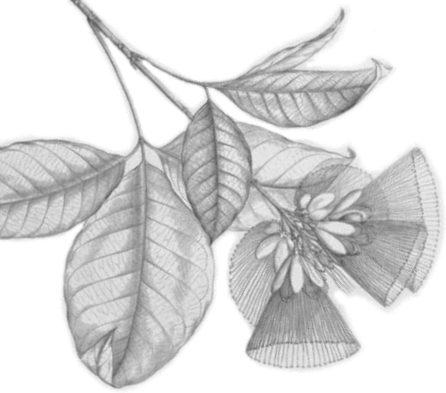

Chemical Characterization and Bioprospecting of Cashew Tree Polysaccharide of Brazilian Cerrado (*Anacardium othonianum* Rizz.), Anacardiaceae

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ABSTRACT:

The use of biodegradable polymers arouses great biotechnological interest because their chemical composition favors the interaction with biological systems, allowing applications in health and environment besides are easily decomposed. This work presents the first effort of extraction, characterization and proposition of the polysaccharide of *Anacardium othonianum* Rizz. (PEJU-GO) as material with potential for biotechnological applications. Based on scanning electron microscopy, infrared spectra and thermogravimetric analysis, the polysaccharide of *A. othonianum* Rizz. is a microporous structure, with numerous intramolecular interactions due to the presence of polar groups that give the material great thermal stability. In addition to the thin layer chromatographic data, the analysis of the chemical composition demonstrates the existence of a galactomannan type structure, with low protein content. The presence of chalcones and flavonoid compounds were also detected. The polysaccharide was able to immobilize Horseradish peroxidase with 75% efficiency over an extended pH range and presented storage and operational stabilities.

Keywords: Polysaccharide; Galactomannan; Immobilization.

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Biodegradable polymers have generated significant interest in research and industrial communities, since their degradation linked to their renewable origins promotes a reduced accumulation of waste and environmental stress, and decomposes more easily throughout industrial processing (Choong & Focattis 2016). In recent years, a great worldwide interest in the development of "green" technologies has started, because they allow the use of products with a lower environmental impact and, in this scenario, plants represent potential sources for the supply of exuded gums in a sustainable process that promotes the removal of the material without causing the death of the individual.

In general, the production of gummy exudate is a plant defense mechanism (Andrade et al. 2013), and has colloidal properties, thickening functions (binding to water molecules), gelling agents (network construction, involving linkage zones), emulsifiers, stabilizers and binders (Marques & Xavier-Filho 1991) and biodegradable. In addition, the use of polysaccharides exuded from plants as a support for enzyme immobilization has showed attractive since this material is easily recovered in simple precipitations in organic solvents, such as ethanol (Silva et al. 2010).

The Anacardiaceae Family was introduced on Africa and India by the Portuguese in the 16th century and is now widespread throughout the tropical regions of the globe. It is a botanical family represented by 70 genera and about 600 species of trees or shrubs, known for being fruitful and presenting good quality wood (Ceruks et al. 2007). India is currently the world's leading producer and exporter of cashew nuts, followed by Vietnam and Brazil. In Brazil the states that stand out are Ceará, followed by Rio Grande do Norte and Piauí (Chaves et al. 2010).

Anacardium occidentale gum and polysaccharide were extensively characterized (De Pinto et al. 1995; Silva et al. 2009). They have been explored in several ways, also showing the potential of this botanical family for the production of films (Silva et al. 2016; Ribeiro et al. 2016), stabilizers with antioxidant action (Botrel et al. 2017), supports for enzyme immobilization (Silva et al. 2010) and for drug-delivery system (Ribeiro et al. 2016).

In the Cerrado, the second largest Brazilian biome in extension (present in approximately 22% of the country), the Cerrado-arboreal cashew (*Anacardium othonianum* Rizz.) (Silva et al. 2001) occurs. The production of gummy exudates by the plant in large quantities is promoted when cortex is attacked, serving to seal the cut and prevent dehydration (Buckeridge et al. 2000). This species has a great ability of establishment and development in environments of water stress, dystrophic soils and with high aluminum content, typical conditions of the Cerrado (Naves 1999).

The scientific literature has described the nutritional potential of *Anacardium othonianum* nuts as a complementary source of proteins (Sousa et al. 2011) and their nutshells as antifungal agents in the composition of nanoparticles (Bonatto & Silva 2014). So far, the physico-chemical properties, as well as the conditions for the exploitation of gummy exudates and the polysaccharides were not reported to this species yet. This paper is the first effort aimed at extracting, characterizing gum and polysaccharide from *A. othonianum*, besides presenting the possibility of using this material as a biodegradable support for immobilization of peroxidase.

