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Antibacterial and antioxidant properties of phenolic-rich extracts from apple (*Malus domestica* cv. Gala)

[1*]Raphaelli, C. O., [1]Dannenberg, G., [1]Dalmazo, G. O., [1]Pereira, E. S., [1]Radünz, M., [2]Vizzotto, M., [1]Fiorentini, A. M., [3]Gandra, E. A. and [1]Nora, L.

[1]Departamento de Ciência e Tecnologia Agroindustrial, Universidade Federal de Pelotas, 96010-900 Caixa Postal 354, Pelotas, RS, Brazil
[2]Embrapa Clima Temperado, 96010-971 Rodovia BR 392, km 78, Pelotas - RS, Brazil
[3]Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, 96010-900 Caixa Postal 354, Pelotas, RS, Brazil

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Abstract

Phytochemicals such as phenolic compounds have antimicrobial properties and are present in apples. In the present work, the antibacterial activity of apple extracts was evaluated against *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* Typhimurium and *Staphylococcus aureus*. Initially, acetone-ethanol extracts of the Gala apple were purified and fractionated by solid phase extraction to obtain an unfractionated phenolic extract (UPE) and four phenolic fractions (PFs). The identification of phenolic compounds was performed by liquid chromatography-mass spectrometry. The antibacterial activity was determined through the disk diffusion method using the minimum inhibitory concentration and the minimum bactericidal concentration. The antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl, and the total phenolic content was estimated using the Folin-Ciocalteau reagent. The UPE and PF contained flavonoid derivatives including quercetin, rutin, epicatechin, malic acid, chlorogenic acid and phloretin. *L. monocytogenes* was susceptible to the UPE and to all PFs (except PF III), resulting in inhibition zones with diameters ranging from 3.75 mm to 14.68 mm. *S. aureus* was susceptible to nearly all PFs, except PF I and III. PF III effectively inhibited the growth of Gram-negative bacteria, which could be attributed to its prominent antioxidant activity (763.3 µg/mg fraction) and phenolic content (459.3 µg/mg fraction). However, PF IV produced inhibition zones that were similar in size but with higher antioxidant activity (1226.8 µg/mg) and phenolic content (620.6 µg/mg). All the fractions and the UPE exhibited high phenolic content and antioxidant activity. PF I, PF II, PF IV, and UPE showed strong activity against *L. monocytogenes*. Antibacterial activity was observed in all fractions, with fraction IV and the UPE having the broadest spectrum of action, as indicated by their ability to inhibit all Gram-positive bacteria tested. Therefore, the phenolic compounds detected in apples have the potential to be used as natural antibacterial agents and/or antioxidants in the food and pharmaceutical industry.

Introduction

Apples (*Malus domestica* Borkh.) which belong to the Rosaceae family are amongst the most diverse and ubiquitously cultivated fruit species (Park *et al*., 2006). This fruit is a significant part of the human diet due to its large production scale, with 84.6 million tons produced in 2014 (FAO, 2015). In addition, frequent consumption of apples has been associated with beneficial effects against risks, markers and aetiologies of cancer, cardiovascular diseases, asthma, and Alzheimer's disease (Hyson, 2011). Some of these beneficial properties arise from the phenolic-rich composition of apples, such as catechin, epicatechin, rutin, phloridzin and chlorogenic acid (Francini and Sebastiani, 2013). Many phytochemicals, such as phenolic compounds, possess medicinal properties and antimicrobial activities against fungi, bacteria and yeasts (Alberto *et al*., 2006).

In the last few decades, the attention of markets towards new products that possess nutraceutical properties (i.e., capable of decreasing the risk of

*Corresponding author.
Email: chirleraphaelli@hotmail.com

diseases) has boosted scientific research focused on characterising molecules in food products and their derivatives, including fruits. The development of functional foods with health-beneficial properties and the extension of food shelf life are the main goals of food science research (Francini and Sebastiani, 2013).

Contamination with microorganisms and food oxidation are important causes of food spoilage. Food spoilage can alter the sensorial attributes of products (e.g., aroma, colour and flavour; Sohaib *et al*., 2017), and the presence of pathogenic bacteria can compromise the safety of food. Microbial spoilage may manifest itself as visible growth (slime, colonies), as textural changes resulting from the degradation of polymers, or as off-odours and off-flavours (Gram *et al*., 2002). More importantly, microbial spoilage presents health risk for consumers (Møretrø and Langsrud, 2017).

