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Characterization of phenolic compounds, antioxidant and antibacterial potential the extract of acerola bagasse flour

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ABSTRACT. The agro-industrial waste from fruit presents as a promising source for the extraction of active principles with biological activity. This study evaluated the antibacterial and antioxidant activities of the extract of acerola bagasse flour (ABF) and characterized phenolic compounds by high-performance liquid chromatography. The antioxidant activity was evaluated by the free-radical scavenging activity using the ABTS+ procedure and by β-carotene/linoleic acid system. The antibacterial activity was evaluated by agar-well diffusion method, using the microorganisms *Listeria monocytogenes* ATCC 19117, *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 15442 and *Salmonella cholerasuis* ATCC 6539. In ABF extract were identified phenolic compounds, in order of increasing concentration: quercetin, *p*-cumárico acid, gallic acid, epigallocatechin gallate, catechin, syringic acid and epicatechin. This extract showed antioxidant potential and bactericidal activity for both gram-negative and gram-positive strains, presenting potential to be used in the food industry.

Keywords: *Malpighia emarginata*, fruit residue, free radical, antimicrobial.

Caracterização dos compostos fenólicos, potencial antioxidante e antibacteriano do extrato de farinha de bagaço de acerola

RESUMO. Resíduos agroindustriais de frutas apresentam-se como uma fonte promissora para a extração de princípios ativos com atividades biológicas. Neste estudo, avaliaram-se as atividades antioxidante e antibacteriana do extrato de farinha de bagaço de acerola (FBA) e caracterizaram-se os compostos fenólicos por cromatografia líquida de alta eficiência. A atividade antioxidante foi avaliada pelo método de sequestro de radicais livres ABTS e pelo sistema β-caroteno/ácido linoleico. A atividade antibacteriana foi avaliada pela técnica de difusão cavidade em ágar, utilizando os microrganismos *Listeria monocytogenes* ATCC 19117, *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 15442 e *Salmonella cholerasuis* ATCC 6539. No extrato da FBA, foram identificados os compostos fenólicos, em ordem crescente de concentração: quercetina, ácido *p*-cumárico, ácido gálico, galato de epicatequina, catequina, ácido siríngico e epicatequina. Esse extrato apresentou potencial antioxidante e atividade antibacteriana para as bactérias gram-negativas e gram-positivas, apresentando potencial para ser utilizado na indústria de alimentos.

Palavras-chave: *Malpighia emarginata*, resíduo de fruta, radical livre, antimicrobiano.

Introduction

The agro-industrial wastes from fruits are natural sources of bioactive substances, widely recognized by its health promoting properties and technological applications, such as antioxidant and antimicrobial (Jorge & Malacrida, 2008), often surpassing the content of the fruit pulp. Among these bioactive substances, protrude phenolic compounds such as flavonoids and tannins, which have high antioxidant and antibacterial capacity (Doughari, El-Mahmood, & Tyoyina, 2008; Sun et al., 2012).

Natural extracts that have antioxidant activity are of great interest to the food industry since the presence of free radicals leads to oxidation lipids, causing undesirable sensory changes in food with unpleasant odor and flavor and thus, the decrease in shelf life (Mohamed, Pineda, & Aguilar, 2007). Furthermore, it is known that synthetic antioxidants used in industries such as butyl hydroxy anisole (BHA), 2,6-di-tert-butyl-4-hydroxytoluene (BHT), tercbutil- hydroquinone (TBHQ) and propyl gallate (PG), show up toxic and carcinogenic effects in animal experiments (Andrade, Cardoso, Batista, Mallet, & Machado, 2012). Already in biological systems, the production of free radicals is associated with several factors such as DNA mutation, protein oxidation and lipid peroxidation, which is directly related to the formation of atheroma, atherosclerosis,

cancer, and diabetes (Santos et al., 2010; Silva, Costa, Santana, & Koblitz, 2010a).

