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Extraction, Isolation and Chemical Modification of the Anacardic Acids from Cashew Nut Shell (*Anacardium occidentale* L.) and Biological Assay

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Abstract

The objective of this study was to isolate the Anacardic Acid (AA), present in the Cashew Nutshell Liquid (CNSL) of the species *Anacardium occidentale* L., to carry out chemical modifications, and to analyze the antioxidant and antibacterial potential of the derivatives. CNSL was obtained using hexane as solvent, in 14% yield. The anacardic acids were purified by the chromatographic process and analyzed by High Performance Liquid Chromatography (HPLC) and ^{1}H and ^{13}C NMR, characterizing the anacardic acids monoene (29.32%), diene (27.06%) and triene (43.62%). Acetylated AA was produced by acetylation reaction. The products, together with the CNSL and the anacardic acid mixture were evaluated for antioxidant potential by DPPH free radical capture method. The results showed that acetylation decrease the antioxidant potential of natural CNSL (597.29 ppm), which are more efficient in inhibiting radicals than the mixture of AAs (> 1000 ppm) and acetylated AA (> 1000 ppm). The antibacterial activity against the standard strains of *Escherichia coli* and *Staphylococcus aureus* were also observed, which were sensitive at concentrations equal to and greater than 78,125 ppm for CNSL and for the mixture of anacardic acids. Acetylated AA presented lower antibacterial activity. Free AAs maintain the *in vitro* antibacterial activity of the CNSL in the strains evaluated and the phenolic hydroxyl is considered important for activity. Thus, the present work made possible a study of the substances presents in the liquid of cashew nuts of the species *Anacardium occidentale* L. contributing to the expansion of the research arsenal in the area.

Introduction

Anacardium occidentale L. belongs to the Anacardiaceae family, which possess about 60 genera and 400 species. The same occurs in regions with a tropical climate. It is a native plant of the fields of the northeast Brazilian coast and can be found also in North of South America and part of Central America. In Brazil, the states that stand out for their production are Ceará, followed by Rio Grande do Norte and Piauí. Brazil or all North of South America and part of Central America are the centers of origin of this species [1], being the fruit also produced in tropical countries like India, Mozambique, Tanzania and Kenya [2].

Cashew production is an economic and social activity of great importance for the Brazilian Northeast, which guarantees income for more than 150 thousand people in the state of Ceará and generates foreign exchange of more than 140 million dollars annually. This culture also produces raw material for various industries that employ people, thus improving the economy of the region and the country [3]. About 90% of the cashew nut sold in

Brazil is directed abroad, with an average of 80% imported by the United States, 6% by the Netherlands and 4% by Canada [4].

In 2017, Brazil, the world's leading producer, produced about 144,580 tons of cashews corresponding to a harvested area of 564,456 hectares, being northeastern region responsible for 98.84% of production. Thus, the cashew tree culture has great socioeconomic importance for Brazil and can be attributed to two main factors: the cashew industry and the peduncle industry [5].

Cashew nuts represent the main economic product from cashew culture, and the shell that surrounds the nut is discarded like residue, having little or no economic value. However, the shells contains the cashew nut liquid, CNSL which have in their composition phenolic components, named as anacardic acid, cardanol and cardol. Anacardic acids are ortho-hydroxy-benzoic acids with a lateral chain of fifteen-carbon atoms biosynthesized from fatty acids and constitute about 70 to 90% of the liquid that is extracted from cashew nut shells [6].

This liquid (CNSL) has constituents with potential applications in activities such as antifungal, antibacterial, among others. Cashew cultivation also has many advantages, since it allows several applications such as the production of sweets from pseudo fruit, the use of bagasse as animal feed and from the shell of the chestnut can be extracted the CNSL with applications in the manufacture of lubricants, cosmetics, paints, also having a wide application in the chemical industry [2,7,8].

