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Study of antimicrobial activity and atomic force microscopy imaging of the action mechanism of cashew tree gum

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ABSTRACT

The aim of this work was to evaluate the antimicrobial potential of two grades of cashew tree gum (crude and purified) against eight microorganisms and to analyze the mechanism of cashew tree gum antimicrobial action via atomic force microscopy (AFM) imaging. The results indicated strong antimicrobial properties of pure cashew tree gum against all tested microorganisms, except for *Candida albicans* and *Lactobacillus acidophilus*. On the other hand crude cashew gum showed antimicrobial activity only against Gram-positive bacteria (MRSA, MSSA, *Listeria innocua* and *Enterococcus faecium*). Atomic force microscopy imaging showed that pure cashew tree gum lead to bacterial cell collapse. In conclusion cashew tree gum presented relevant antimicrobial activity against most of the studied bacteria, and the purification of the cashew gum affected its antimicrobial spectrum.

1. Introduction

Cashew tree gum (CG), is an exudate from *Anacardiaceae* species, such as the cashew tree *Anacardium occidentale* L. from north-eastern Brazil, which is known as a gum producer. Gum exudation generally occurs by wounding the tree during pruning (Torquato et al., 2004).

Cashew gum is water-soluble branched acidic heteropolysaccharide which forms a solution with low viscosity, and is precipitated by organic polar solvents such as ethanol. It is similar in structure to gum arabic, with the polysaccharide chains being composed of arabinogalactans with a variety of side chains, including glucuronic, galacturonic and anacardic acid residues, which may be associated to its antimicrobial activity (Marques, Albuquerque, & Xavier-Filho, 1992). The proportion of monosaccharides in the cashew gum varies, depending on the source, age of the tree, time of exudation and climatic conditions. However, the reported chemical composition has shown that hydrolysis of cashew tree gum results in 70% galactose, 11% glucose, 6% glucuronic acid, 5% arabinose, 4% rhamnose and 1% mannose (Silva, Santiago, Purcena, & Fernandes, 2010).

According to Kubo, Muroi, and Kubo (1995) and Marques et al. (1992), cashew gum has antimicrobial activity against several microorganisms, among which are *Aspergillus flavus*, *Bacillus subtilis*, *Serratia marcescens* and methicillin-resistant *Staphylococcus aureus* (MRSA). They attributed the antimicrobial activity to the presence of anacardic acid, which has a carboxyl group. Moreover, Torquato et al. (2004) stated that cashew gum loses its anacardic acid with high temperature, turning the acid's carboxyl group into cardanol, thus at high temperatures, e.g. autoclaving, the anacardic acid concentration decreases from 78.0 to 8.8 mg per 100 g of cashew gum. The loss of 88% of the anacardic acid is coincident with a decrease in the gum's antimicrobial activity, leading to the hypothesis that this acid is responsible for the activity.

Nowadays, it is possible to control pathogenic microorganisms in food by using synthetic antimicrobials. However, the increase of bacterial resistance to conventional antimicrobial agents creates a need to search for new substances with antimicrobial activity, especially substances of natural origin. Therefore, several edible plants have been studied as sources of new antimicrobial agents (Torquato et al., 2004; Muroi & Kubo, 1996). Cashew gum has been studied for various applications in, for example, the agro-food, paper, cosmetic and pharmaceutical industries due to their potential use as thickening and gelling agents and emulsion stabilizers (Silva et al., 2010). Their usefulness comes about because they are inexpensive, non-toxic, biodegradable and are already being used as binder

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and gelling agent in drug formulations (Gyedu-Akoto et al., 2007; Ofori-Kwakye, Asantewaa, & Kipo, 2010).

Atomic force microscopy (AFM) is a highly suitable tool for the study of bacteria and its great advantage is that samples do not require fixation, conductive coating, or to be imaged in vacuum. Applications of AFM imaging studies include imaging of bacterial nanostructures, genetic variation and the study of antibacterial effects, making this technique extremely advantageous for the imaging of small highly delicate structures on bacteria (Eaton & West, 2010; Eaton, Fernandes, Pereira, Pintado, & Xavier Malcata, 2008; Fernandes, Eaton, Gomes, Pintado, & Xavier Malcata, 2009).

