

Short communication

Phenolic profiles, antioxidant activity and *in vitro* antiviral properties of apple pomace

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ABSTRACT

Methanolic and acetonetic extracts of apple pomace were evaluated for phenolic profiles, antioxidant properties and antiviral effect against herpes simplex virus type 1 (HSV-1) and 2 (HSV-2). Acetone extraction yielded the higher amounts of phenolic compounds. The extraction method influenced the phenolic composition although antioxidant activity correlated weakly with phenols concentration. Among the polyphenols analysed, quercetin glycosides were the most important family, followed by dihydrochalcones. Apple pomace extracts were able to inhibit both HSV-1 and HSV-2 replication in Vero cells by more than 50%, at non-cytotoxic concentrations. Selectivity indexes (SI) ranged from 9.5 to 12.2.

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1. Introduction

Apple pomace is a by-product of the apple cider processing industry and represents about 20–35% of the original fruits. Disposal of apple pomace may present an added cost to beverage industry: on the one hand, its use as fertilizer (compost) without any pre-treatment or in landfills may not continue to be acceptable for evident environmental concerns. On the other hand, this product is a poor animal feed because of its low protein content and its seasonally-restricted availability. For these reasons, exploration of potential alternative uses for this apple waste is needed.

According to epidemiologic studies, diets rich in fruits and vegetables have been associated with reduced risk of developing chronic diseases, such as cardiovascular disease, cancer, diabetes and Alzheimer's disease (Block, Patterson, & Subar, 1992; Hertog, Feskens, Hollman, Katan, & Kromhout, 1993). Phytochemical compounds, particularly polyphenols, have been suggested to be responsible for the health benefits in most of these foods. Apple extracts have been shown to have potent antioxidant activity and antiproliferative activity against human cancer cells (Boyer, Brown, & Liu, 2004; Leontowicz et al., 2002).

Previous studies indicating its phenolic content and high antioxidant activity aim to regard apple pomace as a valuable source

of natural antioxidants and bioactive compounds (Foo & Lu, 1999; Lu & Foo, 1997, 2000; Ćetković et al., 2008).

Herpes simplex virus type 1 (HSV-1) and 2 (HSV-2) (family *Herpesviridae*) are responsible for a broad spectrum of clinical symptoms in humans, varying from mild skin vesicular lesions to severe manifestations such as encephalitis, conjunctivitis, eczema, pneumonia and hepatitis (Whitley, Kimberlin, & Roizman, 1998). Flavonoid-rich extracts and fractions from fruits have been found to inhibit acyclovir-resistant strains of these viruses (Cos, Berghe, Bruyne, & Vlietinck, 2003; Goncalves et al., 2001). However, scarce information is available on antiviral activity of apple pomace. In this work, we present the phenolic profiles and antioxidant abilities of two (methanolic and acetonetic) extracts from apple pomace, and preliminary data on their inhibitory activity against HSV-1 and HSV-2 *in vitro* propagation.

2. Materials and methods

2.1. Materials

2.1.1. Apple pomace

All apple pomace used throughout this work was generated from a mixture of cider apples from El Gaitero S.A. cellar (Villaviciosa, Asturias, Spain). The fresh apple pomace was oven-dried in convection at 60 °C until it reached a constant weight. The dried pomace was then homogenised, ground to 0.5 mm particles with a Cyclotek 1093 (Foss-Tekator) and vacuum stored.

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2.1.2. Viruses and cells

HSV-1 and HSV-2 clinical isolates were kindly provided by Dr. María Oña (Virology Department, Asturias Central Hospital). Both herpes viruses were propagated in green monkey kidney (Vero) cells (ECACC No. 84113001) and titrated by microscopical visualisation of cytopathic effect (CPE), using the endpoint dilution method described by Reed and Muench (1938). Vero cells were grown in Dulbecco modified Eagle's essential minimal medium (DMEM) supplied with 10% foetal bovine serum. Cells were maintained in DMEM with serum concentration reduced to 2%.

