Phytochemical characterization, antioxidant potential and antimicrobial activity of *Averrhoa carambola* L. (Oxalidaceae) against multiresistant pathogens

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Abstract

The objective of this work was to perform the phytochemical characterization, to determine total phenols, antioxidant (AAO%) and antimicrobial potential of the ethanolic extracts of carambola. The phytochemical study was carried out through a qualitative analysis of the chemical constituents and quantitative determination of the phenol content. By the Folin-Ciocalteu test. Qualitative and quantitative antioxidant tests were performed using the DPPH method (2,2 diphenyl-1-picryl-hydrazila) and iron reduction (FRAP). The minimum inhibitory concentration (MIC) was determined by microdilution in 96-well plates. The presence of pyrogallic tannins, steroids and saponins has been identified. The highest total phenol content, quantified in the samples, was found in the stem bark (0.0866 mgEAG/g) and in the fruit (0.0734 mgEAG/g). In the antioxidant evaluation, the extracts of the green fruit bagasse (AAO% 71.9%,) and stem bark at 50 μg/mL (AAO% 94%) with CE50 23.7 μg/mL. Leaf extracts, stem bark, ripe fruit bagasse and green fruit bagasse presented MICs of 100 μg/mL against multiresistant pathogenic bacteria and fungi.

Keywords: phytochemical screening, total phenols, phenolic compounds, carambola.

Caracterização fitoquímica, potencial antioxidante e atividade antimicrobiana de *Averrhoa carambola* L. (Oxalidaceae) frente a patógenos multirresistentes

Resumo

O objetivo desse trabalho foi realizar a caracterização fitoquímica, determinar fenóis totais, potencial antioxidante (AAO%) e antimicrobiano dos extratos etanólicos de carambola O estudo fitoquímico foi realizado por meio de análise qualitativa dos constituintes químicos e determinação quantitativa do teor de fenóis totais pelo teste de Folin-Ciocâlteu. Os testes antioxidantes qualitativos e quantitativos foram realizados pelo método do DPPH (2,2 difenil-1-picril-hidrazila) e redução do ferro (FRAP). A concentração inibitória mínima (CIM) foi determinada por microdiluição em placas de 96 poços. Foi identificada a presença de taninos pirogálicos, esteroides e saponinas. O maior teor de fenóis totais, quantificado nas amostras, foi encontrado na casca do caule (0,0866 mg EAG/g) e no fruto (0,0734 mg EAG/g). Na avaliação antioxidante destacaram-se a 500 μg/mL os extratos do bagaço do fruto verde (AAO% 71,9%), e casca do caule a 50 μg/mL (AAO% 94%) com CE50 23,7 μg/mL. Os extratos das folhas, casca do caule, bagaço do fruto e bagaço verde apresentaram CIM de 100 μg/mL contra multirresistentes patogênicos e fungos.

Palavras-chave: triagem fitoquímica, fenóis totais, compostos fenólicos, carambola.

1. Introduction

*Averrhoa carambola* L. (Oxalidaceae) is an evergreen tree typical of tropical regions being popularly known as carambola (Oliveira et al., 2011). In Brazil the caramboleira is cultivated throughout the country especially in hot regions and in domestic orchards for fruit production, consumed both fresh as well as in sweets and juices (Gol et al., 2015). Chemically the carambola is present in your composition flavonoids, alkaloids, saponins, tannins, vitamins C and A, calcium and potassium, these compounds which have been commonly studied by being related to antioxidant activity
and antimicrobial (Khanam et al., 2015). Once the high consumption of natural herbal products for therapeutic purposes has been associated with decreasing incidence of chronic diseases and infectious diseases (Kuhn et al., 2019; Mallmann et al., 2018), this study aimed to perform phytochemical characterization, determining phenols overall, potential antioxidant and antimicrobial activity of etanólicos extracts from different parts of one of the most consumed fruit species in the country, star fruit, as a possible source of antioxidant and natural antimicrobial.

