2)glucoside (**10**), as described before (Ito et al., 2000). The presence of schaftoside (apigenin-6-*C*-glucoside-8-*C*-arabinoside), described by Louati et al. (2003), was not noticed. The possible occurrence of isorhamnetin derivatives already reported (Louati et al., 2003) was also checked by extraction of the ion – MSn at *m/z* 315 (Extracted Ion Chromatogram, EIC), which characterises their deprotonated aglycone ion. However, we could not detect any of these derivatives.

All of the described flavonoids were found in loquat leaves, as well as in peel, with the exception of the two *C*-glycosides (**5** and **10**) and of quercetin-3-*O*-glucoside (**15**) in the latter. As far as we know, these flavonoids are reported for the first time in loquat peel. In the flesh no flavonoid was observed. The presence of cyanidin-glucoside, described by Koba et al. (2007), or of any other anthocyanin in the studied loquat materials was also not observed (detection at 545 nm, positive MS mode).

3.2. Quantitative analysis

Considering that for the determination of the phenolic variation for a given species, not only the qualitative characterisation of its constituents is important but also the knowledge of their amounts and ratios, the phenolic compounds present in loquat leaves, peel and flesh were quantified (Table 3). Hydromethanolic extracts were used for LC–MS qualitative analysis, but in order to obtain a purified phenolic extract, without other compounds than phenols, SPE methodology was used, which allows us to obtain a better and clearer quantification and a better correlation between chemical composition and antioxidant activity. Within each matrix (leaf, peel or flesh) a similar qualitative phenolic profile was found for all cultivars, but not a quantitative one. The total average phenolic content of loquat leaves was 64 mg/kg, significantly lower than that of peels (1337 mg/kg) and flesh (1668 mg/kg). In a general way, 'Mizauto' cultivar material presented the highest phenolic content. The relative amount of phenolic acids in peel (ca. 94% of total phenolic compounds) was significantly higher than in leaves (ca. 68%). The content of flavonoid compounds was lower, the leaves being the material that presented the highest amounts (ca. 33% of total phenolic compounds), which was significantly higher than that of peels (ca. 8%). In flesh only phenolic acids were determined.

The presence of flavonoids in loquat leaves and peel, in comparison with their absence from the flesh, is not surprising as these compounds act as UV filters, protecting some fragile cell structures, such as chloroplasts, from UV radiation. In fact, these filters consist mainly of flavonols and are located in the skins of fruits and leaves (Macheix et al., 1990).

The major compounds in leaves were 5-caffeoylquinic (**3**) and 5-feruloylquinic acids (**9**), but their relative amounts depended on the cultivar (Fig. 2): in 'Nectar de Cristal', 'Mizuho' and 'Mizumo' 5-caffeoylquinic acid (**3**) was the main constituent (ca. 34%), followed by 5-feruloylquinic acid (**9**) (ca. 17%), while in 'Centenária', 'NE-3' and 'Mizauto' 5-feruloylquinic (**9**) was present in the highest amount (ca. 31%) and 5-caffeoylquinic (**3**) was the second highest (ca. 22%). The highest amounts in flavonoids were found for the pair kaempferol-3-*O*-neohsperidoside (**14**) plus quercetin-3-*O*-glucoside (**15**), except for 'Nectar de Cristal' and 'Centenária', in which kaempferol-3-*O*-rhamnoside (**18**) was the major constituent. In general, naringenin-6,8-di-*C*-glucoside (**5**) and naringenin-8-*C*-rhamnosyl(1 → 2)glucoside (**10**) were minor compounds, present in vestigial quantities (Table 3).

As in leaves, in peel phenolic acids were clearly the main compounds (Fig. 3). 3-Caffeoylquinic (**1**), 3-*p*-coumaroylquinic (**2**), 5-caffeoylquinic (**3**) and 5-feruloylquinic acids (**9**) were the predominant compounds. 'Nectar de Cristal', 'Centenária', 'Mizauto', and 'Mizumo' presented 3-caffeoylquinic acid (**1**) as the major constituent (ca. 53%), followed by 5-caffeoylquinic acid (**3**) (ca. 28%). The other two cultivars exhibited particular phenolic profiles. 'NE-3' evidenced 5-feruloylquinic acid (**9**) as the major compound (ca. 83%). In 'Mizuho' this was also the major constituent (ca. 49%), but 3-caffeoylquinic acid (**1**) (ca. 17%) and 5-caffeoylquinic acid (**5**) (ca. 18%) appeared in similar relative amounts, distinct from the other cultivars. Peel presented small amounts of flavonoids, except 'Mizuho' cultivar, that exhibited notable quantities of the pair quercetin-3-*O*-neohsperidoside (**11**) plus quercetin-3-*O*-sambubioside (**12**) (ca. 6%) and quercetin-3-*O*-rhamnoside (**17**) (ca. 5%). Sinapoyl glucoside (**4**) was the minor compound, appearing in trace quantities (Table 3).