2.1.3. Reagents

Folin–Ciocalteu reagent was supplied by Merck (Darmstadt, Germany). 2,2'-Diphenyl- β -picrylhydrazyl (DPPH), 2,4,6-tris-(2-pyridyl)-s-triazine (TPTZ), ascorbic acid, ferric chloride and sodium acetate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Polyphenol standards were supplied as follows: gallic acid, protocatechuic acid, (+)-catechin, (–)-epicatechin, phloridzin, hydroxycinnamic acids (*p*-coumaric acid, caffeic acid and chlorogenic acid) and rutin, by Sigma (St. Louis, MO, USA); procyanidin B2 and quercetin glycosides (hyperin, isoquercitrin, avicularin and quercitrin) by Extrasynthèse (Genay, France), and reynoutrin, by Apin Chemicals (Abingdon, UK). DMEM and foetal bovine serum were purchased from Sigma (St. Louis, MO, USA).

2.2. Methods

2.2.1. Extractions

Apple pomace (10 g) was extracted with 100 mL of 70% acetone (acetonic extract, AE) or 80% methanol (methanolic extract, ME) in darkness, by magnetic stirring at 20 °C. The extracts were filtered through no. 1 Whatman paper on a vacuum-aided Buchner funnel and concentrated to dryness by rotary evaporation at 30 °C, under reduced pressure. The solid residue was dissolved in distilled water and lyophilised. The total extraction process was done in duplicate.

2.2.2. Determination of total phenolic content

The contents of total phenolics in samples were analysed by the Folin–Ciocalteu colorimetric method. The appropriate extracts dilutions were oxidised with Folin–Ciocalteu reagent and the reaction was stopped with sodium carbonate. After 30 min incubation, the absorbance of the resulting blue product was measured at 750 nm with a Perkin–Elmer Lambda 35 spectrophotometer. Gallic acid was used as standard.

2.2.3. Liquid chromatographic analysis of phenolic compounds

HPLC analysis was performed according to the method validated by our group (Suárez, Palacios, Fraga, & Rodríguez, 2005). We used a Waters system equipped with a 717 automatic injector, provided with a column oven, two pumps (model 510), a diode array detector (model 2996) and Millennium software v.3.1 data module. Separation of polyphenols was carried out on a reversed-phase Nucleosil 120 C₁₈ (250 × 4.6 mm ID, 3 μ m) column from Teknokroma (Barcelona, Spain) thermostated at 25 °C, and a flow rate of 0.8 mL/min was used. The elution solvents were aqueous 2% acetic acid (solvent A) and 100% methanol (solvent B). The samples were eluted according to the following gradient: a linear step from 0% to 45% of solvent B in 55 min and a final isocratic step of 20 min. Fifty microlitre of each organic extract were injected into the HPLC after filtration through a 0.22 μ m PVDF membrane. Quantitation was performed at 313 nm for the hydroxycinnamic acids, 355 nm for the flavonol glycosides and 280 nm for the rest of phenolic compounds. Quantitation was performed according to the external standard method. For compounds lacking of standards, or those whose standard amount at our disposal was too small, the quantification was achieved from similar compounds:

thus, phloretin-2'-xyloglucoside and the unknown dihydrochalcones were quantified as phloridzin and flavonol glycosides as quercitrin.

2.2.4. Radical-scavenging activity (DPPH)

The free radical-scavenging activity of apple pomace extracts was measured using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method. A 40 μ L aliquot of diluted sample was added to 1.460 mL of DPPH solution in methanol (1.0×10^{-4} M) and the reaction mixture was kept in the dark for 240 min. The absorption was read at 515 nm relative to control (100%) and the scavenging effect percentage was expressed as $I = (1 - (A_{515} \text{ of sample} / A_{515} \text{ of control})) \times 100$. The antioxidant capacity was expressed as ascorbic acid equivalents (g ascorbic acid/kg dry matter), calculated from the following equation: $I = 0.2376C - 0.3392$ ($R^2 = 0.997$).