Similarly, natural extracts that present antimicrobial activity are also extremely important, due to the fact that many microorganisms, such as bacteria, commonly involved in food outbreaks presented resistance, not only to the antibiotics already pre-set, as well as the last generation, causing growing global public health problem (Silva et al., 2010b).

Research has been undertaken in order to find natural substances for these purposes, which will reduce the negative effects of free radicals and microorganisms causing of great damage to the food industry. Plant extracts have shown effectiveness as antioxidants (Silva et al., 2010a; Rabelo, Costa, Libório, & Almeida, 2014) and some have also demonstrated antimicrobial activity (Silva et al., 2010b; Costa et al., 2013; Teles & Costa, 2014).

In this context, the use of agro-industrial residues from fruits presents itself very promising for active principle extraction with different beneficial biological activities and can be used as antioxidants and antibacterial. By discarding these residues, secondary metabolites of great aggregated value with possible applications in pharmaceutical and food industries, are also eliminated. For example, the acerola bagasse originated at the juice processing, which according to Marques, Corrêa, Lino, Abreu and Simão (2013), is rich in phenolic compounds, with content records of 10.82 g 100 g^{-1} of dry matter, however these phenolic compounds were not yet identified.

Given the above, in the present study the objective was to evaluate the antioxidant and antibacterial activities the extract of acerola bagasse flour (ABF) and determine the phenolic compounds by high efficiency liquid chromatography (HPLC), aiming to find possible alternative to the food industry, as well as adding value to the fruit.

Material and methods

Preparation of acerola bagasse flour

Acerola *Malpighia emarginata* DC. (BRS 238 Frutacor) bagasse was obtained from plants grown in the municipality of Perdões, Minas Gerais States, Brazil, used for pulp extraction, and the residual bagasse was provided in three batches by a fruit pulp industry. For the preparation of acerola bagasse flour (ABF), acerola bagasse (4 kg) was frozen at -18 °C and lyophilized in glass containers protected from light for 7 days to obtain 450 g of dry bagasse. After lyophilization, acerola bagasse was homogenized using mortar and pestle, was passed in sieves and most flour particles were retained on sieves sized 40 mesh (0.425 mm) to 80 mesh (0.180 mm), thus, classified as fine and then placed in a hermetically sealed flask, protected from light in a refrigerator at 4°C.

Obtention of the extract

To obtain the methanol extract of acerola bagasse flour (ABF), 1 g of acerola bagasse lyophilized powder was transferred to a 250 mL Erlenmeyer and then added 50 mL of 50% methanol solution in three repetitions. Afterwards, it covered with a ground glass joint and put on a hot plate at 80 °C. After boiling for 15 minutes, the extract was filtered in filter paper and collected to a 250 mL becker. The residue was once again put on an Erlenmeyer and this process repeated for two more times. After the third filtration, the becker was taken to the hot plate to evaporate the methanol until the volume reaches 25 mL (Association of Official Analytical Chemistry [AOAC], 2012) and used to measure the antioxidant activity. For the chromatographic analysis and antimicrobial activity, the extract was frozen, lyophilized and subsequently solubilized in ultrapure water obtained from a Milli-Q system. All analyzes were performed in triplicate.

Antioxidant activity

The methodology used to measure the antioxidant activity in the extract by ABTS method was developed by Rufino, Alves, Brito, & Morais (2007). Four different dilutions were made from the obtained extract for the assays and subsequent construction of analytical curves. Six point analytical curves were made with trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (100 to 2,000 μmol L^{-1}) in addition to tests for comparison with the patterns BHT (butylhydroxytoluene–synthetic antioxidant) and with rutin and quercetin, that are flavonoids with proven antioxidant activity; these standards were prepared at a concentration of 200 mg L^{-1}.