MATERIALS AND METHODS

MATERIALS

Horseradish peroxidase Type IV (E.C. 1.11.1.7), pirogallol, hydrogen peroxide, monobasic sodium phosphate and carbohydrate markers were purchased from Sigma-Aldrich (USA). The Thin Layer Chromatography plates were ordered from Alugram® Sil G/UV (Germany).

THE EXTRACTION AND PURIFICATION OF THE Anacardium othonianum POLYSACCHARIDE

The gum *in natura* (G) was obtained through incisions in the trunks of 24 individuals of cashew trees implanted in an arboretum in Goiânia, Goiás (Brazil), at the geographical coordinates 16º35'59.1" south latitude, 49º16'47.1" longitude west of Greenwich, 730 m of altitude, with an area of 6.400m^2. The incisions, 10 cm long and 2 cm deep, were performed in triplicate on each tree branch in February (room temperature in 26 °C and 13 mm precipitation) and September (room temperature in 25.7 °C, without precipitation) of 2016 (Evaporimetric Station 2016). After 15 days, the exudate nodes were collected with a spatula and stored in an amber bottle. We deposited the exsicata of the species *A. othonianum* Rizz. in the Herbarium of the Universidade Estadual de Goiás, by the number 10,993.

The incisions exudate nodules were ground and dissolved in 20% (w/v) distilled water and the mixture maintained at room temperature (26 °C) for 24 h for complete dissolution. We filtered this mixture using nylon (90 threads) and then added absolute ethanol, in the ratio of 1:3. We kept the suspension at room temperature (26 °C) for 24 h. After decantation, we discarded the supernatant, washed the precipitate with absolute ethanol, and filtered through nylon (110 threads). This procedure yielded 75% polysaccharide, named as PEJU-GO. The polysaccharide was air dried, protected from sunlight exposure, crushed and stored in hermetically sealed bottles at 4 °C until use, according Rinaudo and Milas (1991).

CHARACTERIZATION OF Anacardium othonianum POLYSACCHARIDE (PEJU-GO)

The morphological analysis of PEJU-GO was performed by scanning electron microscopy (Shimadzu, model SSx 550, Japan), with magnifications from 50 to 1000x. We used the thin layer chromatography (TLC) to identify previously the carbohydrate composition. For this assay we dissolved 10 mg m^{-1} PEJU-GO samples in hydrochloric acid 6% (v/v) and shaken it for 2 h, followed by autoclaving. About 30 μL of the following solutions were applied: 10 mg mL^{-1} PEJU-GO dissolved in 6% HCl and 5 mg mL^{-1} of carbohydrate markers (glucose, sucrose, mannose, galactose). We performed the TLC according to Chung et al. (1995) using as the mobile phase a solution of n-butanol / methanol / water (4: 2: 1). After running, the plates were oven dried at 70 °C and then sprayed with solution containing 7.5 mL of 85% (v/v) phosphoric acid, 1.0 mL of aniline, 1.0 g of diphenylamine in 50 mL of acetone. We observed the appearance of the bands after incubation of the plates in oven at 90 °C for about 30 min. The content of soluble proteins in PEJU-GO samples was determined by the method of Bradford (1976), using bovine serum albumin as standard and spectrophotometric readings at 595 nm (Kazuaki, Japan). We tested the presence of flavonoids chalcones and isoflavones according to Radi and Terrones (2007). For 80 mL of 70% (v/v) ethanol we added 8 g of PEJU-GO, boiled for 5 min and filtered on qualitative filter paper moistened with 70% (v/v) ethanol. This material, identified as an extract, was used for the characterization reactions.

We analyzed PEJU-GO samples by infrared spectrometry (FTIR) with KBr tablets (Bomn FT-IR model MB100, USA) in the range of 4000 to 500 cm^{-1}. We compared the bands in the spectra to characterize the chemical groups present in the samples, according to Silverstein et al. (2000). The determination of the crystalline phases of the samples was evaluated by X-ray diffraction (XRD) measurements on a D8 Discover diffractometer (Bruker, Germany), under a rotation of 15 rpm and a range of 2° from 5° to 70° and a step of 0.02º (Pang et al. 2012). The thermal stability of PEJU-GO was evaluated by thermogravimetric analysis, using the method of Lomonaco et al. (2012). Samples were subjected to heating ramps of 25 ºC to 500 ºC at the rate of 3 ºC min^{-1} using DTG-60H (Shimadzu, Japan).