The antimicrobial activity of extracts containing concentrated polyphenols may be an interesting subject of study (Albayrak *et al*., 2010). Antimicrobial agents, including food preservatives, have been used to inhibit the growth of food-borne bacteria and extend the shelf life of processed foods. Many extracts from plants, herbs, and spices possess antimicrobial functions, and could be used as a source for antimicrobial agents that prevent food spoilage and inhibit the growth of pathogens (Bagamboula *et al*., 2003; Albayrak *et al*., 2010).

Phenolic extracts from fruits as well as synthetic phenolics are used as antioxidants or additives in the food, pharmaceutical and cosmetic industries. The direct addition of natural compounds to foods is the most common method of phenolic application. Dipping, spraying and coating foods with active solutions prior to packaging are effective techniques to control food spoilage (Lucera *et al*., 2012).

Many plant polyphenols are known to possess antimicrobial properties (Puupponen-Pimiä *et al*., 2001). The increase in the use of antibiotics in clinical medicine has led to the growing incidence of bacterial resistance, thereby prompting the search for new active compounds against multi-drug resistant pathogens. In this context, phenolic fractions from apple extracts have been tested against Gram-positive and Gram-negative bacterial strains. However, the effect of concentrated phenolic extracts (unfractionated or fractionated through solid phase extraction) from apples on pathogens is still poorly known. In the present work, the antioxidant activities and phenolic contents of apple extracts obtained by solid phase extraction were evaluated and their antibacterial activities against *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Staphylococcus aureus* were investigated.

Materials and methods

Materials

Samples of *Malus domestica* cv. Gala were obtained directly from the producer, the Randon Agrosilvopastoril S.A. (RASIP), from orchards in the municipality of Vacaria, state of Rio Grande do Sul, Brazil. *L. monocytogenes* (ATCC 7644), *S. aureus* (ATCC 6538), *E. coli* (ATCC 8739), and *S.* Typhimurium (ATCC 14028) were obtained from the Food Microbiology Laboratory of the Federal University of Pelotas, Brazil. The bacterial strains were cultivated in brain heart infusion broth (BHI-Acumedia®) and the following agars were used: chromogenic (CR-Oxoid®), Baird-Parker (BP-Oxoid®), mannitol lysine crystal violet brilliant green (MLCB-Oxoid®), eosin methylene blue (EMB-Oxoid®) and Müeller-Hinton (MH-Oxoid®). All the following chemicals, standards, and reagents used were of analytical grade and acquired from Sigma–Aldrich® (USA); methanol, hydrochloric acid, sodium hydroxide, acetone, ethanol, dimethyl sulfoxide (DMSO), ethyl acetate, acetonitrile, Folin-Ciocalteau reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate and chlorogenic acid.

Methods

Solid phase extraction

Refrigerated whole apple fruits were sliced (approximately 3 mm in thickness) and 50 g sliced fruits were submerged in 150 mL 1:3 solution of 100% acetone and absolute ethanol, and stored at 8 ± 2°C in the dark. For phenolic extraction, the samples were blended (Ultra-turrax® IKA® Werke GmbH and Co, Staufen, Germany) for 6 min and centrifuged (Eppendorf 5810 R) at 1,792 g at 0°C for 25 min. The crude extract was filtered and concentrated by evaporation under vacuum (Rotavapor® R II Brand Buchi) for 90 min at 40°C for complete removal of the solvent. During all the processes, the samples were stored at 8 ± 2°C in the dark. The crude apple extracts were further fractionated through solid phase extraction (SPE). Following this, the crude apple extracts were diluted in water at a ratio of 1:4 (50 mL extract in 200 mL ultrapure water). The solution was purified and fractionated through SPE at 10 mm Hg on a 35 cm^3 Sep-Pak C-18 Vac cartridge (Part no. WAT043345-Waters Association, Milford, MA, USA) containing 10 g silica following the methodology adapted by Vizzotto *et al*. (2014). This

process resulted in the production of an unfractionated phenolic extract (UPE) and four phenolic fractions (PF). The PFs and UPE were diluted in water to final concentrations of 1 mg/mL (PF I, II, and IV), 50 mg/mL (PF III), and 500 mg/mL (UPE).