Anacardic acids from CNSL present a series of biological activities as antitumor, antiacne, antibacterial, antifungal, molluscocida and ability to inhibit the enzymes tyrosinase, prostaglandinasintaseandlipooxigenase.Thesephenoliccompounds isolated from *Anacardium occidentale* L., showed considerable inhibitory activity against Gram-positive Bacteria *Streptococcus mutans, Brevibacterium ammoniagenes, Staphylococcus aureus, Bacillus subtilis* and *Propionibacterium acnes* [9-12].

Anacardic acids also have antioxidant, antigenotoxic and cytostatic properties, as well as the ability to inhibit growth of protozoan and parasites [13-16]. According to [17], the anacardic acid present in commercial cashew juice has antitumor potential, and may be advantageous in the control of these tumors, if consumed for prolonged periods.

A significant advance in the research for natural products, especially plants and microorganisms, has been unleashing the discovery of severe substances currently used for various treatments [18-22]. Most (60%) of anticancer drugs introduced in therapy in the last decades have their origin in natural products [23]. In a more recent study [24], which analyzed the total number of official drugs registered between January 1991 and December 2014, 113 (83%) of the 136 registered drugs for the fight against cancer are natural or derived from them.

Thus, it is evident that research that seeks to deepen and consolidate knowledge about different methods of obtaining, characterizing and confirming biologically active properties demonstrated by natural compounds should be continuously encouraged. In This sense, the present study carried out the extraction of bioactive compounds from agroindustrial residues (cashew nut shell) and investigated the antibacterial and antioxydant ability of their constituents in the natural form, isolated and after chemical modification of their structure.

Experimental

CNSL Extraction

Cashew nuts obtained in West Potiguar, Rio Grande do Norte, Brazil, were cut to separation of the bark and almond. Then, the husks were extracted in a soxhlet system using 440 g of husks and 1200 mL of hexane as solvent, remaining in the system for 4 hours at approximately 80°C. After extraction, the mixture was concentrated on rotary evaporator under reduced pressure at 60°C and the CNSL obtained (adaptado de [14].

Purification of Anacardic Acid

The obtained CNSL was subjected to the column chromatography process, using silica gel as adsorbent to extract the Anacardic Acids (AAs) present in the mixture. To do this, the 3 mL volume of the CNSL was added to the chromatographic column using as adsorbent silica gel (Φ = 0.063 - 0.2mm), of 10cm long and 5 cm wide. To separate the constituents of the CNSL, 200mL of the hexane and acetic acid mixture (1%) were used first. Then 200 ml of the chloroform and acetic acid mixture (1%) and finally 300 ml of dichloromethane/ethanol (1:1) and acetic acid (1%) were added. The samples were collected in Erlenmeyer with 30ml each. After collection, each sample was concentrated on a rotary evaporator and performed CCD. The fractions with the same retention factor (Rf) were pooled and concentrated in a rotary evaporator under reduced pressure.

Acetylation of Anacardic Acids

From the purification of AAs, the acetylation reaction was carried out using 200µL of AA, 2 mL of acetic anhydride and 200mg of sodium acetate. The mixture was refluxed at 80°C for 2 hours. Then, a solution of 5% HCl was cooled and added to the medium until pH was getting between 4-5. Extraction was performed with 4x10mL of hexane shaking vigorously. Lastly, any traces of water in the organic phase were removed with sodium sulfate and solvent eliminated in a rotary evaporator.

Purification and Characterization of Derivatives

Thin Layer Chromatography (TLC)

For thin-layer chromatography, silica gel 60 (Φ = 2-25µm) chromatograms on T-6145 polyesters from the brand SIGMA CHEMICAL CO with a layer of 250 µm thickness and dimensions

of 10x5 cm were used. Glass plates coated with an approximately 0.5 mm thick layer of silica gel 60 (Φ = 0.004-0.005 mm) code 1094 of the VETEC brand were also used. Ethyl acetate, chloroform and acetic acid (1%) of the Synth brand PA quality were used as the eluent. After elution of the substances on the plates, they were revealed by spraying with vanillin solution ($C_8H_8O_3$, 5.0g) and perchloric acid ($HClO_4$, 0.75 mol/L, 100mL) in ethanol (C_2H_5OH, 100mL) followed by heating at 100°C with thermal blower for approximately 1 min [25].