ACTIVITY AND IMMOBILIZATION OF PEROXIDASE (Horseradish peroxidase, HRP)

We determined the peroxidase activity spectrophotometrically by the method of Halpin and Lee (1987), using spectrophotometer Kasuaki UV/VIS model IL-592, Japan**.** For 2.4 ml of pyrogallol in 0.1 mol L^{-1} sodium phosphate buffer, pH 6.0, we added 0.1 ml of the enzyme solution into the test tubes. After addition of 0.5 mL hydrogen peroxide (H_2O_2) at 0.05 mol L^{-1} the absorbance was measured

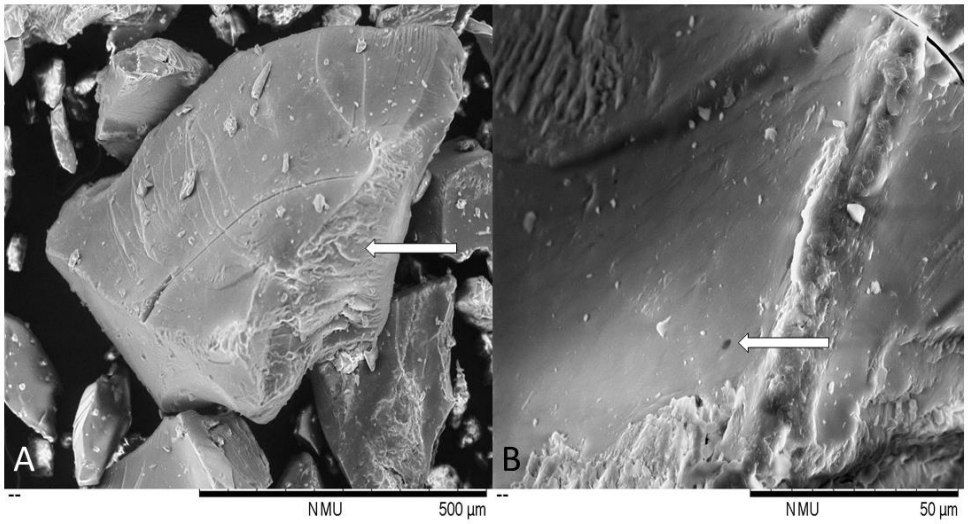
after 1 min at 420 nm. We adopt one enzyme unit (U) as the amount of peroxidase capable to produce increments of 0.1 absorbance / min of reaction under the assay conditions.

HRP immobilization was physically adsorbed using 250 μL of enzymatic solution (72.5 U) on 10 mg of PEJU-GO, under gentle agitation (720 rpm) for 2 h, at 4 °C. Then we precipitated PEJU-GO/HRP with absolute ethanol at 4 °C, centrifuged and tested for immobilized enzymatic activity. The activity test consisted of adding to the PEJU-GO/HRP complex 1.4 mL of sodium phosphate buffer 0.1 mol L^{-1} pH 6.0, 0.5 mL of hydrogen peroxide 0.05 mol L^{-1} and 1.0 mL pyrogallol 0.07 mol L^{-1}. After 1 min of reaction the reading was performed at 420 nm and activity of HRP was calculated as the same pattern for the soluble enzyme.

RESULTS AND DISCUSSION

Scanning electron microscopy showed that PEJU-GO presented irregular and crystalline porous surface (Figure 1), with extensive and amorphous mass, presence of reliefs or depressions, similar to the morphological aspect of *Anacardium occidentale* polysaccharide found by Gowthamarajan et al. (2011). The thickness, its morphology (especially the crystallinity) and the reliefs presented by the PEJU-GO favor the application of this material in biotechnological processes, such as enzyme immobilization techniques, due to its microporosity and the stability characteristics.

Figure 1. Electron micrography of Cerrado-arboreal cashew polysaccharide (*A. othonianum* Rizz.) Magnifications at 200x (A) and 1500x (B), respectively. 1A: the arrow refers to the reliefs and depressions. 1B: evidence of porous structure.



Source: Own Authors.

The thin layer chromatography showed that, compared to the carbohydrates tested as standard, we visualized only the presence of galactose and mannose in both gum and polysaccharide. This suggests that PEJU-GO belongs to the polysaccharide group of galactomannans (Azero & Andrade 1999). Rodrigues et al. (1993) found similar results in the analysis of cashew gum and polysaccharide of *Anacardium occidentale*.