Identification of phenolic compounds

The identification of phenolic compounds in the unfractionated phenolic extract (UPE) and its fractions (PF) was performed using a Xevo UPLC-QTOF-MSN equipment. The analysis was performed in an Acquity UPLC system (Waters), coupled to a Quadrupole Time of Flight (QTOF, Waters) system belonging to the Brazilian Agricultural Research Corporation-EMBRAPA. Chromatographic runs were performed on a Waters Acquity UPLC BEH column (150 mm × 2.1 mm × 1.7 µm), at a fixed temperature of 40°C, with the mobile phases of 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), its gradient varied as follows: 0 - 15 min (2% - 95%) of B; 15.1 - 17 min (100%) of B; 17.1 - 19.1 min (2%) of B. A flow rate of 0.4 mL/min and injection volume of 5 µL were used. High Resolution Mass Conditions-Xevo-QToF. The ESI- mode was acquired in the range of 110 – 1,180 Da, at a fixed source temperature at 120°C, desolvation temperature of 350°C, desalting gas flow of 500 L/h, extraction cone of 0 V and 5 V, 20 kV sample sampling cone, and 2.6 kV capillary voltage. At low scan, the collision energy was 5 eV. At high scan, the collision energy ramp was 20 - 40 ramp eV (trap). The ESI positive mode was acquired in the range of 110 – 1,180 Da, fixed source temperature at 120°C, desolvation temperature of 350°C, desolvation gas flow of 500 L/h, extraction cone of 0.5 V, sampling cone of 32 V, and capillary voltage of 3.2 kV. At low scan, the collision energy was 5 eV (trap). At high scan, the collision energy ramp was 20 - 40 ramp eV (trap). Leucine enkephalin was used as a lock mass. The acquisition mode was MSE. The instrument was controlled by a Masslynx 4.1 software (Waters Corporation).

Antibacterial activity

The antibacterial activities of the fractions (PF I, PF II, PF III, and PF IV) and UPE were evaluated through a modified disk-diffusion assay (CLSI, 2015). All tests were performed in triplicate, and water was used as a control. Bacteria were activated in BHI broth for 12 h. The concentration of the bacterial culture was adjusted to 1.5×10^8 CFU/mL (0.5 McFarland) in peptone water (Acumedia®). The inoculum was spread uniformly on the surface of plates (Cralplast®, 90 mm × 15 mm) containing 4 mm of MH agar (pH 7 ± 0.2); using a sterile swab (Absorve®). A sterile paper disk (Laborclin®) 6 mm in diameter was placed at the centre of each plate, upon which 10 µL UPE or fractions was placed. The plates were incubated at 37°C and the diameters of inhibition zones around the paper disk were measured after 24 h using a digital pachymeter (King Tools®). Sterile water was used as a negative control, and the diameters of the inhibition zones defined the sensitivity of the bacteria to the fractions and the UPE.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined following the method described by Cabral *et al*. (2009) with minor modifications. MIC was determined by testing the activity of the samples (100 µL) at three different concentrations: undiluted, 1:100 (1 µL sample in 99 µL DMSO), and 1:1,000 (0.1 µL sample in 99.9 µL DMSO). The absorbance of solutions containing the sample and bacteria was measured at 620 nm at the time of preparation and 24 h after incubation (37°C) using a spectrophotometer (Biochrom EZ Read 400). The lowest sample concentration at which there was no bacterial growth in the culture medium was considered as the MIC.

MBC was determined from the results obtained in MIC. Aliquots of 10 µL that showed no visible bacterial growth were seeded and incubated for 24 h at 37°C in BHI agar. The lowest concentration at which no bacterial growth occurred was considered as MBC.

Antioxidant activity and total phenolic content

For the assessment of antioxidant activity, 0.01 g freeze-dried sample was mixed (in a vortex mixer) with 5 mL ultrapure water in a screw-cap tube. The antioxidant activity was quantified through a modified version of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical method described by Brand-Williams *et al*. (1995). Briefly, 20 µL of either the UPE or PF was added to 280 µL DPPH and allowed to react for 4 h in the dark at 25°C. Absorbance was measured at 515 nm using a UV-Vis spectrophotometer (SpectraMax 190 Microplate Reader, Molecular Devices). Methanol (95%) was used as a control. Antioxidant activity was estimated as micrograms of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) per mg of PF or UPE. The antioxidant activity of each sample was obtained by comparing the absorbance values of the samples to those from a standard curve ($R^2 = 0.9882$), obtained by allowing DPPH to react with Trolox in methanol at concentrations ranging from 0 to 300 µg/mL. All the analyses were performed in triplicate. For the measurement of phenolic content, 0.1 g freeze-dried sample was mixed (in a vortex

