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Chemical characterization, antimicrobial and antioxidant activity of sugar-apple (*Annona squamosa* L.) pulp extract

Caracterización química, actividad antimicrobiana y antioxidante del extracto de pulpa de manzana de azúcar (*Annona squamosa* L.)

ABSTRACT

Synthetic preservatives are widely present in processed foods, but most of them have carcinogenic potential, requiring the development of new natural alternatives such as fruit extracts, for microbial control. The objective of the study was to evaluate the chemical characterization, antioxidant, and antimicrobial activity of the sugar apple pulp (*Annona squamosa* L.). Physicochemical characteristics were evaluated, an extract was prepared, and its antioxidant activity by DPPH method and antimicrobial by disk diffusion. Minimal inhibitory concentration and minimum bactericidal concentration against strains of *Salmonella typhimurium*, *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus* were evaluated. The physicochemical analysis revealed that sugar apple pulp had 75.0% moisture, 3.0% ash, 4.0% protein, 0.2% lipids, 3.3% fibers, and 14.5% carbohydrates. The antioxidant activity of the extract by the DPPH method was 20.6%. The pulp extract from the sugar apple had inhibition zone for *Staphylococcus aureus*, satisfactory inhibitory effect against *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella Typhimurium*, but did not present a bactericidal effect. Sugar apple pulp presents adequate levels of nutrients and potential for food application due to its microbiological activity and antioxidant properties.

Keywords: *Annona squamosa* L; Bactericidal; 2,2-difenil-1-picril-hidrazila; Pulp extract, *Staphylococcus aureus*.

RESUMEN

Los conservantes sintéticos están ampliamente presentes en los alimentos procesados, pero la mayoría tienen potencial carcinogénico, lo que requiere el desarrollo de nuevas alternativas naturales para el control microbiano, como los extractos de frutas. El objetivo del estudio fue evaluar la caracterización química, la actividad antioxidante y antimicrobiana de la pulpa de manzana de azúcar (*Annona squamosa* L.). Se evaluaron las características fisicoquímicas, y se evaluó su actividad antioxidante mediante el método DPPH y antimicrobiano por difusión en disco, concentración inhibitoria mínima y concentración bactericida mínima contra cepas de *Salmonella Typhimurium*, *Escherichia*

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coli, *Listeria monocytogenes* y *Staphylococcus aureus*. El análisis fisicoquímico reveló que la pulpa de manzana de azúcar tiene 75.0% de humedad, 3.0% de cenizas, 4.0% de proteínas, 0.2% de lípidos, 3.3% de fibras y 14.5% de carbohidratos. La actividad antioxidante del extracto por el método DPPH fue del 20.6%. El extracto de pulpa de la manzana de azúcar tenía zona de inhibición para *Staphylococcus aureus*, efecto inhibitorio satisfactorio contra *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* y *Salmonella Typhimurium*, pero no presenta efecto bactericida. La pulpa de manzana de azúcar presenta niveles adecuados de nutrientes y potencial para la aplicación de alimentos debido a su actividad microbiológica y propiedades antioxidantes.

Palabras clave: *Annona squamosa* L.; Bactericida; 2,2-difenil-1-picril-hidrazila; Extracto de la pulpa; *Staphylococcus aureus*.

INTRODUCTION

Over time, there has been a change in food consumption patterns. The population is seeking more healthy, nutritious, sustainable, and ecologically correct food, and looking to replace industrialized products. Synthetic preservatives, like nitrite, may have carcinogenic potential when used in concentrations higher than that allowed by regulations¹. Because of this potential health hazard, viable and natural alternatives to substitute for chemical agents are being explored. Bioactive compounds from leaves, flowers, fruits, and seeds, which have proven antioxidant and antimicrobial activity and may be used to replace chemical preservatives, have been proposed.

The sugar apple (*Annona squamosa* L.) has the following bioactive compounds: diterpenes, alkaloids, and acetogenins in its composition, which confer potential antioxidant, antimicrobial, cytotoxic activity, antitumor, epilepsy, pesticide, vermicide, antimicrobial, immunosuppressant, dysuria, fever, antiemetic, and antimalarial properties^{2,3,4,5,6,7,8}. The sugar apple belongs to the Annonaceae family, which is represented by 120 genera with 2000 distinct species of plants. Its pulp has a white color, it is soft, sweet, with a pleasant aroma and very nutritious. It is rich in vitamins and minerals, mostly vitamins C and complex B2.