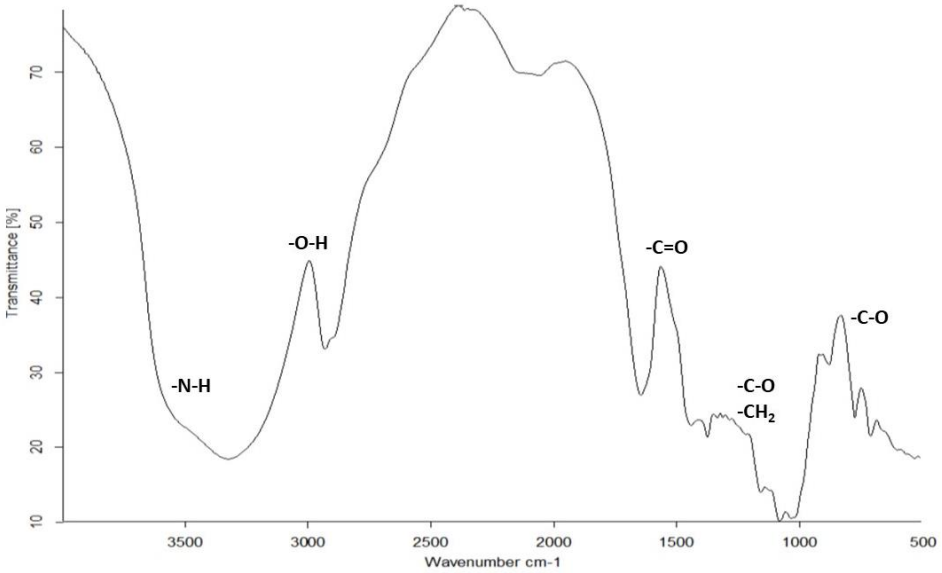
A predictable fact is the content of proteins in gums composition. The analyzes showed a 2.1 x 10^{-6} g mL^{-1} protein in PEJU-GO. These exudates are related to plant defense against mechanical injury or caused by microorganisms (Vilela & Ravetta 2005). Several authors have reported the presence of proteins in these exudates, especially those related to plant defense, such as arabinogalactan proteins, proline-rich proteins and glycine-rich proteins present in *Acacia* species (Arabic gum) (Churms & Stephen 1984; Beltrán et al. 2005; Grubb & Abel 2006).

In addition to the defense role, proteins may be important in the composition of PEJU-GO as aggregating agents for polysaccharide chains, since the protein fraction undergoes co-precipitation with ethanol (Ramesh & Tharanathan 1999). The fact that the integrity of the chain depends on protein-mediated interactions has important implications in the application of gums, since the structural integrity has the same susceptibilities that the proteins present. This integrity by proteins enables the polysaccharide to have a higher proportion of solubility due to acquired surface stability (Prapajati et al. 2013).

For the tests with PEJU-GO, we found the presence of flavonols, chalcones and isoflavones, similar to that found by Godinho et al. (2015) for another species of cashew nut, the *Anacardium humile* St. Hil. The development of green and/or black coloration in the presence of 2% ferric chloride and yellow in the presence of 5% sodium hydroxide confirmed those results. As a medicinal property, flavonoids have antioxidant, anti-inflammatory, anticancer functions (Godinho et al. 2015). Moreover, these compounds also present activity against many infectious (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, and other age-related diseases.

As can be seen in Figure 2, the Infrared spectrum of PEJU-GO shows bands characteristic of polysaccharides, such as those presented in the 3000 to 2840 cm^{-1} range (OH stretch) similar to that found by Monteiro et al. (2015) for *Anacardium occidentale*. Study with Arabic gum (*Acacia senegal*) performed by Daoub et al. (2016) also showed bands close to 2926 cm^{-1}, indicating for this material the presence of sugars such as galactose, arabinose and rhamnose, which for the PEJU-GO can be confirmed by the infrared results and by the TLC technique.

Figure 2. Infrared spectrum of *Anacardium othonianum* polyssaccharide (PEJU-GO).