2. Materials and Methods

2.1. Plant sample

The plant material (bark of the stems, leaves, fruits in State of maturation, unripe fruit and ripe fruit) was identified in the Herbarium of the Environment Institute of Alagoas-IMA, deposited with the registry number 54401.

2.2. Preparation of the extract

The sample was weighed and macerated in ethanol at room temperature for 72 hours. The extract was filtered, evaporated in route-Rotary evaporator coupled to vacuum pump for removal of the solvent, so it was retrieved from the ethanolic extract which has been stored and maintained under refrigeration.

2.3. Qualitative analysis of antioxidant activity

The extracts were analyzed by thin-layer chromatography (CCD) using default positive comparison routine. The plates were eluted with increasing polarity solvents and, after drying, were sprayed with 0.4 mol/L solution of DPPH radical in MeOH. The plates were observed until the appearance of yellow under purple coloring Fund, indicative of possible antioxidant activity.

2.4. DPPH method (1.1-diphenyl-2-picrilhidrazil)

The antioxidant potential of the ethanolic extract of propolis was determined by fotocolorimétrico in vitro method held through the kidnapping of free radicals, which was using the DPPH (1.1-diphenyl-2-picril-hidrazil). This analysis is based on ability that the antioxidant compounds have to donate a Proton to the DPPH forming stable resonance structures, stabilizing so called free radical (Brand-Williams et al., 1990; Sánchez, 2002). The extracts were tested at concentrations of 100-500μg/mL (leaf, fruit pulp, pulp from the fruit pulp of ripe fruit and green) and at concentrations of 0.5-50μg/mL (bark of the stem). For each concentration, the test was performed in triplicate. In 3 ml of each sample were plus 0.1 mL of ethanolic solution of DPPH free radical, and incubated for 30 minutes at room temperature, away from light. White used the samples in each of the dilutions. Elapsed time reading the absorvâncias was held in 517 nm (spectrophotometer) of samples with DPPH against your specific white. How control was used a rate of 0.1 mL of ethanolic solution of DPPH added 3 ml of ethanol. To evaluate the captadora of free radical activity, the percentage of inhibition was based on equation: % inhibition = [(control absorbance-absorbance of sample)/ control absorbance] x 100 (Silva et al., 2019; Mensor et al., 2001).

2.5. Calculation of EC$_{50}$

The values of total antioxidant activity (AAO%) and concentrations (500, 250, 150, 50, 10 and 5 μg/mL $^{-1}$) were related used the “Excel for Windows” program, obtaining, to extract the equation of the line. The resolution of this equation (replacing the Y-value per 50) resulted in the EC$_{50}$ value, that is the concentration required to produce half (50%) of maximum effect estimated at 100% for A. carambola extracts (Silva et al., 2019).

2.6. Ferric-reducing Antioxidant Power (FRAP) assay

The assay was performed according to the method described by Rufino et al. (2006), which is based on the reduction of a ferric tripyridyl triazine complex to its dark blue ferrous form, in the absence and presence of antioxidants. Briefly, the FRAP reagent is prepared by mixing 2.5 mL of a solution of 10 mmol L$^{-1}$ TPTZ in 40 mmol L$^{-1}$ HCl, and adding 2.5 mL of 20 mmol L$^{-1}$ FeCl$_3$ and 25 mL of 0.30 mol L$^{-1}$ acetate buffer (pH=3.6), after which the reagent is heated to 37 °C. Sample aliquots (90 μL) were mixed with 270 μL of distilled water and 2.7 mL of FRAP reagent and incubated at 37 °C for 30 min. The absorbance of the reaction mixture was measured at 595 nm and a calibration curve was prepared with Trollox$^®$ (5–1000 μmol L$^{-1}$). The results are expressed as TEACFRAP, i.e., Trollox Equivalent Antioxidant Capacities calculated with respect to the original FRAP in mmol of Trollox g$^{-1}$.