High Performance Liquid Chromatography (HPLC)

The equipment used was of the brand Shimadzu, with bomb L201147, equipped with column C18 and detector UV-Vis Shimadzu SPD-M20A. The binary water/acetonitrile mixture with 1.8 mL flow was used as the mobile phase.

Infrared (IV)

Spectrometry in the IV region was used to characterize the substances obtained. IR spectra were obtained using Perkin Elmer Spectrometer (SPECTRUM 100) FT-IR.

Antioxidant Activity

The antioxidant potential test was performed by the ability to capture 2,2-diphenyl-1-picrylhydrazyl free radicals described by (Brand-Williams et al., 1995) [26] with some modifications.

Initially, 20 mg of each substance was added into 2 mL of methanol (separately), yielding 10000 ppm solutions. Subsequently they were diluted in the respective concentrations: 100; 200; 400; 600; 800 and 1000 ppm. 1 mL of each concentration was added to the test tubes together with 1 mL of 60 μmol.L^{-1} DPPH methanolic solution. The samples were left to stand for 30 minutes in the absence of light. After this period, they were analyzed in spectrophotometer of the brand TEKNA model T2000 using wavelength of 520 nm. Methanol was used as the control. The process was performed in triplicate.

After reading the absorbances of the samples, a graph was created using the excel software. The values of the percentage of inhibition of each concentration were used in order to verify the linearity of the obtained results. Finally, the ability of each substance to neutralize 50% of the DPPH free radical (I%) was calculated by the equation 1.

Equation 1. Mathematical representation used to evaluate the antioxidant potential of CNSL, AAs and derivatives.

$$IP(\%) = 100\left(1 - \frac{\text{Abs (sample)}}{\text{Abs (DPPH)}}\right)$$

At where:

IP% = Percentage inhibition of DPPH radical; Abs (sample) = Absorption relative to sample analyzed; Abs (DPPH) = Absorption relative to standard DPPH solution.

Antibacterial Activity

Preparation of the Culture Medium

The methodology used followed the standardization method of [27] with some modifications. For the preparation of the culture medium the mass of 35 g of the Mueller Hinton-Broth medium (MH) was measured and added to an Erlenmeyer flask along with 1 L of distilled water under gentle heating until complete homogenization of the medium. Then the medium was taken to the autoclave (121°C, 1.5atm for 20min) for sterilization. Replication of the bacteria (Strains Standard: *Escherichia coli*, *Staphylococcus aureus*) was performed in medium (MH) for a period of 24 hours at a temperature of 35 ° C. After this time, the bacterial suspension was diluted saline solution (0.85% NaCl) until they reached a value between 0.08 and 0.1 absorbance in a spectrophotometer at 625 nm (corresponding to 0.5 McFarland). For the test, 96-well microplates were used where 90 μL of the Mueller Hinton-Broth broth and 10 μL of the diluted bacterial suspension were added to each well. The derivatives were prepared at concentrations of 2500 ppm: 5 mg/mL, using as solvent 10% (v/v) Dimethylsulfoxide (DMSO) in water. Then, 100 μL of the solution was added to the first wells of each column already containing the broth and the bacterial suspension, homogenizing and diluting to the other wells. After this process, the microplates were taken to the greenhouse for a period of 24 hours at 35°C. After the 24-hour period, 25 μL of resazurin was added to each well. The microplates were kept in the oven for 1 hour after addition of resazurin and after that time the reading was performed. The wells that acquired pink staining indicated the presence of bacteria, whereas those that acquired blue staining meant that there was inhibition of the bacteria. The negative control was performed using only 10% (v/v) Dimethylsulfoxide Solution (DMSO) in water.

Results and Discussions

After extraction, the sample resulted in 61.657 g of fresh CNSL corresponding to a yield of 14%. [28] obtained a yield of 30% CNSL by the method of heating the nuts at 40°C for 7 days. The yield obtained in this study was lower than that of the author, but with a much shorter CNSL, 4 hours [29]. carried out the extraction of the CNSL from cashew nuts in the soxhlet system for 4 hours using ethyl alcohol as the solvent to obtain a yield of 40%. Therefore, it can be seen that the yield obtained in the present study was not satisfactory.