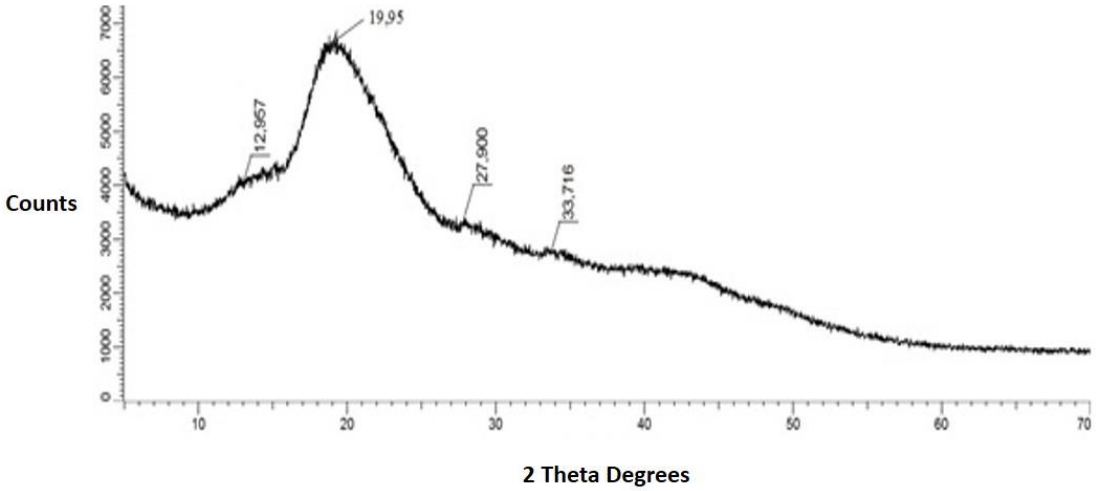
Source: Own Authors.

The bands present in the range of 1870 to 1540 cm^{-1} are related to the presence of groups -C=O, (Sekkal et al. 2003). The region along 750-950 cm^{-1} could be associated to various types of vibration of pyranoside rings and glycosidic bonds, as also visualized by Monteiro and collaborators in the characterization of *Anacardium occidentale* (Monteiro et al. 2015). We also evidenced a stretching between 900 and 675 cm^{-1}, characteristic of stretching hydrogen in aromatic groups. Ritter et al. (2015) found proximal stretches (at 840-700 cm^{-1}) characteristic of chalcones, detected in the PEJU-GO through identification tests. A sharp band at 1614 and 1602 cm^{-1} reflects a carboxyl group elongation (C=O) similar to that identified in *Anacardium occidentale* (Silva et al., 2009) and by Daoub et al. (2016) with the study of gum arabic, suggesting the presence of galactoproteins and amino acids in PEJU-GO. The absorption bands within the range of 1000-1500 cm^{-1}can be attributed to functional groups, such as pyran ring stretch, C-O stretch, O-H in-plane deformation, -CH_2 scissors vibration and C-O-C antisymmetric stretch .Furthermore, at 2900 cm^{-1} it is showed the axial deformation of –CH_2. The band observed in 1700 cm^{-1} could be attributed to a carboxyl group, which was also found by Wang et al. (2017) in the characterization of xanthan gum obtained from a strain *Xathomonas campestris*.

An extremely broad band that appeared at 3400 cm^{-1} resulted from the vibration of O-H or N-H (Wang et al. 2014). This information suggest the presence of polar groups, which would be important to interaction between the gum and phenolic compounds. Besides, in the fingerprint region observed at 1300-500 cm^{-1}, it can be suggested C–O bond stretching, and this result was similar to the found for gum arabic (*Acacia seyal* gum) by Shi et al. (2017).

Infrared spectroscopy has been a useful alternative as a complement to the high-resolution techniques of protein structure. At first, a structure as large as that of a protein should give rise to a large amount of vibrational overlays, thus obscuring the information. However, the repetition of standard structures, which occurs in biomolecules, in particular in proteins, makes their spectrum much simpler, allowing researchers to obtain useful information (Arrondo & Gonai 1999). The typical bands of a protein are based on the N-methyl-acetamide model molecule, where the amide band at 3100 cm^{-1} and 1300 cm^{-1} corresponds to an overlapping of various vibrational modes (Cantor & Schimmel 1980). We observed in the PEJU-GO the appearance of strong band in 1600cm^{-1}, corresponding to the band I of amide group, typical of proteins (Arrondo & Gonai 1999). This band corresponds to the vibration of the amide group of the carbon skeleton. We expected the presence of proteins in polysaccharide since this material is a result of the defense mechanism in the plant. In addition, proteins, mainly arabinogalactans, sustain these carbohydrate chains through galactose and arabinose linkages, mainly (Thude & Classen 2005).

Figure 3. Infrared spectrum of *Anacardium othonianum* polyssaccharide (PEJU-GO).