Previous work reported conflicting results, with Marques et al. (1992) stating that cashew gum possesses antimicrobial activity against *S. aureus* among other microorganisms, while Torquato et al. (2004) reported no antimicrobial activity for cashew gum. However, the two studies used different cashew gum purification techniques that could have a direct effect on the concentration of anacardic acid in the final solution. However, until now, there is no work reported to describe possible mechanisms involved in such antimicrobial activity. As such, the aim of the present work was to evaluate the antimicrobial activity of cashew gum (crude and purified) and analyze its effects via AFM.

2. Materials and methods

2.1. Microorganisms and stock conditions

The microorganisms tested in this study were *Escherichia coli* (ATCC 25922), methicillin-sensitive *S. aureus* (MSSA) (ATCC 25923), methicillin-resistant *S. aureus* (MRSA) (clinical strain from Instituto Português de Oncologia do Porto (IPO), *Listeria innocua* (11288 NCTC), *Pseudomonas aeruginosa* (ATCC 10145), *Enterococcus faecium* (CCUG 34441 – Gutenberg University), *Lactobacillus acidophilus* (CCUG 33386) and *Candida albicans* (CCGU 49242). Growth of *L. innocua*, *E. coli*, MSSA, MRSA, *P. aeruginosa* and *E. faecium* was performed in general media – plate count agar (PCA), from (Merck, Darmstadt, Germany). *L. acidophilus* was grown in Man Rogosa Sharpe Agar (MRS Agar), from (Biokar Diagnostics, Beauvair, France) and *C. albicans* was grown in Sabouraud dextrose agar (SDA), from (Becton Dickinson and Company, Cockeysville, USA).

2.2. Cashew tree gum

Crude samples of exudate of cashew tree gum were collected from native trees at Ilha Grande de Santa Isabel, Piauí, Brazil. The cashew gum was treated with a solution of sodium salt (NaCl) using the method previously described, (Costa, Rodrigues, & Paula, 1996), with few modifications. Nodules free of bark were selected and dissolved in ultrapure water at room temperature to give a 5% (w/v) solution. The solution pH was adjusted to approximately 7.0 by addition of diluted aqueous NaOH (VETEC, Rio de Janeiro, Brazil). The sodium salt was used to improve the precipitation process of the gum through the substitution of proton of carboxyl group by the ion sodium. The clear solution was successively filtered through sintered glass (coarse grade) and the polysaccharide was precipitated with ethanol (VETEC, Rio de Janeiro, Brazil). Prior to testing both grades (pure and crude) of cashew gum powder were dissolved in distilled water and the resulting solutions were filter sterilized using a 0.22 μm sterile filter (FriLabo – Maia, Portugal). All processes involving CG were carried out entirely at room temperature in order to avoid changes in anarcardic acid content (Torquato et al., 2004).

2.3. MIC and MBC determination

The minimal inhibitory concentration (MIC) testing was performed via a broth microdilution assay in accordance to the Clinical and Laboratory Standards Institute guidelines described in the document M7-A6 (Ferraro et al., 2003). Briefly, in this semi quantitative method bacteria were inoculated, after overnight growth, at 0.5 MacFarland standard (1.5×10^8 CFU/ml) in the appropriate media (Muller-Hinton broth for all microorganisms, except for *L. acidophilus* and *C. albicans* that was used MRS broth and Sabouraud broth, respectively) with crude cashew gum concentrations ranging from 10 to 60 mg/ml and for pure cashew gum ranging from 10 to 50 mg/ml. Two controls were simultaneous assessed: one with 10 mg/ml cashew gum but without inoculum, and another where cashew gum was replaced with sterile water and inoculated with inoculum. Samples were incubated 24 h at 37 °C, with the exception of *L. acidophilus* samples that were incubated 48 h, in a microplate reader (FLUOstar, OPTIMA, BGM Labtech) with optical density being recorded at 660 nm. The MIC was determined by observing the lowest concentration of cashew gum which would inhibit visible growth of bacteria. All assays were performed in triplicate.

The minimal bactericidal concentration (MBC) was determined as described by Fernandes et al. (2008). The MBC were determined as the lowest concentration of cashew gum at which bacterial growth was prevented and the initial viability was reduced by at least 99.9% within 24 h; it was determined by inoculation of 100 μl aliquots of negative tubes (absence of turbidity in MIC determination) on appropriate media, using the plate spread technique.

2.4. Atomic force microscopy

The effect of cashew gum on the bacterial cell surface (MRSA and *E. coli*) was examined by AFM. The control samples were prepared by applying 40 μl of bacterial suspension without treatment (control) onto a clean glass surface, followed by air-drying. The antibacterial assay samples were incubated at concentration of 10 mg/mL of pure cashew gum for the same treatment times as for the antimicrobial assays (see above). The samples were then gently rinsed with deionized water to remove salt crystals, and air-dried again under ambient conditions before analysis. No special care was taken over the drying conditions; however, all samples were prepared at the same time and so were exposed to the same conditions. Samples were examined within 24 h of deposition.