2.2.5. Ferric reducing-antioxidant power (FRAP)

FRAP assay measures the reduction of ferric iron to the ferrous form in the presence of the antioxidant components. The working FRAP reagent was prepared freshly every day by mixing 2.5 mL of TPTZ (10 mM in 40 mM hydrochloric acid), 2.5 mL of ferric chloride (20 mM) and 25 mL of sodium acetate buffer (300 mM, pH 3.6). The FRAP assay was carried out at 37 °C, in 1-cm disposable plastic cells. Nine hundred microlitre of the FRAP reagent were mixed with 90 μ L of water and 30 μ L of apple pomace extract (diluted 1:25 with methanol). After 120 min, the absorbance at 595 nm was measured. The antioxidant capacity of the apple pomaces were expressed as g ascorbic acid/kg dry matter.

2.2.6. Cytotoxicity assay

Vero cells were seeded into 96-well plates at a density of 2×10^4 cells/well, and incubated at 37 °C in 5% CO₂ atmosphere during 48–72 h until confluent monolayers were formed. Increasing concentrations of the test extracts (500–12,000 μ g/mL) were added, with a replicate number of six wells per concentration. Cells were incubated in these conditions with the test extracts during 72 h. After this incubation period, 10 μ L of a PBS-solution containing 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were added into each well and the plates were incubated for another 4 h. Plates were decanted by gently inversion and the produced solid precipitate was dissolved with 100 μ L/well of a solution containing 1 N HCl in anhydrous 2-propanol and the absorbance at 570 nm was determined using a MicroQuant spectrophotometer with a reference wavelength of 620 nm. The cell viability percentage was calculated as the ratio between the mean absorbance of treated with respect to that of untreated wells (Mosmann, 1983).

2.2.7. Antiviral assay

About 96-well plates with confluent cell monolayers were pre-incubated for 1 h with increasing non-cytotoxic concentrations of the extracts in the range of 50–1000 μ g/mL. Six wells were used for each concentration. Afterwards, 10 μ L of a virus dilution containing 100 TCID₅₀ of HSV-1 or HSV-2 were added into each well. Plates were incubated at 37 °C in 5% CO₂ atmosphere and microscopically observed daily for CPE. When at least one CPE focus was observed in every virus control well, the percentage of wells with CPE among treated wells was determined for each treatment concentration (del Barrio & Parra, 2000). Acyclovir (ACV) at concentrations varying from 0.5 to 8 μ g/mL served as positive controls.

2.2.8. Statistical analysis

Statistical analyses were performed with the SPSS software package. Differences among extracts were determined using Student *t*-tests. Cytotoxic mean concentration (CC₅₀) and antiviral effective mean concentration (EC₅₀) values were obtained by

regression analysis of the dose–response curves generated from data.

3. Results and discussion

3.1. Phenolic profiles and antioxidant activity

Methanolic (ME) and acetic (AE) extracts from apple pomace were evaluated to compare their total phenolic content, phenolic profiles and antioxidant capacity. An aliquot of each lyophilised extract (30 mg) was dissolved in methanol (10 mL). All data are presented as mean value of three replications and its standard deviation (SD).

Table 1 summarises the concentrations of phenolic compounds, the radical-scavenging activity and reducing power of apple pomace extracts.

In this industrial pomace, a total of 12 phenolic compounds (three phenolic acids and nine flavonoids) were identified by comparison of their retention times and UV–Vis spectra with those of standards. Furthermore, two other unknown compounds exhibit-

Table 1
Phenolic composition and antioxidant activity of apple pomace.