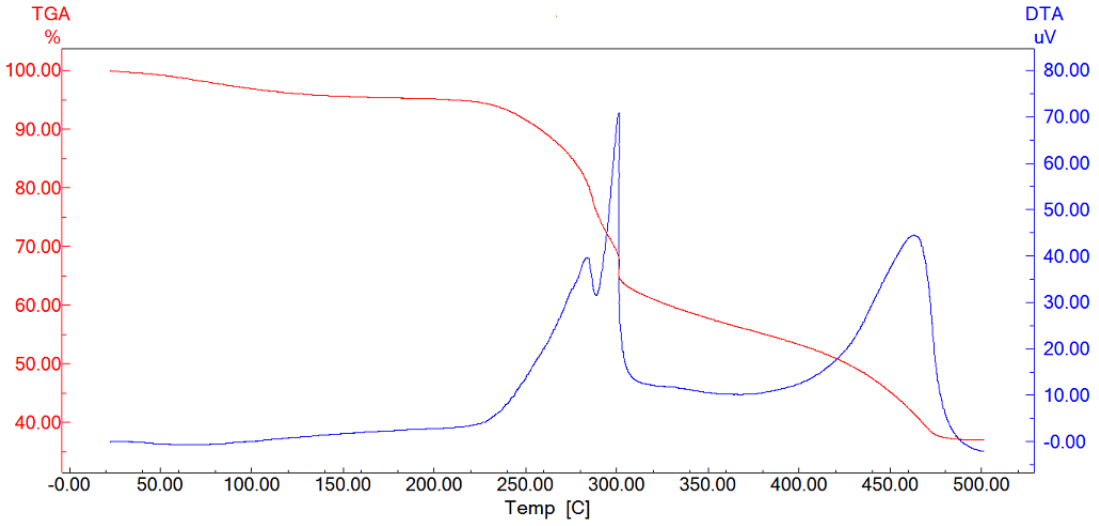
Source: Own Authors.

The crystalline and amorphous forms of the same material may show differences in particle size, particle shape, physicochemical properties, chemical stability, water solubility and also in hygroscopicity (Cano-Chauca et al. 2005). The predominance of large and diffuse peaks in X-ray diffractograms indicates amorphous and partially crystalline materials with considerable and semi-defined noise (Cano-Chauca et al. 2005). In the X-ray diffraction pattern of PEJU-GO (Figure 3) the cause of these semi-defined peaks is due to the high sugar content in the sample. The patterns exhibited a larger, but semi-defined, peak at 19.95° and small and weak peaks at 12.95°, 27.90° and 33.81°,

indicating the amorphous phase of the material (Wu et al. 2012). A similar aspect was observed in analyzes of mango pulp, performed by Harnkarnsujarit and Sanguansri (2011) and in characterizations of galactomannans, performed by Xu et al. (2007), with peaks recognized in 19.86º and 22.56º. This might be related to the close packing of PEJU-GO molecules, resulted to the strong intermolecular interactions between the polar groups, as the hydrogen bonding. The small sharp peaks in 27.90° and 33.81° might reflect the presence of low crystalline sites in the PEJU structure (Cozic et al. 2009; Wolkers et al. 2004).

In the thermal analysis (Figure 4) the PEJU-GO polysaccharide decomposition occurred in two stages, the first at 280-300 °C (maximum at 300 °C) and the second at 300-490 °C (maximum at 430 °C). A similar result was obtained by Monthé and Rao (1999) in which the first degradation event was found to occur above 200 °C and the second around 300 °C, probably due to the depolymerization with the formation of water, CO and CH_4 (Zohuriaan & Shokrolahi 2004). This result shows that the material has great thermal stability and this is an advantage, since it is a natural polymer, which should be considered for studies aimed at the biotechnological applications of PEJU-GO. In this perspective, the water solubility of cashew gum makes this material a very attractive support for enzyme immobilization, since its recovery can be easily achieved by simple precipitation with polar organic solvents, such as ethanol (Silva et al. 2010).

Figure 4. Thermogravimetric analysis of *Anacardium othonianum* polysaccharide (PEJU-GO). In red are the thermogravimetry data. In blue are the differential thermal analysis.