2.7. Determination of phenolic compounds

The ethanolic extract obtained was used for the determination of the levels of total phenolics, by Spectrophotometric method using Folin-Ciocalteau reagent, according methodology described and the calibration curve constructed with Gallic acid standards (10 to 350 μg/mL) and expressed as mg of EAG (Gallic acid equivalents) per g of extract (Scherer and Godoy, 2014).

2.8. Antimicrobial activity test

The antimicrobial activity was analyzed by the method of microdiluição in broth (Ferreira et al., 2006) with varying statements on final concentrations between 500 to 50 μg/mL. We used standard strains of Staphylococcus aureus ATCC 29213 (MRSA), Staphylococcus saprophyticus ATCC 25352, Enterococcus faecalis ATCC 29212, Enterobacter aerogenes ATCC 13048, Klebsiella pneumoniae ATCC BAA-1705 (KPC), Proteus mirabilis ATCC 25933, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Acinetobacter baumannii ATCC 17978, and clinical isolates of S. aureus, E. aerogenes, K. pneumoniae, E. coli, P. aeruginosa and A. baumannii with multidrug resistance profile obtained from samples clinics. The fungi examined were Candida albicans ATCC 200955 and C. krusei ATCC 200917. The Minimum inhibitory concentration was defined as the lowest concentration able to inhibit microbial growth. Statistical analyses were performed using the Assistat program (7.6). The variance analysis
of the experiment was determined using the Dunnett test at the level of 5% probability.

2.9. Phytochemical analysis of the extract

To perform the phytochemical screening stage was based on the methodology proposed by Matos and Matos (1989) which has been crafted with some adaptations in order to carry out prospecting the following allelochemicals: phenols, pyrogalic tannins, philophane tannins, anthocyanin and anthocyanidin, flavones, flavonols, xanthones, chalcones, aurones, flavononois, leucoantocianidinas, catechins, flavonones, flavonols, xanthoids, steroids and triterpenoids saponins. Of each extract obtained and used in bioassays from 35 mL to the phytochemical prospecting, which have been split into seven portions of 3 mL test tubes numbered and identified according to each type of extract and one 10 mL portion in beakers labeled. Heated the beaker in a Bain-Marie by means of a hot plate with agitation until the total evaporation from the liquid, which was used in tests for steroids, and triterpenoid saponins.

3. Results and Discussion

The present study has shown that saponins, tannins and pyrogalic steroids presented himself as the most frequent among the compounds studied, in various parts of vegetables star fruit (Table 1). The great interest around these classes of secondary metabolites is the correlation that these compounds have with various biological activities, such as antioxidant and antimicrobial activities among other (Kuhn et al., 2019; Mallmann et al., 2018; Milani et al., 2012). A diet abundant in some of these compounds is able to act in the prevention of various diseases. Compounds such as saponins are related to action detergent and emulsifier, expectorant and diuretic (Kaneshima et al., 2016), anti-inflammatory (Xiong et al., 2015), antifungal (Woldemichael and Wink, 2001). The tannins have astringent, antidiarrheal drug effects, antimicrobial, antiseptic and the flavonoids, abundant in fruits, have anti-inflammatory properties, antibacterial, antifungal, antioxidant and anticancer (Kaneshima et al., 2016). The presence of these compounds in the extracts corroborates with the antioxidant and antimicrobial potential presented by various parties studied the A. carambola.

Regarding the antioxidant potential, all the extracts were active, the stem bark was highlighted by presenting AAO% 94.05 to 50 μg/mL and EC₅₀ 28.5 ± 2.9 μg/mL, the other extracts presented AAO% > 59% at 500 μg/mL. All values of R² (coefficient of determination) were higher than 0.9 (Figure 1). Through Dunnett’s test, it was possible to verify the existence of a significant difference (P < 0.05) between the antioxidant potential of the stem bark and the other plant extracts evaluated.