Both CNSL and AAs were evaluated in High Performance Liquid Chromatography. Figure 1 shows, respectively, the chromatogram of the CNSL analysis, where it is possible to identify at least 6 major products and the chromatogram referring

to the fraction containing AAs obtained from the column in which only 3 major components are identified.

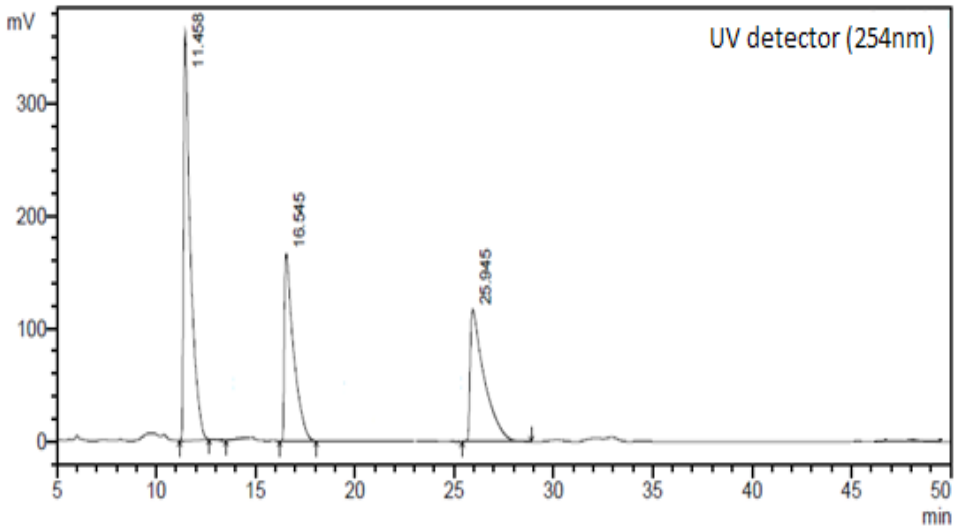

Figure 1: Chromatogram (HPLC) of the AAs analysis of the cashew nut shell liquid after extraction and purification, showing the AAs as a mixture of the triene (11.45min), diene (16.54min) and monoene (25.94min) constituents.

After the analysis by HPLC, proving the separation of the components of the CNSL *in nature*, the characterization of the AAs mixture was performed by ^{13}C and ^{1}H Nuclear Magnetic Resonance (NMR). The one-dimensional carbon spectra (Figure 2) show a signal with chemical shift of 175 ppm corresponding to the carboxylic acid carbonyl, evidencing the presence of the anacardic acid.