AFM was carried out with a Veeco Multimode IVa atomic force microscope (Veeco, Santa Barbara, CA) equipped with a j-type scanner (ca. $100 \times 100 \times 5\ \mu m^3$). Bacterial morphology studies were carried out in tapping mode in air, using fresh silicon cantilevers with a resonant frequency approximately 300 kHz (AppNano, Santa Clara, CA, USA). Two independently prepared samples were analyzed, and three or more areas were analyzed for each sample, but only characteristic images are shown here. Images were analyzed with Gwyddion software (Nečas, Klapetek, & Anderson, 2012). Root mean square roughness (Rq), and cell heights are quoted as means from at least 10 cells; Rq was measured in flattened 40 nm × 40 nm areas in the center of the cell.

3. Results and discussion

3.1. MIC and MBC determination

Analysis of the results obtained (Table 1) showed that both crude and pure cashew gum were capable of inhibiting the growth of most of the microorganisms studied, with pure CG inhibiting all studied microorganisms with the exception of *C. albicans* and *L. acidophilus*, while crude CG only inhibited the Gram-positive bacteria

Table 1
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of pure and crude CG upon the studied microorganisms. Values in mg/ml. ND – not detected. All assays were done in triplicate.

	MIC		MBC	
	Pure CG	Crude CG	Pure CG	Crude CG
E. coli	30	ND	ND	ND
MRSA	30	40	ND	50
MSSA	30	40	ND	50
L. innocua	30	40	ND	50
P. aeruginosa	20	ND	ND	ND
E. faecium	30	40	ND	50
C. albicans	ND	ND	ND	ND
L. acidophilus	ND	60	ND	ND

studied (MRSA, MSSA, *L. innocua*, *E. faecium* and *L. acidophilus*). The absence of MIC signifies that the CG was not capable of inhibiting the microorganisms at the tested concentrations. Contrary to the results reported by Torquato et al. (2004), antimicrobial activity was not observed against yeasts (*C. albicans*), in this study.

The inhibited bacteria presented for pure gum MIC values of 30 mg/ml with exception being *P. aeruginosa* with a MIC of 20 mg/ml, for crude gum *L. acidophilus* presented a MIC value of 60 mg/ml, for the Gram-positive microorganisms (MRSA, MSSA, *L. innocua*, *E. faecium*) were presented a MIC of 40 mg/ml, the remaining microorganisms (*E. coli*, *P. aeruginosa* and *C. albicans*) did not present MIC value. Similar MIC values were obtained among several microorganisms because, the range of concentrations tested was limited, thus allowing for different microorganism to present the same MIC. On the other hand, for the tested concentrations, MBC values were only found for crude CG with MSSA, MRSA, *L. innocua* and *E. faecium* presenting a MBC of 50 mg/ml, because of the range of tested CG.

Our results are in line with those previously reported by Marques et al. (1992) and Muroi and Kubo (1996). These authors reported that cashew gum presented antimicrobial activity against several microorganisms, among which are *A. flavus*, *B. subtilis*, *S. marcescens* and MRSA. Also, Kubo et al. (1995) reported a narrow spectrum of activity for anacardic acid with its effect targeting Gram-positive bacteria thus, since anacardic acid is thought to be the active agent of CG gum, our results are in line with those reported by these authors. It is relevant to notice that in all these studies there was no reference of antimicrobial activity against *E. coli* and *P. aeruginosa* that were inhibited in our study at 30 and 20 mg/ml of pure gum, respectively, possibly because different concentrations and cashew tree gum composition were involved.

The differences in behavior between pure and crude CG may be due to the purification process. Optical density results showed that all studied microorganisms (data not shown) presented similar behaviors when inhibited by different concentrations. All organisms showed similar behavior to *E. coli* and MRSA (shown in Fig. 1), with the only exception being *L. acidophilus* for pure gum. In this case, there was almost no difference between the positive control and highest gum concentration, thus indicating no inhibitory effect upon this bacteria species (Fig. 2), in agreement witht MIC results shown in Table 1.