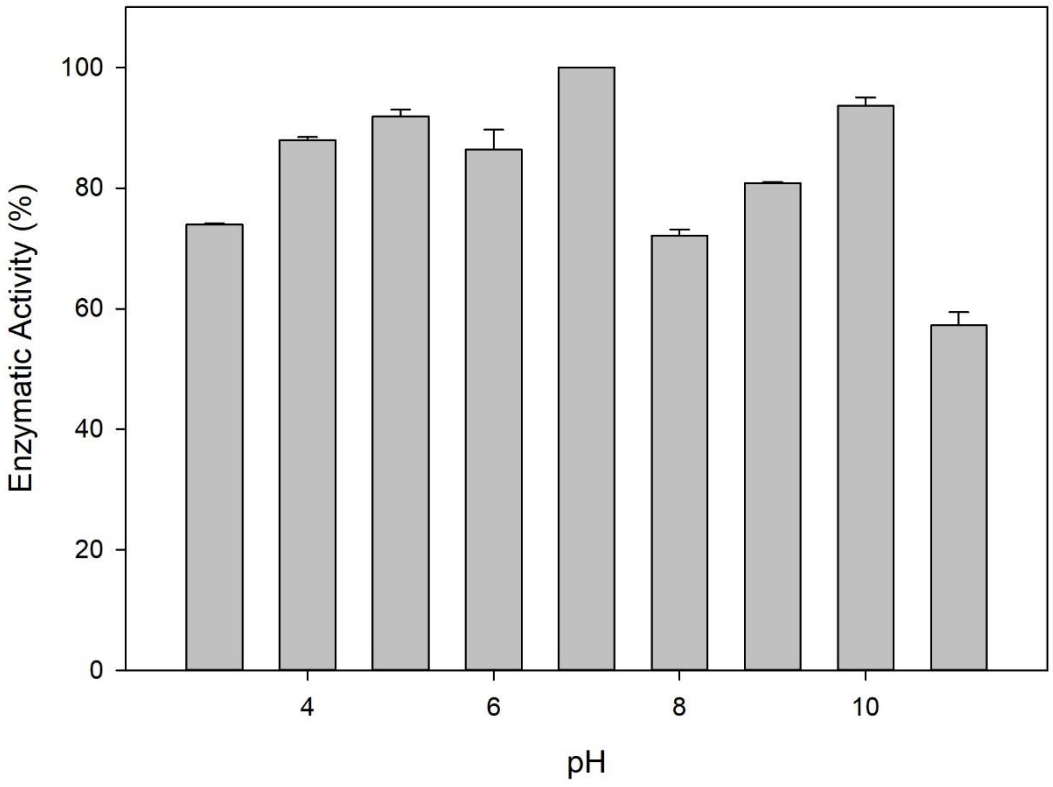


Source: Own Authors.

Evaluating the immobilization of HRP in PEJU-GO, the adsorption method retained 21 U of active peroxidase, which corresponds to 75% yield. This yield is remarkable due to the structure presented through electron microscopy, in which the enzyme binds to the support through electrostatic attractions established between the opposing charges present, both on the surface of the support (for PEJU-GO, porous and with reliefs) and the enzyme. In the case of the physical adsorption, the enzymes are bound to the matrix through the exchange of ions, a process involving hydrogen bonding, van der Waals forces and hydrophobic interactions (Gashtasbi et al. 2014). This process of physical adsorption between HRP and PEJU-GO can occur by two steps: (1) the enzyme molecules diffuse from the bulk solution to the outer surfaces of the PEJU-GO and adsorb on the hydrophobic sites easily accessible on the surface and (2) the enzyme molecules diffuse through the pores of the polysaccharide (Niu et al. 2013).

Under this way, we predict the total adsorption capacity of the final product by the texture and chemistry of the polymer (Na & Lee 2017). Thus, the influence of pH in this process is determinant and for the PEJU-GO-HRP complex is evidenced in Figure 5.

Figure 5. Effect of pH over Horseradish peroxidase (HRP) immobilization on *Anacardium othonianum* polysaccharide (PEJU-GO). Data of immobilized HRP were expressed in percentage.

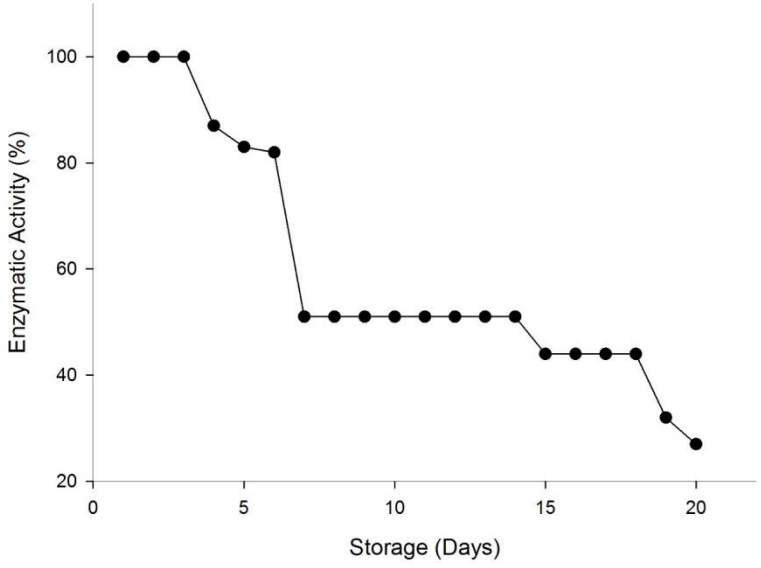


Source: Own Authors.