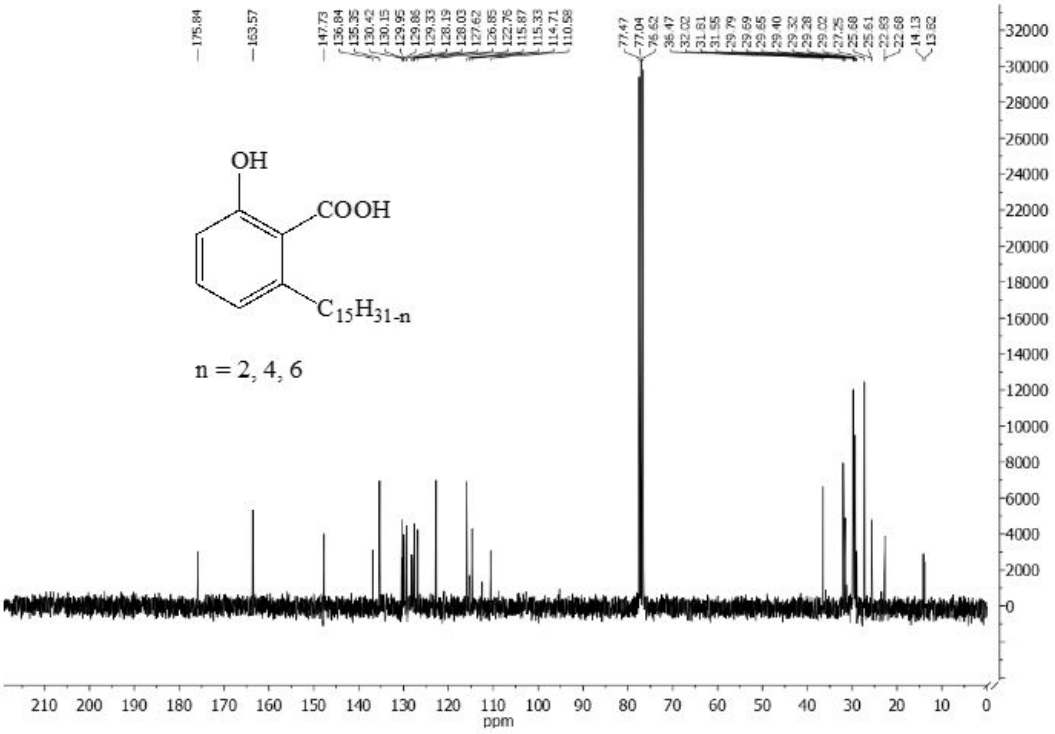

Figure 2: ^{13}C NMR spectrum of anacardic acids isolated from CNSL.

The ^{1}H NMR spectrum (Figure 3) showed the characteristic signals of the olefinic double bonds (4.8-6.0 ppm), as well as the H bound to the aromatic ring (6.5 to 7.5 ppm). The composition of AAs (29.32%), diene (27.06%) and triene (43.62%) were determined by standardization of the chromatogram areas and the stationary phase characteristic used (reverse phase, C18), these being the most abundant anacardic acids. Triene showed higher yield followed by monoene and diene with very close yields. As in the analysis by HPLC was used reverse phase column triene was the first compound characterized.

Morais et al., [30] Performed the characterization of the anacardic acid components present in the CNSL obtaining yields of 17.13, 17.77 and 28% for the monoene, diene and triene components, respectively. Therefore, the yields obtained in this work were quite similar.