The methodology used to measure the antioxidant activity in the extract by β-carotene/linoleic acid method was developed by Rufino et al. (2007). The test was applied to the extract, at a concentration of 10,000 mg L^{-1}. For the preparation of the β-carotene/linoleic acid solution system, 50 μL of β-carotene diluted in chloroform (20 g L^{-1}) were used, to which 40 μl of linoleic acid were added, as well as 530 μL of tween 20 (emulsifier) and for solubilization, 1 mL of chloroform. In a flask covered with aluminum for protection against light, chloroform was evaporated in a rotary-evaporator and 100 mL of oxygen saturated water (distilled water treated with oxygen for 30 min.) were added,

and the combination was agitated until the solution system presented a yellow-orange coloration. In test tubes, 2.5 mL of that solution system were added to 0.2 mL of each sample dilution used for the test. Control tubes were made containing 2.5 mL of the solution system with 0.2 mL of 2,6-di-tert-butyl-p-cresol (BHT), quercetin and rutin, all at the concentration of 200 mg L^{-1}. In laboratory tests, it was found that the concentration of 200 mg L^{-1} BHT is the one that provides the greatest protection for the system, when compared to others; therefore, its use is suggested. After homogenization, their readings were taken in a spectrophotometer at 470 nm, using water to calibrate the spectrophotometer; this was considered to be the reading at time zero (initial). The tubes were placed in a water bath, at 40°C and readings were taken after 2h.

Antibacterial activity

Evaluation of antibacterial activity was performed by using agar well diffusion method, with *Listeria monocytogenes* ATCC 19117 (gram-positive), *Escherichia coli* ATCC 11229 (gram-negative), *Salmonella cholerasuis* ATCC 6539 (gram-negative) and *Pseudomonas aeruginosa* ATCC 15442 (gram-negative) and the method used was the diffusion in cavity Agar. The ABF extract concentrations evaluated were: 500; 250; 125; 62.5; 31.25; 15.62; 7.81; 3.90, 1.95 μg mL^{-1}, inoculating 10 µL of ABF extract for each concentration and for control treatment (without ABF extract) was inoculated 10 µL of distilled water. As a comparison standard 10 µL of a 100 μg mL^{-1} chloramphenicol antibiotic solution were inoculated (National Committee for Clinical Laboratory Standards [NCCLS], 2003; Pereira et al., 2008). From the diameters of the inhibition zones obtained, one can evaluate the sensitivity profile of bacteria against the different concentrations of the ABF extract. The minimum inhibitory concentration was defined as the lowest concentration of ABF extract that caused inhibition zone.

Identification and quantification of phenolic compounds

The high performance liquid chromatography (HPLC) was performed using a Shimadzu UHPLC chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with two LC-20AT high-pressure pumps, an SPD-M20A UV-vis detector, a CTO-20AC oven, a CBM-20A interface, and an automatic injector with an SIL-20A auto sampler. Separations were performed using a Shim-pack VP-ODS-C18 (250 mm × 4.6 mm) column, connected to a Shim-pack Column Holder (10 mm × 4.6 mm) pre-column (Shimadzu, Japan). The phenolic standards used were gallic acid, catechin, epigallocatechin gallate, epicatechin, syringic acid, *p*-coumaric acid, ferulic acid, salicylic acid, resveratrol and quercetin all obtained from Sigma-Aldrich (St. Louis, MO, USA). The stock standard solutions were prepared in methanol (HPLC grade; Sigma-Aldrich, USA).

The mobile phase consisted of the following solutions: 2% acetic acid in water (A) and methanol: water:acetic acid (70:28:2 v v^{-1} v^{-1}) (B). Analyses were performed for a total time of 65 min. at 40 °C, flux of 1 mL min^{-1}, wavelength of 280 nm, and injection volume of 20 μL in a gradient-type system (100% solvent A from 0.01 to 5 min.; 70% solvent A from 5 to 25 min.; 60% solvent A from 25 to 43 min.; 55% solvent A from 43 to 50 min.; and 0% solvent A for 10 min.) until the end of the run. Solvent A was increased to 100%, seeking to maintain a balanced column. Acetic acid and methanol (HPLC grade; Sigma-Aldrich, USA) were used in the preparation of the mobile phase.