The antioxidant potential of carambola against the DPPH radical was higher than that presented by Cinnamomum camphora (500 μg/mL AAO% 7.11%; EC₅₀ 12942 μg/mL) and Terminalia brasiliensis (AAO% 6.0%) (Cansian et al., 2010; Sousa et al., 2007), and the results obtained in pineapple, cashew, passion fruit and mango residues (5.63 ± 0.25; 68.60 ± 0.23; 10.29 ± 0.44 and 33.03 ± 2.40 μmol et/g of dry matter, respectively) (Infante et al., 2013). The production and concentration of phytoantioxidants can vary and also depends on environmental conditions. They can be induced or regulated by stress conditions such as high radiation, temperature, mineral imbalance or even pathogenic attacks subjected to each part of the studied fruit (Neill et al., 2002; Wilmes et al., 2011). In the antioxidant assays DPPH Trolox and FRAP Trolox The plant samples also presented antioxidant activity, with determination of the calibration curve of the synthetic Trolox pattern (y =-0.006x + 0.6882, R² = 0.99; 0. 000704x + 0.0144, r² = 0.99) respectively (Table 2), highlighting the stem bark of this fruit.

By determining the calibration curve of the synthetic gallic acid pattern (y = 7.701x + 0.0131 and R² = 0.99) It was possible to quantify the total phenols in all the extracts (Table 2). The stem bark presented the highest

Table 1. Classes of secondary metabolites detected in extracts of Averrhoa carambola.

| Class of compounds               | Ethanolic Extract |
|---------------------------------|-------------------|
|                                 | Green Fruit Bagasse | Ripe fruit bagasse | Leaf | Stem bark | Fruit pulp |
| Phenols                         | A                 | A                 | A    | A         | A          |
| Pyrogallic tannins              | P                 | P                 | P    | P         | A          |
| Philophane tannins              | A                 | A                 | A    | A         | A          |
| Anthocyanin and anthocyanidin   | A                 | A                 | A    | A         | A          |
| Chalcones and aurones           | A                 | A                 | A    | A         | A          |
| Flavononols                     | A                 | A                 | A    | P         | P          |
| Leucoanthocyanidins             | A                 | A                 | A    | A         | A          |
| Catechins                       | P                 | A                 | A    | P         | P          |
| Flavonones                      | A                 | A                 | A    | A         | P          |
| Flavones, flavonols and xanthones | P               | A                 | A    | A         | A          |
| Steroids                        | P                 | P                 | P    | P         | A          |
| Triterpenoids                   | A                 | A                 | A    | A         | A          |
| Saponins                        | P                 | P                 | P    | P         | P          |

Absent (A), Presence (P).
index of phenols with 3891.87 mg of eaq/g of crude extract, which is consistent with its expressive antioxidant potential. These results were higher than those presented by blueberry (7.26), strawberry (7.72) and blackberry (13.22) (Silva et al., 2011), Jenipap (15.43±0.70) and umbu (19.71±1.47), (Omena et al., 2012), pineapple (5.63 ± 0.25) and cashew (10.29 ± 0.44) (Infante et al., 2013).

The extracts of stem bark, leaves and fruits of *A. carambola* showed antibacterial and antifungal activity. The lowest inhibitory concentration presented by the extracts was 100 μg/mL (Table 3), considered strong antibacterial and antifungal activity, because according to Sartoratto et al. (2004), a strong activity of plant extracts would be for mic values between 50-500 μg/mL. The crude leaf extract presented a broad spectrum of action against Gram-positive and Gram-negative bacteria, such as *S. aureus* ATCC 29213 MRSA, *S. aureus* 6 MRSA, *S. aureus* 10 MRSA, *S. aureus* 12 MRSA, *E. faecalis* ATCC 29212, *K. pneumoniae* 8 ESBL and *A. baumannii* 2 MBL, even in the face of multidrug-resistance mechanisms. However, it did not present antifungal activity in relation to the other extracts. The extracts of the bagasse of the ripe fruit and the bark presented antistaphylococcal action, as well as the extracts of the leaf. The extract of the green fruit bagasse when compared to that of the ripe bagasse, was restricted only to the antifungal activity.