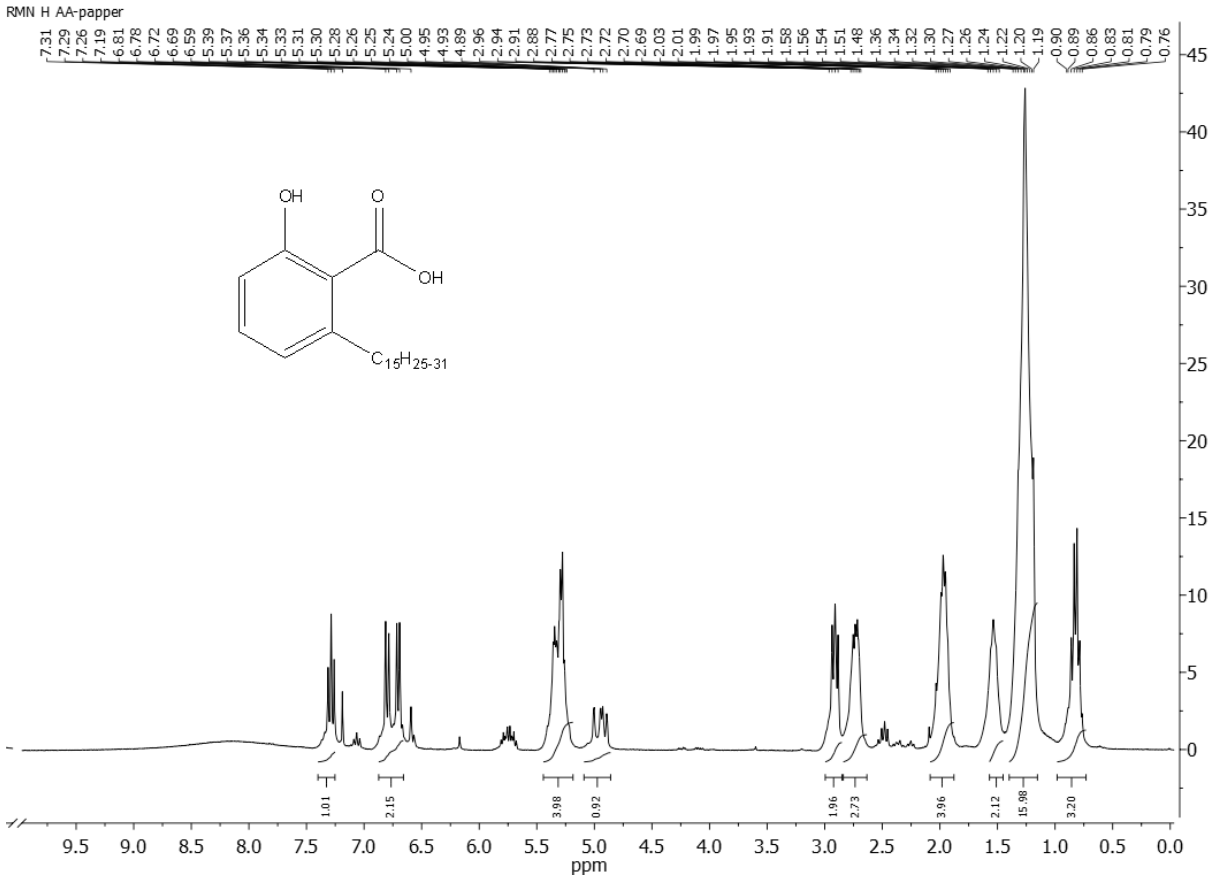

Figure 3: ^{1}H-NMR spectrum of anacardic acid isolated from cashew nuts.

The mixture of AAs was also analyzed in infrared, resulting the spectrum shown in Figure 4, where it is possible to verify the intense absorptions at 1645 cm^{-1}, consistent with the signal concerning the carboxylic acid carbonyl ortho to the hydroxy group (-OH). This spatial arrangement between the atoms enables the formation of intramolecular hydrogen bonding by causing the carbonyl group signal to reduce its double bond character and exhibit lower wavelength stretching than that found for carboxylic acids in general (1680-1715 cm^{-1}).

It is also possible to verify the distortion and overlap between 2500 and 3400 cm-^{1}, generated due to the hydrogen bonds in substances with the carboxylic acid function, which also helps to denote the presence of the carboxylic acid function in the structure.

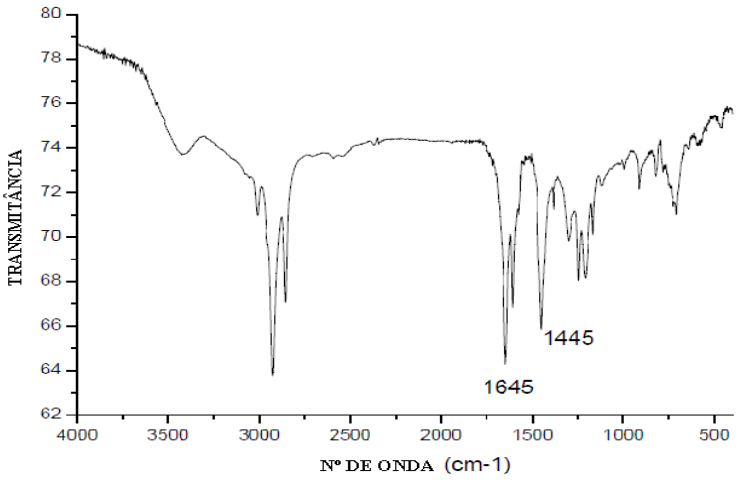

Figure 4: Infrared spectroscopy spectrum of the mixture of AAs.

The acetylation reaction of AAs is shown in Figure 5. The product of the acetylation reaction of AAs was characterized by infrared spectroscopy (Figure 6). The signal at approximately 1760 cm^{-1} is compatible with the absorption relative to the stretching of the carbonyl group of the ester function.