Considering the potential harm of chemical preservatives and the potential action of the sugar apple pulp concerning pathogenic microorganisms, the objective of this study was to evaluate the chemical characterization, antioxidant and antimicrobial activity of the sugar apple pulp.

MATERIAL AND METHODS

Sample acquisition and preparation

One hundred sugar-apples were purchased a warehouse and general store company in São Paulo (CEAGESP), Brazil. The fruits were at the mature ripening stage.

The fruit pulp was manually separated from the bark and seeds, and then kept at -18 °C to produce pellets and stored in polyethylene plastic bags in an ultra-freezer - 80 °C until use. For the physical-chemical analyses, the fresh pulp was used, while for the antioxidant and antimicrobial activity analysis samples were dried in air circulation kiln (Cienlab) at 35 °C for five days and subsequently prepared in an ethanolic extract.

Preparation of ethanolic extract

The extract was prepared in the Natural Processing Products laboratory, at the Federal University of Pelotas, Capão do Leão, Brazil, according to previous extraction tests. After drying samples in a laboratory oven, an ethanolic extract was prepared with 25.0 g of pulp (wet basis) and 200 mL of ethanol and sonicated (Ultra Sonic Cleaner - Unique) for 30 mins at 60 °C. Afterward, the extract was filtered, and the solvent was evaporated.

Physical-chemical analysis

The physical-chemical analyses of sugar apple pulp were carried out in the Bromatology laboratory of the Faculty of Nutrition of the Federal University of Pelotas in the city of Pelotas, Brazil. The contents of moisture, ash, crude fibers, crude proteins, and crude lipids were determined by the AACC standards⁹. Carbohydrate content was calculated by difference.

Antioxidant activity

The antioxidant activity of the extract was performed according to the method described by Brand-Williams et al.¹⁰ using the Spectrophotometric DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. Afterward, a solution was prepared with 0.10 g ethanolic extract and 10 mL of methanol. This solution rested for 24 h and, after this period, was subjected to centrifugation at 4200 rpm at 25 °C for 15 min. An aliquot of the supernatant was then removed for analyses.

Antimicrobial activity

For the experiment, commercially acquired standard strains of the following bacterial species were used: *Salmonella Typhimurium* (ATCC 13311), *Escherichia coli* O157:H7 (ATCC 43895), *Listeria monocytogenes* (ATCC 7644) and *Staphylococcus aureus* (ATCC 10832). Suspension media assayed were brain heart infusion broth (BHI) and glycerol (propane-1, 2, 3-triol) in a proportion of 3:1 (v:v). To perform the reactivation, a strap was transferred to Tryptic Soy Broth (TSB) and incubated in a laboratory oven for 24 h at 37 °C. Afterward, it was striated on Petri dishes with selective means and incubated for 24 h at 37 °C for the isolation of the colonies. From this bacterial growth in Petri dishes, a lift was extracted and resuspended in saline solution (NaCl 0.8%), which was standardized at the 0.500 concentration on the McFarland scale (1.5×10^8 UFC mL⁻¹). All trials were performed in triplicate.

Disk diffusion analysis was performed according to the protocol proposed by CSLI¹¹. A saline solution was prepared containing bacterial growth that was striated in Petri dishes with a swab containing Muller-Hinton Agar. Then, 10.0 ml of the extract was collected (125 mg/mL) and placed on the plates with sterilized filter paper discs with 6 mm diameter on the surface, and subsequently incubated for 24 h at 37 °C. Measurement of the inhibition zone was done with the ruler in three measurements, equidistant and expressed in centimeters.

The minimum inhibitory concentration was carried out according to the method proposed by Cabral et al.⁸ Microtitration plates with 96 wells containing in each well 100 µL of BHI broth, 100 µL of Inoculum (80.0 µL of BHI broth and 20.0 µL of saline water with bacterial growth) and the pulp extract in four concentrations: 351 mg mL⁻¹, 69.0 mg mL⁻¹, 3.40 mg mL⁻¹, and 0.340 mg mL⁻¹ were used.

After the sample was prepared, the microtitration plates were evaluated with a microplate reader (Biochrom EZ Read 400) at 620 nm. It was then incubated for 24 h at

37 °C, and after that, a new reading was performed with a spectrophotometer. The minimum inhibitory concentration was the lowest concentration in which bacterial growth in the culture medium was not present.