The studies of Liu et al. (2002) and Kalaiarasan and Palvannan (2013) report the stabilization of the enzymatic activity of HRP in the presence of polysaccharides (such as dextran and sodium alginate), resulting in the retention of active HRP in oscillating pH and temperature. The use of polysaccharides as additives may increase catalytic efficiency by forming a protective layer in the active center of the enzyme to restrain the attack of free phenoxy radicals formed in the catalytic cycle (Kalaiarasan & Palvannan 2013). The use of PEJU-GO as a support for enzyme immobilization like HRP is reinforced, since there is great versatility in the behavior of the immobilized enzyme complex (66% enzymatic activity at pH 3), neutral (65% enzymatic activity at pH 7) and alkaline medium (70% enzymatic activity at pH 10), which allows its application in different reactional environments.

The stability of the PEJU-GO/HRP complex shows about 50% of enzymatic activity remained after 15 days (Figure 6). Niu et al. (2013) observed similar results for the same conditions in fibrous membranes. This suggest that polysaccharides play fundamental roles in the enzymatic stability through electrostatic, dipole-ionic or hydrophobic interactions.

Figure 6. Operational stability of *Anacardium othonianum* polysaccharide/Horseradish peroxidase complex (PEJU-GO/HRP).



Source: Own Authors.

From the commercial point of view, the applicability and reuse of immobilized complexes are important factors for the development of biocatalysts. Unlike the free enzyme, the immobilized enzyme, being easily separated from the reactive solution and reused, reduces the cost of operations in practical applications. Table 01 shows the operational stability of the PEJU-GO/HRP complex. After

three repeated runs, PEJU-GO/HRP by the physical adsorption method and without addition of enzymatic stabilizers retained about 18% of its initial activity.

Table 1. Storage stability (shelf-life) of *Anacardium othonianum* polysaccharide/Horseradish peroxidase complex (PEJU-GO/HRP). No stabilizer was used in this experiment.

NUMBER OF USES	REMAINING ACTIVITY (%)	
	SOLUBLE HRP	PEJU-GO/HRP
1	100	100
2	8,9	65
3	0,93	18
4	0,58	6

Source: Own Authors.

CONCLUSIONS

The set of informations acquired by this present paper indicates that gums exuded from Brazilian Cerrado plants are alternative sources of polysaccharides for biotechnological applications. These allows low cost and extraction in a sustainable way.

This work represents new alternative applications of exuded gum polysaccharides, verified through characterization reactions. The PEJU-GO shows promise as support of peroxidase immobilization, by several factors: it is a polysaccharide stable under storage conditions, it is able to act in different reactional media, it is biodegradable, and it is extracted in a sustainable manner. This set of advantages reduces the negative impact of chemical analyzes on the environment and analytical laboratories.

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Caracterização Química e Bioprospecção de Polissacarídeo de Caju Arbóreo do Cerrado (*Anacardium othonianum* Rizz), Anacardiaceae

RESUMO:

O uso de polímeros biodegradáveis desperta grande interesse biotecnológico porque sua composição química favorece a interação com sistemas biológicos, permitindo aplicações na saúde e ambiente, além

de serem facilmente decompostos. Este trabalho apresenta o primeiro esforço de extração, caracterização e proposição do polissacarídeo de *Anacardium othonianum* Rizz. (PEJU-GO), como material com potencial para aplicações biotecnológicas. Baseado na microscopia eletrônica de varredura, espectros de infravermelho e análise termogravimétrica, o polissacarídeo de *A. othonianum* Rizz. é uma estrutura microporosa, com inúmeras interações intramoleculares devido a presença de grupos polares que conferem ao material grande estabilidade térmica. Somada aos dados de cromatografia em camada delgada, a análise da composição química demonstra a existência de uma estrutura tipo galactomanana, com baixo teor protéico. Também foram detectadas a presença de chalconas, e compostos flavonoides. O polissacarídeo foi capaz de imobilizar peroxidase de Horseradish com eficiência de 75%, numa faixa extensa de pH e apresentou estabilidade ao armazenamento e operacional.

Palavras-Chave: Polissacarídeo; Galactomanana; Imobilização.

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