mixer) with 5 mL 95% methanol in a screw-cap tube. The PFs and UPEs were quantified in triplicate through the Folin-Ciocalteau method adapted from Swain and Hillis (1959). Absorbance was measured at 725 nm (SpectraMax 190 Microplate Reader, Molecular Devices). Prior to measurement, 95% methanol was used as a blank control. Chlorogenic acid was dissolved in 95% methanol at concentrations ratios of 0 to 400 mg/mL; the resulting solutions were used to obtain a standard curve ($R^2 = 0.9993$). The total phenolic concentrations were expressed as chlorogenic acid equivalents.

Statistical analysis

All assays were conducted in triplicate, and statistical analyses (descriptive and comparison of means) were performed using the statistical analysis system Winstat version 2.11.

Results

The unfractionated phenolic extract (UPE) and fractions (PFs) were concentrated through SPE, which minimises the risk of phenolic degradation. In this simple, effective and versatile method, components of interest are concentrated and separated from other species by applying the sample mixture to an appropriate solid sorbent and selectively eluting the desired components (Michalkiewicz *et al*., 2008). This reduces the use of solvents and allows more effective separation of compounds. After being purified and fractionated, the compounds were identified by UPLC-QTOF-MSN. Due to the presence of flavonoid derivatives such as quercetin, hydroxycinnamic acid and anthraquinone floretin, the PFs and UPE have been shown to be promising antimicrobials, based on substances in previous reports from the same species or genus (Table 1).

The results from the action of UPEs and PFs against pathogenic bacteria based on the inhibition zones are shown in Table 2. Gram-positive bacteria were more sensitive than Gram-negative bacteria, except PF III, for which the inhibition zones were significantly higher with *S.* Typhimurium and *E. coli*. *L. monocytogenes* was susceptible to UPEs and to all PFs (except PF III), resulting in inhibition zones with diameters ranging from 3.75 mm to 14.68 mm. *S. aureus* was susceptible to PF II and PF IV. According to the previously defined classification for active and non-active extracts, PF IV exhibited antibacterial activity against *L. monocytogenes* and *S.* Typhimurium. PF IV and UPE also showed activity against both *L. monocytogenes* and *S. aureus*. Extracts containing phenolic compounds such as procyanidin, rutin, quercetin and floretin-2'-O-glycoside, demonstrated antimicrobial activity against Gram-positive bacteria (Cabral *et al*., 2009).

The minimum inhibitory concentrations (MIC)

Table 1. Phenolic compounds identified in apple extracts as obtained by solid phase extraction.

Phenolic compound		PF I[a]	PF II[a]	PF III[a]	PF IV[a]	UPE[b]
Name	Empirical formula	Retention time (min)				
Malic acid α-D-glucoside	$C_{10}H_{15}O_{10}$	0.99				
Glucosyl-O-pentosyl-O-glucoside	$C_{16}H_{23}O_{14}$	1.44				
Oligomers of hexoses	$C_{18}H2_9O_{15}$	1.67				
Carboxybenzoate	C_8H_5O4	3.46				
Procyanidin B dimer	$C_{30}H_{25}O_{12}$	-	2.76	2.76	2.76	2.76
Chlorogenic acid 5-caffeoylquinic acid	$C_{16}H_{17}O_9$	-	3.05	3.05	3.05	3.05
Procyanidin B dimer isomer	$C_{30}H_{25}O_{12}$	-	3.25	3.25	3.25	3.25
Amygdalin	$C_{20}H_{26}NO_{11}$	-	3.35	3.35	3.35	3.35
Epicatechin	$C_{15}H_{13}O_6$	-	3.51	3.51	3.51	3.51
4-*p*-coumaroylquinic	$C_{24}H_{37}O_{12}$		3.59	3.59	3.59	3.59
Procyanidin B trimer	$C4_5H_{37}O_{18}$	-	-	3.74	3.74	3.74
Rutin	$C_{27}H2_9O_{16}$	-		4.12	4.12	4.12
Quercetin hexoside	$C_{21}H1_9O_{12}$	-		4.24	4.24	4.24
Quercetin derivative	$C_{27}H_{27}O_{16}$	-		4.46	4.46	4.46
Quercetin-O-α-L-arabinofuranoside	$C_{20}H_{17}O_{11}$	-		4.62	4.62	4.62
Phloretin-2′-O-(2″-O-xylosyl)-glucoside	$C_{26}H31O_{14}$	-		4.76	4.76	4.76
Phloretin-2′-O-(2″-O-xylosyl) glucoside isomer	$C_{26}H31O_{14}$	-		4.82	4.82	4.82
Phlorentin-2'-O-glucoside	$C_{21}H_{23}O_{10}$	-		5.19	5.19	5.19