The extracts showed activity, even in the face of multidrug-resistant strains, demonstrating that the resistance of wild strains did not interfere with the activity of plant extracts of the carambola. This result is promising due to

| Ethanolic Extract       | *DPPH Trolox* μmol ET/g amostra | **FRAP Trolox** μmol ET/g amostra | ***TFT** mg EAG/g amostra |
|-------------------------|----------------------------------|-----------------------------------|--------------------------|
| Fruit pulp              | 429.55±151.08                    | 7106.72± 649.12                   | 143.91±13.16             |
| Green fruit Bagasse     | 198.44±65.43                     | 4738.12±1019.33                   | 97.90±16.51              |
| Ripe fruit bagasse      | 225.11±18.35                     | 5284.43±1141.30                   | 214.92±9.80              |
| Stem Bark               | 1971.77±10.71                    | 79932.29±269.06                   | 3891.87±81.39            |
| Leaf                    | 1040.66±23.09                    | 11162.20±2395.23                  | 470.76±3.01              |

*Antioxidant activity DPPH with synthetic Trolox pattern (Mmol trolox equivalent/g sample). **Reduction of FRAP iron (Mmol trolox equivalent/g sample). ***Total phenolic content TFT (mg equivalent of galic acid/g of dry extract). Mean ± standard deviation (n = 3). ET - Ethanol extract; EAG - Gallic acid equivalents.
the increasing pace of resistance occurring in different pathogens, representing a major therapeutic challenge (Rossi, 2011). The extracts with antistaphylococcal action may represent an alternative for the discovery of new antibiotics, since vancomycin is one of the few therapeutic options for methicillin-resistant Staphylococcus aureus (MRSA) (Gardete and Tomasz, 2014).

Enterococcus may be the cause of at least 10% of hospital infections, with high levels of resistance (Prieto et al., 2016), so antimicrobial action studies of plant extracts have been carried out (Costa et al., 2010). The extracts of the leaf and the bagasse of the ripe fruit of the carambola were effective against a wild strain of E. Faecalis, which represents about 85 to 90% of the Enterococcos isolated in the clinic (Prieto et al., 2016).

Gram-negative bacteria have been a frequent problem in the hospital environment because they have the ability to develop mechanisms of mainly enzymatic resistance. The treatment of infections caused by strains of K. pneumoniae is difficult due to the existence of strains carrying plasmids, which encode enzymes known as beta-lactamases (ESBL), thus conferring resistance to beta-lactam. The extracts of the leaves and the A. carambola bark showed activity against K. pneumoniae which is the main species producing ESBL responsible for 2 to 5% of the nosocomial infections, mainly respiratory and urinary as reported by Scarpate and Cossatis (2009).

The antimicrobial activity assays also inhibited the growth of A. baumannii multiresistant producer of metallo-beta-lactamases, a carbapenemic inhibitory enzyme, through the leaf extract of A. carambola. A. baumannii has become an important pathogen in recent years due to the increasing number of hospital outbreaks (Cirino et al., 2008). The activity against fungi of medical interest was observed in the extracts of the peel and fruits, including the extract of the bagasse of the green fruit that did not present antibacterial action (Table 3). Therefore, the importance of the antifungal activity detected, because the resistance based on different mechanisms continues to grow and exacerbate the need for new treatments against Candida infections.

### 4. Conclusion

Under the conditions of this study, our results showed that A. carambola has antioxidant and antimicrobial activities. The activities presented may be related to the presence of phenolic substances in extracts. It is of great importance the isolation of the chemical constituents of this species, so that they can be tested in experimental models, and thus determine the compounds responsible for the biological activities of extracts.

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