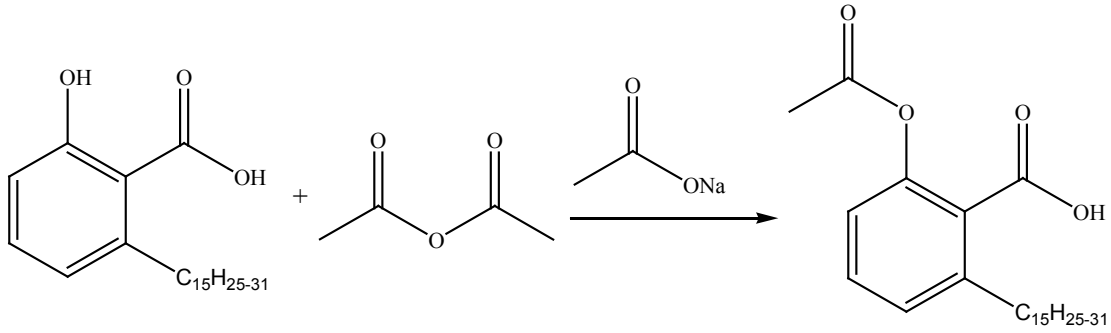

Figure 5: Chemical reaction of AA acetylation.

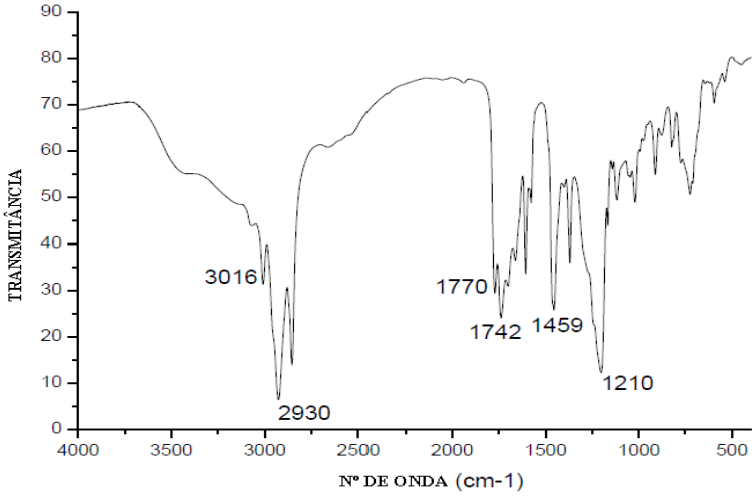

Figure 6: Infrared product of the AA acetylation reaction.

The evaluation of the antioxidant capacity demonstrated a better action for the *in natura* CNSL, as can be observed in Table 1.

SAMPLE	IC_{50} (ppm)
CNSL	597.29
AAs	> 1000
Acetylated AA	> 1000

Table 1: Percentage of each sample to inhibit 50% of the DPPH free radical.

The CNSL was the substance that presented better antioxidant activity in relation to the others [30]. carried out the antioxidant activity of the major anacardic acids (monoene, diene and triene) using the free radical capture method of DPPH obtaining IC_{50} values of 2060, 1780 and 810 ppm, respectively. Thus, the values obtained in the antioxidant activity of the mixture of anacardic acids is compatible, since the analysis was performed from the mixture of the three acids. The reduction in the ability to inhibit radicals observed when analyzing the IC_{50} of CNSL and the isolated AAs is possibly related to the lower content of phenolic compounds in the sample evaluated, since phenolic compounds are recognized for their ability to inhibit the propagation of radicals [31].

Antibacterial Activity

Figure 7 shows the results of the antibacterial activity of the analyzed substances (CNSL, AAs, and acetylated AA) against the standard strains of *Escherichia coli* and *Staphylococcus aureus*. Since the wells with blue staining indicate that there was inhibition of bacteria by the action of the substance, already the wells with pink staining demonstrate that there was the growth of bacteria and therefore there is no antibacterial action.