[a]PF: phenolic fractions obtained by solid phase extraction; [b](UPE): Unfractionated phenolic extract.

Table 2. Inhibition zones (mm) obtained through the agar diffusion method following exposure of pathogenic bacteria cultures to apple phenolic extracts.

Treatment	Inhibition zone (mm)*			
	Gram-positive		Gram-negative	
	L. monocytogenes ATCC 7644	*S. aureus* ATCC 6538	*E. coli* ATCC 8739	*S.* Typhimurium ATCC 14028
PF I	3.75 ± 0.89^{Ac}	n.d.	n.d.	4.59 ± 0.34^{Ab}
PF II	6.13 ± 0.07^{Bb}	10.93 ± 0.16^{Ac}	n.d.	n.d.
PF III	n.d.	n.d.	7.5 ± 0.6^{Aa}	7.0 ± 0.0^{Aa}
PF IV	14.65 ± 0.33^{Ba}	16.19 ± 0.05^{Ab}	n.d.	n.d.
UPE	14.68 ± 0.26^{Ba}	17.68 ± 0.09^{Aa}	n.d.	n.d.

Data are means ± standard deviations of three replicates (n = 3). n.d.: not detected. Means with similar small letter superscripts in a column, and capital superscripts in a row did not differ significantly by Tukey's test ($p \leq 0,05$). PF: phenolic fraction obtained by solid phase extraction (SPE). UPE: Unfractionated phenolic extract.

Table 3. Minimum inhibitory concentration (MIC) of phenolic fractions and unfractionated phenolic extract of apple against pathogenic bacteria.

Bacterium	MIC*				
	Phenolic Fractions (PF)				Unfractionated phenolic extract (UPE)
	I	II	III	IV	
S. aureus ATCC 6538	0.05	0.05	0.05	0.05	0.05
E. coli ATCC 8739	0.5	0.5	0.05	n.d.	0.05
L. monocytogenes ATCC 7644	0.5	0.05	n.d.	50	50
S. Typhimurium ATCC 14028	0.05	0.05	0.05	0.05	0.5

*n = 4; n.d.: not detected.

Table 4. Minimum bactericidal concentration (MBC) of phenolic fractions and unfractionated phenolic extract of apple against pathogenic bacteria.

Bacterium	MBC				
	Phenolic Fractions (PF)				Unfractionated phenolic extract (UPE)
	I	II	III	IV	
S. aureus ATCC 6538	+	+	+	+	+
E. coli ATCC 8739	+	+	+	+	+
L. monocytogenes ATCC 7644	+	+	+	+	+
S. Typhimurium ATCC 14028	+	+	+	+	+

- : inhibition of bacterium; + : growth of bacterium.

against pathogenic bacteria are shown in Table 3. An extract is considered to have a strong activity when the value of MIC is lower or equal to 0.5 mg/mL. An extract with MIC between 0.6 and 1.5 mg/mL is considered to have moderate activity, and an extract with MIC above 1.6 mg/mL is considered to have low activity (Cabral *et al*., 2009). Considering this classification, all the PFs and UPEs showed strong activity against *S. aureus* and *S.* Typhimurium. Against *E. coli* and *L. monocytogenes*, nearly all the PFs, except PF IV, had strong activities. The UPE had strong activity against *S. aureus*, *E. coli* and *S.* Typhimurium, but not against *L. monocytogenes*.

The MIC is the lowest concentration of a chemical required to prevent visible growth of bacterium. All the fractions and the purified extract exhibited inhibitory activity but did not promote bacterial death. Despite their strong antimicrobial activity as evidenced by their low MIC, the UPE and the PFs did not promote bacterial cell death as determined through the MBC. This outcome could be attributed to the low extract concentrations used in the present work (Table 4).