3.2. Atomic force microscopy

We report here for the first time the effect of cashew gum upon cell structure of both Gram-positive bacteria – MRSA and Gram-negative bacteria – *E. coli*. Morphological characterization of MRSA and *E. coli* cells are shown in Fig. 3. The images obtained showed typically rod-shaped cells with pili or fimbria extended on the glass surface, for the *E. coli*, and cocci-shaped cells for MRSA. After

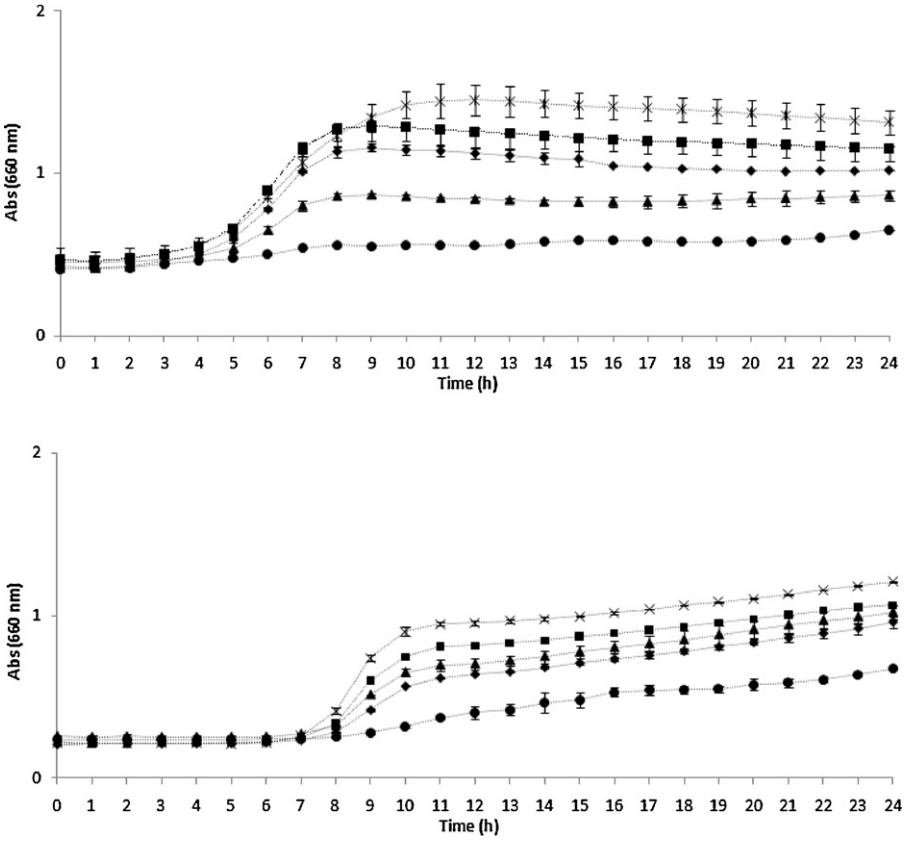

Fig. 1. Influence of pure CG on the growth of MRSA (a) and *E. coli* (b) at (•) 30 mg/ml, (▲) 20 mg/ml, (♦) 10 mg/ml, (■) 5 mg/ml; and (×) positive control (without CG).

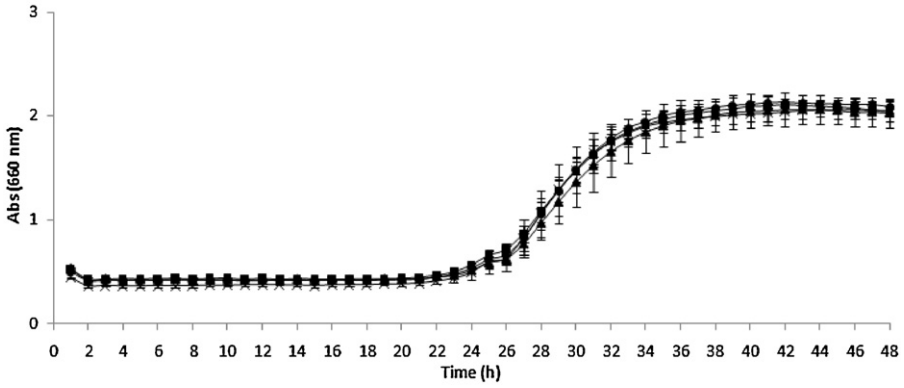

Fig. 2. Influence of pure CG on the growth of *L. acidophilus* at (•) 30 mg/ml, (▲) 20 mg/ml, (⧫) 10 mg/ml; and (×) positive control (without CG).