In loquat flesh, some differences amongst the cultivars phenolic profile were also noticed (Fig. 4). In "Nectar de Cristal", only two compounds were quantified: 3-caffeoylquinic (**1**) (ca. 54%) and 5-feruloylquinic acids (**9**) (ca. 46%). 'Mizumo' presented 5-feruloylquinic acid (**9**) as the major compound (ca. 84%), followed by 5-*p*-coumaroylquinic acid (**7**) (ca. 13%). 5-Caffeoylquinic acid (**3**) was the main compound in 'Centenária' and 'Mizuho' cultivars (ca. 56%), while in 'NE-3' and 'Mizauto' the major constituent was 5-

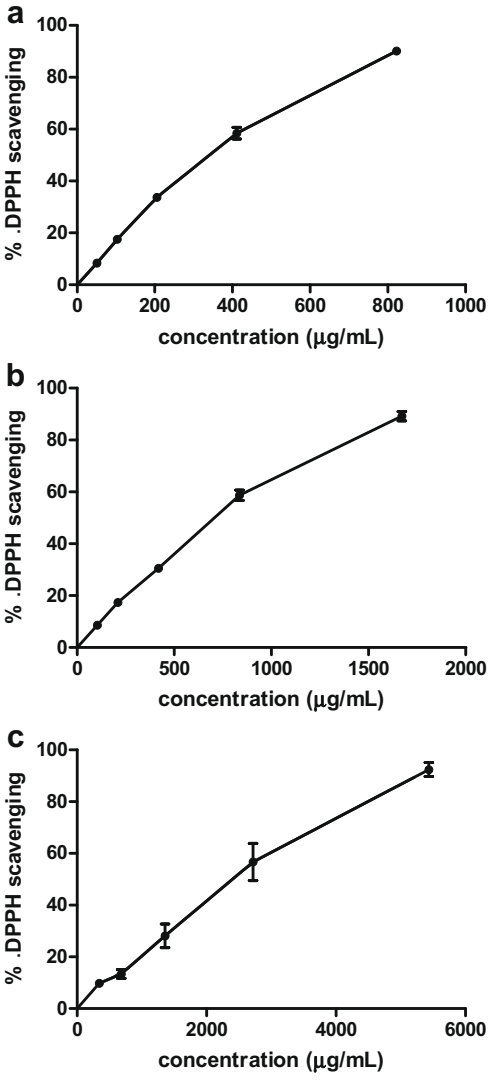

Fig. 5. Effect of loquat material extracts on DPPH reduction: (a) leaves, (b) peel and (c) flesh. Values show mean ± SE from three experiments performed in triplicate.

feruloylquinic acid (**9**) (ca. 54%). In general, sinapoyl glucoside (**4**) was the minor compound.

3.3. Antioxidant activity

For this study, 'Mizauto' cultivar was chosen for its higher phenolic content. The three materials exhibited a concentration-dependent DPPH radical scavenging activity (Fig. 5). The leaves were shown to be the most active material (IC_{50} of 341 μg/ml), followed by the peels (IC_{50} at 706 μg/ml). Flesh clearly showed a lower antioxidant capacity, with an IC_{50} of 2399 μg/ml.

The antioxidant potential exhibited by loquat leaves and fruit is obviously determined by their composition. In fact, both caffeic, *p*-coumaric, ferulic and sinapic acid derivatives and quercetin, kaempferol and naringenin glycosides, have already displayed antioxidant capacity (Agnihotri et al., 2008; Foti, Piattelli, Baratta, & Ruberto, 1996; Fukumoto & Mazza, 2000). However, no correlation was observed between the antioxidant potential of each loquat material and the phenolic content, a fact that has already been noticed for other plant species (Conforti et al., 2008): the total amount of phenolics in the analysed materials follows the order flesh > peel > leaves (Table 3). Nevertheless, the IC_{50} values found in the DPPH assay are ordered as the flavonoid content of the materials: the leaves had the highest relative flavonoid amount (ca. 35%), followed by the peel (ca. 7%) and the flesh did not present flavonoids. So, these results seem to suggest that flavonoids give a greater contribution to the antioxidant activity exhibited by loquat. On the other hand, the role of other components besides phenolics cannot be ignored, as the result of the action of the different compounds present in loquat fruits and leaves, which may comprise synergic and antagonist effects, is still unknown.

In conclusion, according to the results obtained it may be anticipated that the phenolic profile of loquat leaves and fruits is dependent on the cultivar. Additionally, the different vegetal materials exhibit distinct phenolic composition, flavonoids only being found in leaves and peel. Generally, 'Mizauto' materials presented the highest phenolic content, and all of them possess antioxidant capacity. These facts show this cultivar to be more interesting from nutritional and health benefit perspectives, as its consumption renders a higher intake of antioxidant constituents, which may contribute to the prevention of diseases in which free radicals are implicated.

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