Figure 1 shows the antioxidant activities and the phenolic contents of the concentrated apple extracts. PF III and PF IV had high antioxidant activities (763.3 µg and 1226.8 µg of Trolox equivalent per mg of PF, respectively) and phenolic content (459.3 µg and 620.6 µg of chlorogenic acid equivalent per mg of fraction, respectively). The unfractionated phenolic extract had moderate antioxidant activity (814.2 µg/mg).

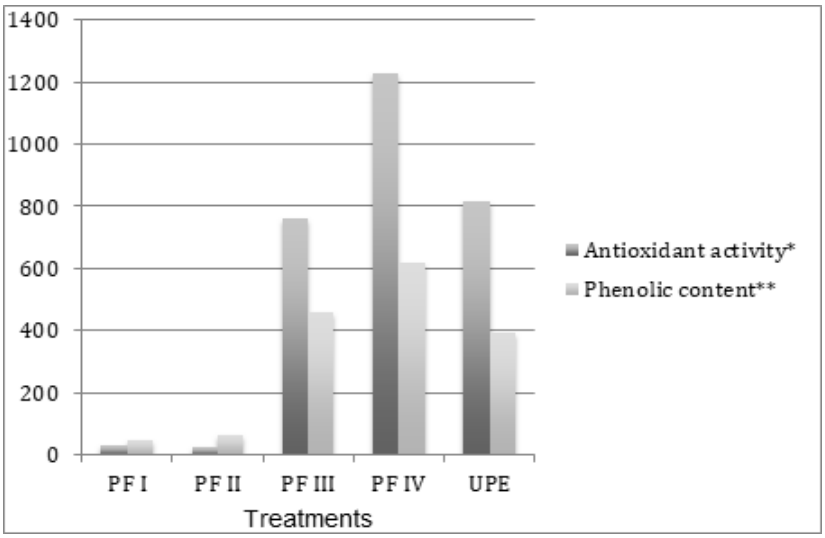

Figure 1. Antioxidant activities and phenolic contents of the apple phenolic fractions (PF) and apple unfractionated phenolic extracts (UPE). Data are means ± standard deviations of three replicates (n = 3); *µg Trolox equivalent per mg of sample; **µg of chlorogenic acid equivalent per mg of sample.

Discussion

Phenolic-rich extracts from apple could serve as a source of antimicrobial agents against food spoilage and pathogens. In the present work, phenolic-rich extracts from apple were characterised in terms of composition, antioxidant activity and phenolic content, and tested against *E. coli*, *L. monocytogenes*, *S.* Typhimurium, and *S. aureus*. These pathogens are amongst the most important agents of food-borne illnesses in humans and animals. The most common condition is bacterial food poisoning, caused by *Salmonella*, *Staphylococcus*, *Clostridium perfringens*, *E. coli*, or *Bacillus cereus*, with common symptoms of gastroenteritis, fever, and weakness (Adley and Ryan, 2016).

Food-borne diseases affect the health and wealth of society; so their detection and control constitute significant components of the overall management of food-borne bacterial pathogens (Billington and Hudson, 2014). Studies show that phenolic compounds, including flavonoids (Panche *et al*., 2016), phenolic acids and polyphenols (Alberto *et al*., 2006), have antimicrobial properties.

However, the available studies were performed with non-purified and/or non-fractionated extracts, and/or missing compound identification, making it difficult to interpret the results. In the present work, the identification of phenolic constituents revealed that the UPE contained the same phytochemicals as PF III and PF IV; PF I contained malic acid α-D-glucoside, glucosyl-O-pentosyl-O-glucoside, oligomers of hexoses and carboxybenzoate, and PF II contained procyanidin B dimer, chlorogenic acid, 5 caffeoylquinic acid, procyanidin B dimer isomer, amygdalin, epicatechin and 4 p coumaroylquinic. From the literature, it is possible to detect several polyphenolic molecules in apples, such as (+) - catechin and (−) - epicatechin (flavan-3-ols or flavonols), phloridzin (dihydrochalcone glycosides), quercetin (flavonols), cyanidin (anthocyanidins), cyanidin-3-O-galactoside (anthocyanins), chlorogenic acid (phenolic acids), and hydroxycinnamates (p-coumaric acid) (Francini and Sebastiani, 2013). Other studies have shown that Gala apples are rich in catechin, epicatechin, rutin, chlorogenic, and caffeic acid (Escarpa and González, 1998), as well as containing elevated amounts of rutin (Minnocci *et al*., 2010).