The minimum bactericidal concentration was carried out according to the method proposed by Cabral et al.¹² From sample wells, 15.0 µL was withdrawn that showed inhibition in the test of minimum inhibitory concentration. These were striated in Petri dishes with BHA agar (Bushnell Haas agar) and were incubated for 24 h at 37 °C. The minimum bactericidal concentration of the plates was considered when there was no bacterial growth in the triplicates.

RESULTS

Physical-chemical analysis and Antioxidant activity

The physical-chemical composition and antioxidant activity of the sugar apple pulp can be observed in table 1. The extract presented 75.0% moisture content, 3.0% ash, 4.0% protein, 0.2% lipid, 3.3% crude fiber, and 14.5% carbohydrates. The percentage of antioxidant activity of the ethanolic extract from the sugar apple pulp was 20.6 %.

Table 1. Physical-chemical composition and antioxidant activity of the sugar apple pulp (*Annona squamosa* L.)

Parameter	%±SD
Moisture	75.0 ± 0.35
Ash	3.0 ± 0.0
Protein	4.0 ± 0.17
Lipids	0.2 ± 0.01
Crude Fibers	3.3 ± 0.01
Carbohydrates	14.5 ± 0.0
Antioxidant activity	20.6 ± 0.0

* Simple arithmetic average of three replicates ± standard deviation.

Antimicrobial activity

The ethanolic extract had a satisfactory inhibition effect for *Staphylococcus aureus* but did not affect *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella Typhimurium* (Table 2). The sample presented a minimum inhibitory concentration of 0.340 mg mL⁻¹ for all bacteria tested (Table 3) and showed no bactericidal effect on the tested concentrations (Table 4).

Table 2. Inhibition zones obtained by the disk diffusion method by application of ethanolic extract from the sugar apple pulp (*Annona squamosa*, L.) for *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* e *Salmonella Typhimurium*.

Bacteria	Inhibition zones (cm*)
<i>Staphylococcus aureus</i>	0.980
<i>Escherichia coli</i>	Nd
<i>Listeria monocytogenes</i>	Nd
<i>Salmonella Typhimurium</i>	Nd

* Simple arithmetic average of three replicates. Nd= not detected.

Table 3. Minimum inhibitory concentration (MIC) of the ethanolic extract of the sugar apple fruit (*Annona squamosa*, L.) for bacteria *Salmonella Typhimurium* m, *Listeria monocytogenes*, *Staphylococcus aureus* e *Escherichia coli*.

Bacteria	Concentrations (mg mL ⁻¹)*
<i>Salmonella Typhimurium</i>	0.340
<i>Listeria monocytogenes</i>	0.340
<i>Staphylococcus aureus</i>	0.340
<i>Escherichia coli</i>	0.340

* Simple arithmetic average of three replicates.

Table 4. Minimum bactericidal concentration (MBC) of the ethanolic extract from the sugar apple pulp (*Annona squamosa* L.) for bacteria *Salmonella Typhimurium*, *Listeria monocytogenes*, *Staphylococcus aureus* e *Escherichia coli*.

Bacteria	Concentration (mg mL ⁻¹)*
<i>Salmonella Typhimurium</i>	Nd**
<i>Listeria monocytogenes</i>	Nd
<i>Staphylococcus aureus</i>	Nd
<i>Escherichia coli</i>	Nd

* The concentration used was the Minimum Inhibitory Concentration;

**Nd – not detected.

DISCUSSION

Physical-chemical analysis

The moisture content of the fruit pulp was 75.0%. This result is superior to that found in atemoya fruits in the study by Santos et al.¹³ The high water content of fruits, combined with low lipids and a high percentage of sugars, are excellent food sources¹⁴. Fiber, ash, and protein content were slightly higher than those found in atemoya fruits in the studies by Santos et al.¹³ and Cruz et al.¹⁵. These small variations are related to the distinct stages of fruit ripening of the studies, as well as the influence of climate and soil.

The fiber and mineral content found in the fruits of *Annona* indicates that it has a potential health benefit, promoting gastrointestinal tract functioning, and may also control diabetes by interfering with the action of digestive enzymes^{16,17}. Thus, the sugar apple can be consumed due to its functional character¹⁸.

Antioxidant activity

The antioxidant potential to control free radical production in fruits is generally good agents in reducing reactive oxygen species due to the presence of phytochemicals such as terpenes, alkaloids, phenols, carotenoids, and anthocyanins¹⁹.