The ABF extract and the standards were filtered through a 0.45-μm nylon membrane (EMD Millipore, USA) and directly injected into the chromatographic system, in three replicates. The phenolic compounds in the extract were identified by comparison with retention times of standards. Quantification was performed by the construction of analytical curves obtained by linear regression using Origin 6.1 computer software (OriginLab, Northampton, MA, USA) and considering the coefficient of determination (R^2) equal to 0.99.

Results and discussion

The yield on the ABF extract was 48 ± 0.01% dry weight.

The antioxidant activity (AA) of the ABF extract by method ABTS was 405.11 μmol trolox L^{-1} g^{-1} (Table 1). It was observed that AA was lower that standards BHT and rutin, representing 22.7 and 27.5% of the activity of these standards, respectively. Already compared to quercetin, the AA of the ABF extract was even lower.

Table 1. Antioxidant activity the extract of acerola bagasse flour by two methods.

	ABTS method μmol trolox L^{-1} g^{-1}	β -carotene / linoleic acid method % inhibition
ABF extract	405.11 ± 1.83	82.17 ± 3.93
BHT	1,787.10 ± 29.40	93.71 ± 1.14
Quercetin	8,076.43 ± 41.08	57.26 ± 2.95
Rutin	1,473.07 ± 21.39	91.20 ± 1.32

Data from three repetitions mean ± standard deviation.

In relation to the AA by the method of β-carotene/linoleic acid, ABF extract showed relatively high AA (Table 1), with superior value than quercetin and slightly lower than BHT and rutin standards.

For the analysis of antibacterial activity were evaluated the following concentrations: 500; 250; 125; 62.5; 31.25; 15.62; 7.81; 3.90, 1.95 0 μg mL^{-1}, but only the concentration of 500 μg mL^{-1} resulted inhibition. Therefore, the inhibitory minimum concentration of the ABF extract was 500 0 μg mL^{-1}, for the bacteria under study. The antibiotic chloramphenicol, used as control, inhibited all bacteria, forming an inhibition zone 14 mm for all tested microorganisms. In the concentration of 0 mg L^{-1} ABF extract the bacteria tested suffered no inhibition. The bacteria *L. monocytogenes* (gram-positive), *E. coli* (gram negative), *P. aeruginosa* (gram negative) and *S. cholerasuis* (gram-negative) showed inhibition zone (mm) 7.5 ± 1.5 (54 % inhibition); 8.5 ± 0.86 (61 % inhibition); 9.3 ± 1.53 (66 % inhibition) and 7.7 ± 1.04 (55 % inhibition), respectively.

It was observed that the ABF extract presented antimicrobial activity greater than 50% compared to the positive control, suggesting that higher concentrations of extract will probably increase the inhibition front of the bacteria tested.

It is known that, in most cases, gram-negative bacteria are less sensitive to antibacterial action than gram-positive bacteria, since cell walls of gram-negative bacteria are rich in polysaccharides which inhibits the penetration of antimicrobial substances (Burt, 2004). However, the ABF extract showed inhibitory effect equal to or higher than presented by gram-positive bacteria.

The antioxidant and antibacterial ability of the ABF extract can be attributed to phenolic compounds in the ABF extract. Thus, in this extract the following phenolic compounds were identified, in mg 100 g^{-1} extract, in order of increasing concentration: quercetin (0.47 ± 0.02), *p*-coumaric acid (3.86 ± 0.21), acid gallic (5.31 ± 0.36), epicatechin gallate (14.60 ± 1.43), catechin (18.13 ± 0.53), syringic acid (59.46 ± 0.18) and epicatechin (146.97 ± 2.39) (Figure 1). The epicatechin had the highest content followed by syringic acid.