The UPE and PFs were concentrated as per the SPE procedure that minimises the risk of phenolic degradation in samples (Michalkiewicz *et al*., 2008). However, studies demonstrating the antimicrobial activity of *Malus domestica* phenolic extracts (concentrated per solid phase extraction) are poorly explored in the literature.

Against *L. monocytogenes*, all the treatments, except PF III, showed antibacterial activity, with inhibition zones ranging from 3.75 mm to 14.68 mm. Against *S. aureus*, all the treatments, except PF I and PF III, showed antibacterial activity, with inhibition zones ranging from 10.93 mm to 17.85 mm. PF IV, which caused large inhibition zones against *L. monocytogenes* and *S. aureus*, had high antioxidant activity (1226.8 µg/mg) and high phenolic content (620.6 µg/mg). UPE, that was more effective against Gram-positive bacteria, also had high antioxidant

activity (814.2 µg/mg) and high phenolic content (390.8 µg/mg). All treatments showed antibacterial activity, prominently against Gram-positive bacteria. The inhibition zones were significantly larger for *S. aureus* than for *L. monocytogenes*. Extracts from "Granny Smith" apples with high phenolic content have been shown to possess high antibacterial activity against *E. coli*, *S. aureus* and *L. monocytogenes* (Alberto *et al*., 2006). In the present work, the Gram-positive bacterial strains tested were more sensitive to the phenolic extracts, which differed from other studies, where Gram-negative bacteria produced larger inhibition halos (Zhang *et al*., 2016).

There have been no studies demonstrating the antimicrobial activity of extracts from apples concentrated through SPE. It should be noted that the composition of PF III was similar to those of PF IV and UPE, with very high amounts of phenolics. Phenolic compounds of nutraceutical importance, such as catechins, epicatechin, epigallocatechin, and epigallocatechin-3-gallate, have been described as potent antimicrobial agents (Sourabh *et al*., 2014). The presence and size of inhibition halos indicate the susceptibility of bacteria to phenolic extracts; halos smaller than 7 mm indicate a non-active extract and those larger than 12 mm indicate an extract with satisfactory antimicrobial activity (Sourabh *et al*., 2014). It could be inferred that the phenolic content of the extracts are directly related to their antimicrobial potential. However, it is important to emphasise that the results of inhibition zones should not be simply compared, since some variables such as the type of extract and the growth medium are not directly related to the antimicrobial potential (Duarte *et al*., 2005).

In a similar study, Shahbazi (2017) did not observe inhibition halos after the incorporation of methanolic extracts of apples (*Malus pumila*) in culture media inoculated with *E. coli*, *S. aureus*, *S.* Typhimurium and *L. monocytogenes*. In a study conducted by Tahera *et al*. (2014), the pulp aqueous extracts of apples from Bangladesh promoted the formation of inhibition halos with diameters of 9 mm (for *Escherichia* spp.), 9.5 mm - 10 mm (for *Staphylococcus* spp.), 9.7 10 mm (for *Listeria* spp.), and 7.8 11 mm (for *Salmonella* spp.). In the present work, UPE and PFs promoted the formation of inhibition halos with diameters of 7.5 mm (for *E. coli*), 10.93 17.68 mm (for *S. aureus*), 3.75 14.68 mm (for *L. monocytogenes*), and 4.59 7.0 mm (for *S.* Typhimurium).

Ethyl acetate extracts from Golden Delicious apple pomace showed good inhibitory activities against *S. aureus* with a minimum inhibition concentration (MIC) of 1.25 mg/mL, and against *E. coli* with a MIC of 2.50 mg/mL (Zhang *et al*., 2016). In the present work, PFs and UPE presented good MICs against all bacterial strains, except PF IV against *E. coli* and PF III against *L. monocytogenes*. Pires *et al*. (2018) showed that hydromethanol extracts of *Malus domestica* Borkh. cv. 'Bravo de Esmolfe' had high MIC against *E. coli* and *L. monocytogenes* (5 mg/mL), indicating a considerable level of antimicrobial activity. Similarly, Shahbazi (2017) obtained MIC values superior to 10 mg/mL for methanolic extracts of *Malus pumila* against *E. coli*, *S. aureus*, *L. monocytogenes*, and *S.* Typhimurium.

