Characterization, antibacterial activity and antibiotic modifying action of the *Caryocar coriaceum* Wittm. pulp and almond fixed oil

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ABSTRACT

In this study the physicochemical characterization of the pulp and almond fixed oil was carried out; their antibacterial activity and aminoglycoside antibiotic modifying action against standard and multi-resistant Gram-positive and -negative bacteria were investigated using the broth microdilution assay. Physical properties such as moisture, pH, acidity, peroxide index, relative density and refractive index indicate stability and chemical quality of the oils. In the GC/MS chemical composition analysis, a high unsaturated fatty acid content and the presence of oleic and palmitic acids were observed in the oils. In the antibacterial assay, more significant results were obtained for *Escherichia coli*, while other standard and multi-resistant strains presented MIC values ≥ 1024 μg/mL. Furthermore, the fixed oils in association with antibiotics were able to significantly improve antibacterial activity against *S. aureus* with a reduction in MICs.

Key words: Pequi, fatty acid, antibacterial, aminoglycosides.

1. Experimental methods

1.1. Plant material and botanical identification

*Caryocar coriaceum* Wittm. fruits were obtained from a Technology Center – CENTEC unit, the Vocational Technology Center - CVTec in the Municipality of Barbalha, Ceará, Brazil. A *C. coriaceum* species exsiccate was sent to the Prisco Bezerra Herbarium of the Federal University of Ceará, identified by Prof. Dr. Lígia Queiroz Matias and deposited under sample number 44523.

1.2. Fixed oil acquisition

The fixed oils were obtained using a discontinuous hydraulic pressing mechanical extraction method using 500g of the pulp and almond separately. Samples were added to a stainless steel cylinder and pressed for approximately 2 h, whose pressure was recorded at 15 T by a manometer. The collected fixed oils yielded 11.10 and 26.80% of the raw material for the pulp and almond, respectively. These were then stored in hermetically sealed amber bottles under refrigeration.

1.3. Physicochemical characterization

Physicochemical analysis was performed on the fixed oils with regards to the following parameters: water content, pH, acidity (as oleic acid), relative density, peroxide index and refraction index at 40 °C (Lutz, 2008).

1.4. Fatty acid analysis

Fatty acids were indirectly determined using their corresponding methyl esters. The oil (0.2 g) was saponified for 30 min under reflux with potassium hydroxide solution in methanol, following the method described by Hartman and Lago (1973).
After adequate treatment and pH adjustment, the free fatty acids were methylated with methanol by acid catalysis in order to yield the respective methyl esters.

The volatile constituent analysis was carried out in a GC/MS, HP, model 5971, using the non-polar fused silica column DB-1 (30 m x 0.25 mm i.d., 0.25 μm film), eluted with helium gas at 8 mL/min and with split mode. Injector and detector temperatures were set to 250 °C and 200 °C, respectively. The column temperature was programmed from 35 °C to 180 °C at 4 °C/min, and then from 180 °C to 250 °C at 10 °C/min. Mass spectra were recorded from 30 to 450 m/z, with an electron beam energy of 70 eV. Injected sample was 1 μL of 5 mg/mL of the oil solution fixed in acetone.

The individual components were identified by matching their mass spectra with those of the database using the library constructed using the spectrometer (Wiley, 229) and NIST 08 using retention indices (IR) as a pre-selection (Alencar et al., 1990), as well as by visually comparing standard fragmentation to that reported in the literature (Adams, 2007).

1.5. Antibacterial analysis

1.5.1. Strains utilized

Standard and multiresistant bacterial strains were used in the analyzes. The standard strains were: *Staphylococcus aureus* SA–ATCC 6538, *Bacillus cereus* BC–ATCC 33018, *Escherichia coli* EC–ATCC 10536, *Pseudomonas aeruginosa* PA–ATCC 9027, *Klebsiella pneumoniae* KP–ATCC 10031, *Shigella flexneri* EC–ATCC 12022 and *Proteus vulgaris* PV–ATCC 13315. The multiresistant strains were: *S. aureus* SA–10 and *E. coli* EC–06 with their resistance profile identified in table 1. These were maintained in blood agar base (Laboratory Difco Ltd, Brazil.) and cultured at 37 °C for 24 h in Heart Infusion Agar (HIA, Difco. Laboratories Ltd.).

1.5.2. Antibiotics

The aminoglycoside drugs amikacin and gentamicin were used in the tests (Sigma Co., St. Louis, USA). All drugs were diluted in sterile water to a concentration of 5000 μL/mL.

1.5.3. Minimum Inhibitory Concentration test

For the Minimum Inhibitory Concentration (MIC) assays, eppendorfs were prepared with 100 μL of the inoculum and 900 μL of the BHI liquid culture medium at a concentration of 10% in 96-well plates were filled in the numerical sense by adding 100 μL of this solution into each well. Subsequently, serial microdilution with 100 μL of the tested substance was performed, varying in concentrations from 1024 μg/mL to 1.0 μL/mL. The plates were taken to an incubator for 24 h at 37 °C. To read the bacterial MIC, 20 μL of resazurin was added to each well, where after 1 h the wells’ color change was noted, where a change from blue to red coloration corresponds to microbial growth and a permanence at blue the absence of growth, as established by CLSI (2008).