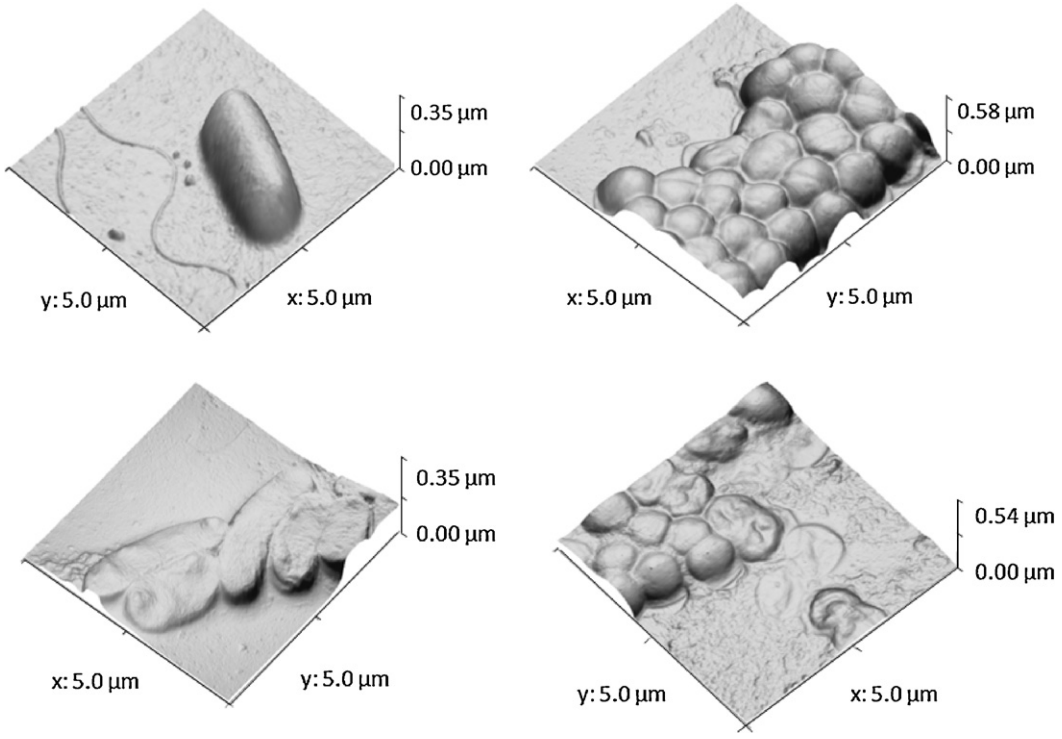

Fig. 3. Tapping mode AFM images of the effect of pure CG on cell morphology. Left: cells of *E. coli*, right: cells of MRSA. Top: control with no CG, bottom: after exposure to 10 mg/ml CG.

cashew gum treatment a range of cell morphologies was observed, including undamaged cells, as observed in the control, as well as cells with obviously altered morphologies.

Comparing images of cells treated with 10 mg/ml of pure cashew gum with those of controls, several differences can be seen: cells collapsed and became rougher and the pili and fimbria observed in the control disappeared after cashew gum treatment in the case of *E. coli*. In the case of MRSA, the cells also collapsed and became rougher, and a large amount of debris was observed close to the collapsed cocci. These observations were confirmed by measurements of cell height and membrane roughness – Table 2. As can be seen, both species showed reduced cell height, and increased membrane roughness after treatment. In addition, in all cases, the standard deviation in the results increased, which can be explained by the fact that some cells showed the effects of treatment, while others did not. This is in contrast to recent AFM studies of *E. coli* and *S. aureus* treatment by the polysaccharide chitosan, in which only the Gram-positive bacteria were affected morphologically (Eaton et al., 2008). Nevertheless, in this work, regardless of the extensive number of damaged cells observed, some undamaged cells could be observed in all samples, in particular when cells are located in the center of clusters, where the gum could not access easily. These results may indicate that cashew gum interacts with cell walls leading to the disruption of the cell wall (lysis) and consequent release of intracellular contents.

Table 2
Measured AFM parameters from *E. coli* ATCC 25922 and MRSA. Rq represents the root mean square roughness, and height represent the maximum cell height. SD represents the standard deviation.