	AE	SD	ME	SD
<i>Benzoic acids (1)</i>				
Protocatechuic acid	134.33	5.32	118.19	3.46
<i>Hydroxycinnamic acids (1)</i>				
Chlorogenic acid	166.12	0.05	170.56	4.74
Caffeic acid	22.27	0.07	19.68	0.72
<i>Procyanidins and flavan-3-ols (1)</i>				
B2*	74.87	0.95	65.67	1.09
Epicatechin	88.45	1.48	88.21	0.87
<i>Dihydrochalcones (1)</i>				
Unknown dihydrochalcone (a)*	13.11	0.35	3.20	0.00
Unknown dihydrochalcone (b)*	88.09	0.01	9.51	0.06
Phloretin-2'-xyloglucoside	170.72	0.11	170.04	0.62
Phloridzin*	380.05	2.72	362.28	1.83
<i>Flavonols (1)</i>				
Hyperin*	253.71	0.59	213.01	0.86
(Rutin + Isoquercitrin)*	122.28	0.10	103.26	1.56
Reynoutrin*	73.97	0.89	59.51	0.26
Avicularin*	185.10	1.66	146.46	0.18
Quercitrin*	131.78	2.69	105.58	0.08
Total phenols (2)*	6.48	0.29	3.63	0.02
DPPH (3)*	9.75	1.15	6.66	0.76
FRAP (3)*	10.87	0.26	7.73	0.26

(1) mg/kg dry weight pomace, (2) g gallic acid/kg dry weight pomace, and (3) g ascorbic acid/kg dry weight pomace.

AE, acetic extract; ME, methanolic extract.

* Significant differences between extracts ($p < 0.05$).

ing spectral characteristics similar to phloridzin ($\lambda_{\max} = 284.0$ nm) were determined. With regard to the phloretin derivatives, the 3-hydroxyphloretin 2'-xyloglucoside and 2'-glucoside had been described in apple products (Lu & Foo, 1997; Tsao, Yang, Young, & Zhu, 2003). The predominant phenolic families were quercetin glycosides followed by dihydrochalcones. Five quercetin glycosides were detected; two flavonols, hyperin and avicularin, represented more than 50% of total flavonols in pomace, hyperin being the predominant. Phloridzin and phloretin-2'-xyloglucoside were the major dihydrochalcones quantified with content values around 20% and 10% of the total phenolic compounds, respectively. Minor compounds found in apple pomace were phenolic acids, epicatechin and procyanidin B2. The range of concentrations of phenolic compounds determined was consistent with previous reports in literature (Price, Prossert, Richetin, & Rhodes, 1999; Schieber, Keller, & Carle, 2001; Van Der Sluis, Dekker, Skrede, & Jongen, 2002).

Aqueous acetone showed to be slightly more efficient than aqueous methanol for phenolic compounds extraction, except for chlorogenic acid. This result agrees with previous reports (Heinonen, Meyer, & Frankel, 1998; Kahkonen, Hopia, & Heinonen, 2001).

It was found that both extracts showed remarkable antioxidant activities, but the acetic extract exerted 1.4 times more radical-scavenging activity and reducing-antioxidant power than the methanolic one.

Significant differences were found among extracts for total phenols (Folin–Ciocalteu method), procyanidin B2, flavonol glycosides, three different dihydrochalcones and antioxidant activities (Table 1).

3.2. Cytotoxic effects and antiviral activity of apple pomace extracts

In order to assess the cytotoxic effects of apple pomace a colorimetric MTT assay was performed after incubating cell cultures with increasing concentrations of the extracts, as previously described (Mosmann, 1983). The CC_{50} values obtained for these extracts on Vero cells were 5494.9 ± 292.4 $\mu\text{g/mL}$ and 7281.8 ± 509.5 $\mu\text{g/mL}$ for AE and ME extracts, respectively. These values correlated well with morphological changes recorded during microscopic examination of treated cultures. These changes consisted of cell rounding, appearance of cytoplasmic inclusions and the loss of monolayer confluence, and became evident with treatment concentration over 7000 $\mu\text{g/mL}$. At 1200 $\mu\text{g/mL}$, the highest concentration used in antiviral assays, the mean cell viability was close to 90% and 80% for AE and ME extracts, respectively (data not shown).