1.5.4. Antibiotic activity modifying effect

Evaluation of the fixed oils as antibiotic activity modifiers were performed according to Coutinho et al. (2014). The tests were performed in triplicates. For each eppendorf, 1162 μL of 10% BHI were used with 150 μL of each strain’s inoculum and the tested substance with volume corresponding to a sub-inhibitory concentration (MIC/8 = 128 μg/mL). Controls were prepared with only 1350 μL of BHI (10%) and 150 μL of bacterial suspension. The plates were filled in numerical order and each well
received 100 μL of the solution. Microdilution was performed with 100 μL of each antibiotic up to the penultimate well and the final volumes were discarded. The plates were then incubated at 37 °C for 24 h and read through the addition of resazurin.

1.5. Statistical analysis

Data analysis was performed using the statistical program GraphPad Prism 5.0. The data were analyzed using a two-way ANOVA test, using the geometric mean of the triplicates as the central data and the standard deviation of the mean. Subsequently a Bonferroni post hoc test was performed (where $P<0.05$ and $P<0.0001$ were considered significant and $P>0.05$ insignificant).

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**Table S1:** Bacterial source and antibiotic resistance profile.

| Bacteria     | Source          | Resistance profile                                                                 |
|--------------|-----------------|------------------------------------------------------------------------------------|
| *S. aureus*  | Surgical wound | Cephalothin, Cephalexin, Cefadroxil, Oxacillin, Penicillin, Ampicillin, Ampicillin + Sulbactam, Amoxicillin, Moxifloxacin, Ciprofloxacin, Levofloxacin, Erythromycin, Clarithromycin Azithromycin and Clindamycin |
| *E. coli*    | Surgical wound | Cephalothin, Cephalexin, Cefadroxil, Ceftriaxone, Cefepime and Ampicillin + Sulbactam |

**Table S2:** Physicochemical properties of the fixed oils of the pulp and almond of *Caryocar coriaceum*.

| Analysis                  | Pulp              | Almond             |
|---------------------------|-------------------|--------------------|
| Water contente (% p/p)    | 0.50±0.50<sup>a</sup> | 0.30±0.70<sup>a</sup> |
| pH                       | 6.32±1.10<sup>a</sup> | 5.05±0.90<sup>a</sup> |
| Acidity (as oleic acid %) | 3.84±0.85<sup>a</sup> | 1.20±0.60<sup>b</sup> |
| Relative density (g/cm³)  | 0.30±0.35<sup>a</sup> | 0.31±0.50<sup>a</sup> |
| Peroxide index (meq/Kg)  | 4.40±0.72<sup>a</sup> | 5.60±0.72<sup>b</sup> |
| Refraction index (40 °C)  | 1.45±0.01<sup>a</sup> | 1.46±0.05<sup>a</sup> |

Results are expressed with means ± S.E.M. (n = 3) of experiments performed in triplicate. Averages in lines of table followed by different letters (a, b) differ statistically (ANOVA and Tukey test – *P* < 0.001).

**Table S3:** Fatty acids identified in the fixed oils of the pulp and almond of *Caryocar coriaceum*.

| Order | Constituents                | RT (min) | Pulp (%) | Almond (%) |
|-------|-----------------------------|----------|----------|------------|
|       | Saturated                   |          |          |            |
| 1     | Palmitic acid (C16:0)       | 27.76    | 27.59*   | 46.27*     |
| 2     | Heneicosanoic acid (C21:0)  | 31.53    | -        | 1.68       |
|       | Unsaturated                 |          |          |            |
| 3     | Linoleic acid (C18:2)       | 31.08    | -        | 4.96       |
| 4     | Oleic acid (C18:1)          | 31.22    | 72.41*   | 48.09*     |

RT: relative retention time (Adams, 2007).
Table S4: Minimum Inhibitory Concentration values - MIC (μg/mL) of the pulp and almond of *Caryocar coriaceum*.

| Bacterial strains                        | MIC (µg/mL) |  |
|------------------------------------------|-------------|--|
|                                          | Pulp        | Almond     |
| *Proteus vulgaris* PV–ATCC 13315          | ≥ 1024      | ≥ 1024     |
| *Klebsiella pneumoniae* KP–ATCC 10031     | ≥ 1024      | ≥ 1024     |
| *Shigella flexneri* EC–ATCC 12022         | ≥ 1024      | ≥ 1024     |
| *Pseudomonas aeruginosa* PA–ATCC 9027     | ≥ 1024      | ≥ 1024     |
| *Escherichia coli* EC–ATCC 10536          | 812.75      | ≥ 1024     |
| *Escherichia coli* EC–06                  | ≥ 1024      | ≥ 1024     |
| *Bacillus cereus* BC–ATCC 33018           | ≥ 1024      | ≥ 1024     |
| *Staphyloccus aureus* SA–ATCC 6538        | ≥ 1024      | ≥ 1024     |
| *Staphyloccus aureus* SA–10               | ≥ 1024      | ≥ 1024     |
Figure S1: Effect of fixed oil of the pulp and almond of Caryocar coriaceum and activity of aminoglycoside antibiotics against strains of Staphylococcus aureus – SA and Escherichia coli – EC. Values represent the geometric mean ± M.S.E. (Mean Standard Error). One-way ANOVA, followed by the Bonferroni test. $P < 0.0001$ and $P < 0.01$ antibiotic + fixed oil vs control (antibiotic). PO: Pulp Oil; AO: Almond Oil.