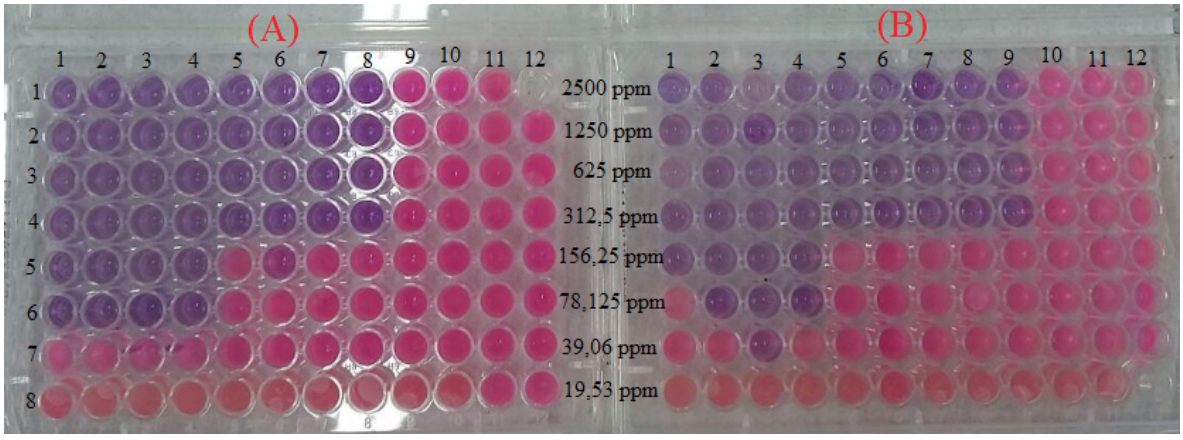

Figure 7: Result of the antibacterial activity of the substances used against the strains (A) *Escherichia coli* and (B) *Staphylococcus aureus*.

Table 2 shows the minimum concentration values tested for each substance, for better visualization, against the standard strains of *Escherichia coli* and *Staphylococcus aureus.*

SAMPLE	CMI (ppm) *Escherichia coli*	CMI (ppm) *Staphylococcus aureus*
CNSL	78.125	78.125
AAs	78.125	78.125
Acetylated AA	312.5	312.5

Table 2: Minimum concentration tested for each sample against the standard strains used.

From the results expressed it is possible to analyze that the samples presented equal values of minimum concentration against the two strains gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*). Since CNSL and the mixture of AAs presented the same MIC (78.125µg/mL). These results are consistent with those reported in the literature that denote the antibacterial potential of CNSL and anacardic acid [11,14].

Conclusion

The present study made it possible to extract, isolate and submit reactions aiming at the structural modification of the anacardic acids present in cashew nuts of the species *Anacardium occidentale* L. as well as carry out the analysis of its antioxidant and antibacterial potential. The isolation of CNSL was performed successfully, as well as the isolation of the mixture of AAs, which presented a yield of 92%. By means of the analyzes of the antioxidant activity with the samples studied (CNSL, AA and acetylated AA) using the free radical capture method of DPPH, it was possible to verify that AAs of the CNSL is a product of lower efficiency in the capture of radicals free, which is possibly related to the reduction of phenolic compounds content in the sample, since in the isolation cardol and cardanol, both phenolic

compounds, are withdrawn from the medium. It was also observed that acetylation of CNSL decreased the antioxidant activity.

In the analysis of the antibacterial activity, the strains used of *Escherichia coli* and *Staphylococcus aureus* were sensitive at concentrations equal to and greater than 78.125 µg mL for CNSL and for the mixture of AAs. Therefore, it is possible to conclude that the isolation of AAs does not compromise the antibacterial activity presented by the *in vitro* CNSL in the strains evaluated, and acetylation nevertheless is not useful for improving activity In order to continue the study, it is proposed to search for other methodologies in order to obtain a higher yield in the CNSL extraction.

Therefore, it is expected that the same will contribute significantly to the expansion of studies on the search for new natural sources for clinical use through the biological analysis of the antioxidant and antibacterial activities of the shells of the species *Anacardium occidentale* L.

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