As part of an ongoing study on the properties of apple pomace, we demonstrated its efficacy in reducing viral replication levels, when assessed against HSV-1 and HSV-2, two enveloped viruses considered as human pathogens of major clinical interest, since

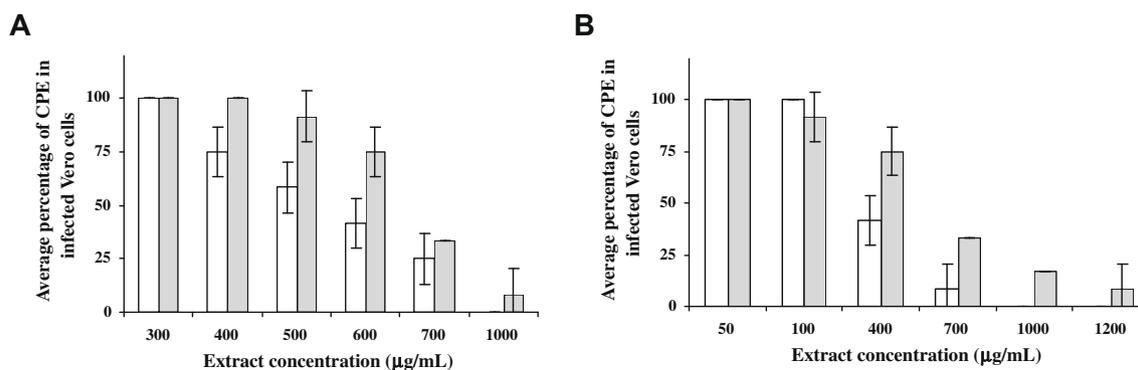


Fig. 1. Average percentage of CPE observed in Vero cells treated with increasing concentrations of acetic (AE, empty bars) and methanolic (ME, dashed bars) extracts, and infected with HSV-1 (A) or HSV-2 (B) clinical isolates. Data are presented as mean of three independent experiments and its standard deviation.

Table 2

Antiviral activity of apple pomace extracts. Data are presented as mean of three independent experiments and its standard deviation.

Extracts	CC ₅₀ ^a (μg ⁺ /mL)	HSV-1		HSV-2	
		CE ₅₀ ^b (μg ⁺ /mL)	SI	CE ₅₀ (μg ⁺ /mL)	SI
AE	5494.9 ± 292.4	576.7 ± 17.2	9.5	450.7 ± 40.8	12.2
ME	7281.8 ± 509.5	710.9 ± 1.6	10.2	629.6 ± 50.7	11.6
ACV ^c	>100	2.8	>35.7	5.1	>19.6

^a Concentration reducing cell viability by 50%.

^b Concentration reducing virus-induced CPE by 50%.

^c Data from an individual experiment.

⁺ μg of lyophilised pomace extract.

they are associated with high morbidity and globally spread human diseases.

Morphological alterations of Vero cells induced by the replication of HSV-1 and HSV-2 were completely prevented when 1000 μg/mL of AE were present in infected cell cultures. Likewise, a concentration of 1200 μg/mL of ME was able to decrease the percentage of CPE by more than 90% (Fig. 1A and B). For each extract and virus species a selectivity index (SI) was calculated by dividing the CC₅₀ by the corresponding EC₅₀ value (Table 2).

The inhibition of virus replication is achieved within extracts concentration ranges which were at least nine orders lower than those of cytotoxic concentrations.

Both, HSV-1 and HSV-2 viruses used in this study were considered ACV-resistant isolates, since EC₅₀ values found for this nucleoside analogue were greater than 2 μg/mL, as has previously been reported (Stranska, van Loon, Polman, & Schuurman, 2002).

The antiherpetic properties of apples pomace must be probably due to the presence of flavonoids which are known to inhibit these viruses (de Bruyne et al., 1999; Khan, Ather, Thompson, & Gambari, 2005).

Taken together, the beneficial properties of these apple residues outlined in this work aim at highlighting the feasibility of its use with medicinal purposes, as a dietary supplement, or included in topical formulations to treat the herpetic skin symptomatic lesions. At present, our group is analysing different types of apple pomaces (single-cultivar and industrial) in order to compare their antioxidant and antiviral activities.

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