The antioxidant activity of phenolic compounds is attributed to the reducing power of aromatic hydroxyl group, which reduces reactive free radicals and produces the radical fenoxila stabilized by resonance. The antioxidant capacity of phenolic structures is influenced by the number and position of hydroxyl groups, as well as by glycosylation positions that are particularly able to bind the iron and copper (Mckay & Blumberg, 2002; Sun, Simonyi, & Sun, 2002; Jung et al., 2003). The flavonoids and tannins contain a variety of hydroxyl groups and show higher antioxidant capacity and elimination of free radicals able of alter the kinetics of lipid peroxidation by modifying the organization of lipid compounds. They stabilize membranes by fluidity, decreasing and preventing the diffusion of free radicals and limiting the peroxidative reaction (Arora, Byrem, Nair, & Strasburg, 2000; Yen, Duh, & Tsai, 2002). These compounds were found in the ABF extract and must have contributed to the antioxidant activity.

While, the antibacterial action of phenolic compounds is related to the inhibition of bacterial enzymes due to their complexation of their substrates, modification microorganisms metabolism, or complexation of metal ions essential for microbial metabolism (Scalbert, 1991). The tannins and flavonoids, such as quercetin, present ability to inactivate enzymes and complexing with extracellular proteins, soluble proteins and with the cell wall of bacteria, setting the probable antimicrobial mechanisms of action. The total rupture of microbial membranes can be given by flavonoid lipophilic character (Mendes et al., 2011).

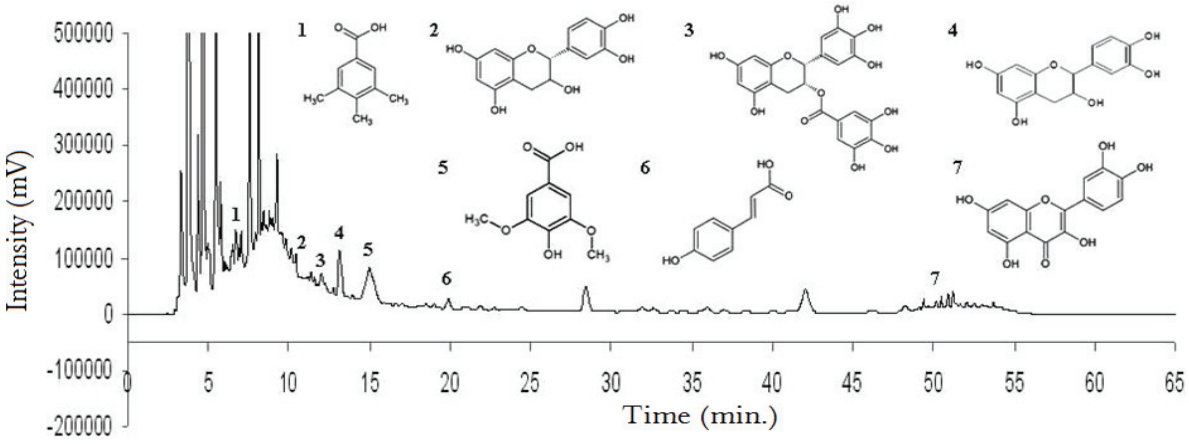

Figure 1. Chromatogram of the extract of acerola bagasse flour with peak identification: 1 - gallic acid (time = 6.541); 2 – catechin (time = 10.419); 3 – epigallocatechin gallate (time = 11.987); 4 – epicatechin (time = 13.139); 5- syringic acid (time = 14.988); 6 – *p*-cumaric acid (time = 19.892) and 7 – quercetin (time = 51.185).

Already the catechin is toxic to microorganisms due to the existence of two hydroxyl groups in its structure (Cowan, 1999). These phenolic compounds were found in the ABF extract and probably contributed to the antibacterial activity.

Conclusion

The ABF extract contains phenolic compounds, in order of increasing concentration: quercetin, *p*-coumaric acid, gallic acid, epicatechin gallate, catechin, epicatechin and syringic acid, and has significant potential to be exploited as an antioxidant and antibacterial agent in the pharmaceutical and food industry.

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