	Rq (nm)	SD	Height (nm)	SD
E. coli	1.6	0.4	290	23
E. coli treated	3.0	2.4	218	40
MRSA	1.7	0.7	438	54
MRSA treated	3.2	2.9	317	127

4. Conclusion

Both crude and pure cashew gum showed antimicrobial activity against all Gram-positive bacteria with MIC of 40 and 30 mg/ml, respectively. However, only pure cashew gum was capable of inhibiting *E. coli* and *P. aeruginosa*. Gram-positive bacteria showed greater susceptibility to cashew gum. AFM imaging proved to be a valuable tool in understanding the antimicrobial mechanism of cashew gum upon bacterial cell. Cashew gum leads to disintegration of the cell structure probably due to the interaction with cell wall.

These results, associated with the structural similarity of cashew tree gum with gum arabic, open the possibility of exploring the use of cashew tree gum in the food and cosmetic industries.

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References

Costa, S. M. O., Rodrigues, J. F., & Paula, R. C. M. D. (1996). Monitorização do processo de purificação de gomas naturais: Goma do cajueiro. *Polímeros: Ciência e Tecnologia*, 49–55.

Eaton, P., & West, P. (2010). *Atomic force microscopy* (1st ed.). Oxford: Oxford University Press., p. 248.

Eaton, P., Fernandes, J. C., Pereira, E., Pintado, M. E., & Xavier Malcata, F. (2008). Atomic force microscopy study of the antibacterial effects of chitosans on *Escherichia coli* and *Staphylococcus aureus*. *Ultramicroscopy*, *108*(10), 1128–1134.

Fernandes, J. C., Eaton, P., Gomes, A. M., Pintado, M. E., & Xavier Malcata, F. (2009). Study of the antibacterial effects of chitosans on *Bacillus cereus* (and its spores) by atomic force microscopy imaging and nanoindentation. *Ultramicroscopy*, *109*(8), 854–860.

Fernandes, J. C., Tavaria, F. K., Soares, J. C., Ramos, O. S., João Monteiro, M., Pintado, M. E., et al. (2008). Antimicrobial effects of chitosans and chitooligosaccharides, upon *Staphylococcus aureus* and *Escherichia coli*, in food model systems. *Food Microbiology*, *25*(7), 922–928.

Ferraro, M. J., Wikler, M. A., Craig, W. A., Dudley, M. N., Eliopoulos, G. M., Hecht, D.W., et al. (2003). *Metodologia dos Testes de Sensibilidade a Agentes Antimicrobianospor Diluiç ão para Bactérias de Crescimento Aeróbico – sixth edition*. Clinical and Laboratory Standards Institute, 26(2), 15–18.

Gyedu-Akoto, E., Oduro, I., Amoah, F. M., Oldham, J. H., Ellis, W. O., & Opoku-Amevaw, K. (2007). Rheological properties of aqueous cashew tree gum solutions. *Scientific Research and Essay*, *2*(10), 458–461.

Kubo, I., Muroi, H., & Kubo, a. (1995). Structural functions of antimicrobial long-chain alcohols and phenols. *Bioorganic & Medicinal Chemistry*, *3*(7), 873–880.

Marques, M. R., Albuquerque, L. M. B., & Xavier-Filho, J. (1992). Antimicrobial and insecticidal activities of cashew tree gum exudate. *Annals of Applied Biology*, *121*(2), 371–377.

Muroi, H., & Kubo, I. (1996). Antibacterial activity of anacardic acid and totarol, alone and in combination with methicillin, against methicillin-resistant *Staphylococcus aureus*. *Journal of Applied Bacteriology*, *80*, 387–394.

Nečas, D., Klapetek, P., & Anderson, C. (2012). Gwyddion: An open-source software for SPM data analysis. *Central European Journal of Physics*, *10*(1), 181–188.

Ofori-Kwakye, K., Asantewaa, Y., & Kipo, S. L. (2010). Physicochemical and binding properties of cashew tree gum in metronidazole tablet formulations. *International Journal of Pharmacy and Pharmaceutical Sciences*, *2*(Suppl. 4), 105–109.

Silva, T. M., Santiago, P. O., Purcena, L. L. A., & Fernandes, K. F. (2010). Study of the cashew gum polysaccharide for the horseradish peroxidase immobilization—Structural characteristics, stability and recovery. *Materials Science and Engineering C*, *30*(4), 526–530.

Torquato, D. S., Ferreira, M. L., Sa, G. C., Brito, E. S., Pinto, G. A. S., & Azevedo, E. H. F. (2004). Evaluation of antimicrobial activity of cashew tree gum. *World Journal of Microbiology and Biotechnology*, *20*(5), 505–507.