The percentage of antioxidant activity against the DPPH radical of the ethanolic extract from the sugar apple pulp was 20.6 %. The low antioxidant activity of the extract may be related to the type of solvent used, and according to other literature, methanol is more effective than ethanol for extracting compounds with antioxidant potential²⁰. However, due to cytotoxicity, alternatives have been sought to use solvents that do not harm consumers' health. However, the value found in the present study is still higher when compared to other studies in the literature that evaluated the percentage of antioxidant activity of aqueous extracts²¹ and ethanol²², possibly due to due to edaphoclimatic differences that alter fruit composition.

Antimicrobial activity

The formation of the halo of bacterial inhibition with the disk diffusion test indicates if the micro-organism is susceptible to the compound. Halos smaller than 0.700 cm are considered non-active for the bacterium and diameters higher than 1.20 cm have an inhibitory effect according to Arora and Kaur²³.

We observed that ethanolic extract had a satisfactory inhibition effect (0.980 cm) for *Staphylococcus aureus*. The zone formed against *S. aureus* was inferior when compared to the study of Aher et al.²⁴. The authors reported the antimicrobial activity of *Annona squamosa* seeds, through an ether oil extract, and presented an inhibition zone of 1.20 cm for *S. aureus* and 1.04 cm for *E. coli*. Our results were also smaller than those reported by Yusha'u et al.²⁵, where halos of 0.600 cm were observed for *S. aureus* in ethanolic extracts of ethanolic *Annona squamosa*. Differences in halo sizes of the present study may be due to the edaphoclimatic factors that alter fruit composition.

An extract should present a minimum inhibitory concentration of up to 0.500 mg mL⁻¹ to be considered as an efficient antimicrobial agent, between 0.600 to 1.50 mg mL⁻¹ for moderate, and above 1.60 mg mL⁻¹ to be considered a weak antimicrobial agent according to Duarte et al.²⁶. The sample presented a minimum inhibitory concentration of 0.340 mg mL⁻¹ for all bacteria tested, thus can be considered an efficient antimicrobial agent. This result was higher than the one found by Almeida et al.²⁷ in ethanolic extract of *Annona vepretorum* for *E. coli* (0.190 mg mL⁻¹) and lower for *S. Typhimurium* and *S. aureus* which presented a MIC of 1.56 mg mL⁻¹. Patel et al.²⁸ evaluated *Annona squamosa* extract and obtained values of 0.920 mg mL⁻¹ and 1.10 mg mL⁻¹ for *E. coli* and *S. aureus*, respectively. Rabêlo et al.²² evaluated an ethanolic extract of *Annona cherimola* Mill. x *A. squamosa* L. and found values of 0.600 mg mL⁻¹ for *S. aureus*. The differences between the values of Mic may be due to differences in sample cultivation, concentrations of the substances, and differences in methods²⁹.

The sample showed no bactericidal effect on the tested concentrations. While a study evaluating extract ethanolic from *Annona vepretorum* found a bactericidal effect for *E. coli* in concentrations of 0.780 mg mL⁻¹, and 6.25 mg mL⁻¹ for *S. Typhimurium* and *S. aureus*.²⁷ Almeida et al.²⁷, in *Annona densicona* extracts, found values of 10.0 mg mL⁻¹ for *E. coli*, *S. Typhimurium*, and *S. aureus*. The study by Barboza et al.³⁰ showed no effect for *S. Typhimurium* and presented concentrations of 0.500 mg/ml for *E. coli* and *S. aureus* in extracts of *Annona mucosa* extracted with dichloromethane.

The absence of the bactericidal effect found in this study may be related to the concentrations of the extract tested, and higher concentrations may be necessary for causing bacterial death.

CONCLUSION

The ethanolic extract studied presented satisfactory results in physicochemical analysis, similar to other studies. The antioxidant activity observed was low, but superior to that previously demonstrated in the literature. The extract also exhibited an inhibition effect for *S. aureus* when applied purely in the disk diffusion test and presented a satisfactory inhibitory effect for *S. aureus*, *L. monocytogenes*, *E. coli*, and *S. Typhimurium*. However, it showed no bactericidal effect on the tested concentrations. Thus, sugar apple pulp extract presents a potential for application in microbiological and antioxidant control. Further studies are needed with distinct concentrations of extract for food application.

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