According to MIC results, all bacterial strains tested in the present work were sensitive to one or more treatments; however, this occurred without bacterial cell death, as determined through the MBC. Apple edible films enriched with apple peel polyphenols were highly effective against *L. monocytogenes* (Du *et al*., 2011). Studies have demonstrated that phenolic extracts of apple peels from the "Royal Gala" and "Granny Smith" varieties inhibited the growth of *E. coli*, *Pseudomonas aeruginosa* and *S. aureus* to a high degree (Yamaguchi *et al*., 2008). Another study demonstrated the effective antimicrobial activity of apple peel extracts against *L. monocytogenes* and *S. aureus* (Friedman *et al*., 2013). Fratianni *et al*. (2011) have also shown that the mechanism underlying the antimicrobial activity of "Annurca" apple extracts may be based on the regulation of cell growth or the inhibition of quorum sensing detection (cell-cell signalling).

S. aureus and *L. monocytogenes* were more sensitive to the treatments. These agents cause food-borne diseases, which are a major problem in public health, being responsible for diseases of varying severity and death worldwide, causing a huge social and economic impact on communities and their health systems. *S. aureus* causes serious infections and can lead to food poisoning by releasing enterotoxins in foods, and toxic shock syndrome, by releasing superantigens into the bloodstream. It is also one of the main causes of hospital infections, being associated with increased mortality rates and longer hospital stays (de Kraker *et al*., 2011). *L. monocytogenes*, usually transmitted by dairy products, causes fever, fatigue, malaise, and may or may not cause nausea, vomiting, pain and diarrhoea. Meningitis, meningoencephalitis, encephalitis and septicaemia might occur in more severe cases (Friedman *et al*., 2013).

All the apple extracts presented high contents of phenolic compounds, in particular PF IV. Other fruits, such as strawberries, have a high phenolic content

and are associated with the inhibition of microbial growth (Oliveira *et al*., 2016). Methanolic extracts of a native Iranian apple cv. Gala, obtained through SPE, yielded a high phenolic content (Faramarzi *et al*., 2014). Fractions from ethanolic extracts of apple peels containing quercetin and epicatechin glycosides, showed high antioxidant activities when combined with other polyphenols such as fluoridine and cyanidin-3-O-galactoside (Sekhon-Loodu *et al*., 2013). Total phenolic content was found to be positively correlated to bacterial inhibition activity in four apple cultivars (Qi, 2003). However, in the present work, antibacterial activity was probably more strongly influenced by the specific phenolic compounds found in the extract than the total amount of phenolics present.

Other studies confirm the microbial activity of phenolic compounds. Chitosan films incorporated with various concentrations of gallic acid increased the antimicrobial activity against *E. coli*, *Salmonella*, *Listeria innocua* and *Bacillus subtilis* (Sun *et al*., 2014). Extracts from the bark and fruit pericarp of mangosteen contain mixtures of phenolic compounds and were active against Gram-positive bacteria, notably *L. monocytogenes* (Palakawong *et al*., 2013.). Phenol-rich (2.12 mg of gallic acid/g to 30.63 mg of GAE/g) fruits, such as *Crataegus oxyacantha* L. (Rosaceae), exhibited antifungal and antimicrobial potential against *E. coli*, *Pseudomonas aeruginosa*, *Salmonella ebony*, *Aspergillus niger*, and *Candida albicans* (Kostić *et al*., 2012).

Conclusion

In conclusion, all the fractions and the unfractionated phenolic extract exhibited high phenolic content and antioxidant activity, in addition to a strong activity against food-borne Gram-positive bacterial strains. All the fractions, except PF I and PF III, had strong activities against *S. aureus*. A strong activity was observed against *L. monocytogenes* with PF I, PF II, and PF IV, as well as with UPE. Antibacterial activity was observed for all treatments, with PF IV and UPE having the broadest spectrum of action. The phenolic compounds detected in the apples show a potential to be used as natural antibacterial agents and/or antioxidants in the food and pharmaceutical industry. Future studies comparing the synergistic effect of concentrated extracts, especially from PF IV and UPE, obtained from different apple varieties, may provide interesting outcomes in the control of food-borne bacteria.

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