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GLENDA QUARESMA RAMOS

ANÁLISE MORFOLÓGICA E QUÍMICA DAS FOLHAS DE *Anacardium occidentale* L.

MACAPÁ

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Amapá, como parte dos requisitos para obtenção do título de Mestre em Ciências Farmacêuticas.

Orientador: Prof. Dr. Henrique Duarte da Fonseca Filho

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Dedico este trabalho

a minhas “vózinhas” ***(in memoriam),***

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por todos os mi

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RESUMO

As plantas representam uma importante fonte de produtos naturais biologicamente ativos e na medicina tradicional. O *Anacardium occidentale* é uma planta muito conhecida de grande uso popular para tratar várias infecções e de alto valor comercial na produção de alimentos. Os estudos morfológicos e químicos são de extrema importância para o melhor conhecimento da espécie e sua produtividade. Dessa forma, este trabalho teve como objetivo analisar os aspectos morfológicos e químicos das folhas de *A. occidentale*. Para as análises morfológicas foram empregadas técnicas de microscopia que vão desde a escala macro até a nano, permitindo assim uma ampla descrição da folha. Foram avaliadas também as propriedades de molhabilidade das folhas por medidas de ângulo de contato. As folhas são do tipo hipoestomática, e possuem uma cutícula altamente estriada com grânulos de cera. Devido a essas características, a face adaxial, apresentou um comportamento hidrofóbico, gerando um ângulo de contato superior a 100°. Medidas de Difração de Raios-X foram realizadas para avaliar a cristalinidade das ceras epicuticulares e os resultados mostraram um pico de alta intensidade em torno 21°. Uma triagem fitoquímica foi realizada com o extrato etanólico e apresentou positivo para alcalóides, saponinas, fenóis e taninos. Análises químicas complementares foram realizadas pelas técnicas de Espectroscopia no Infravermelho por Transformada de Fourier, Absorção Atômica de Chama e Cromatografia Gasosa acoplada ao Espectrômetro de Massas. A composição química da cera e do extrato etanólico mostraram os triterpenos, fitoesteróides e alcanos como os principais compostos das folhas.

Palavras-chave: Anacardiaceae, superfície foliar, microscopia, cromatografia, cera epicuticular.

ABSTRACT

The plants are a significant source of biologically active natural products and in traditional medicine. The *Anacardium occidentale* is a well-known plant of great popular use to treat various infections and of high commercial value in food production. The morphological and chemical studies are extremely important for a better understanding of the species and their productivity. Thus, this study aimed to analyze the morphological and chemical aspects of *A. occidentale* leaves. For morphological analysis, microscopy techniques were used ranging from nano to macro scale, allowing a broad description of the leaf. Also the wettability properties of leaves were evaluated by contact angle measurements. The leaves are hypostomatics and have a highly striated cuticle with wax granules on adaxial surface. Due to these features, the adaxial face presented a hydrophobic behavior, generating a contact angle higher than 100°. X-Ray Diffraction measurements were performed to evaluate the waxes crystallinity and the results showed a peak of high intensity around 21°. A phytochemical screening was performed with the ethanolic extract and it was positive for alkaloids, saponins, phenols and tannins. Additional, chemical analyzes were carried out by Spectroscopy Infrared Fourier Transform, Atomic Absorption and Flame Gas Chromatography and Mass Spectrometer techniques. The chemical composition of the wax and ethanolic extract showed the triterpens, phytosterols and alkanes as the main compounds of the leaves.

Keywords: Anacardiaceae, leaf surface, microscopy, chromatography, epicuticular wax.

SUMÁRIO

1 INTRODUÇÃO

O Brasil é o país que detém a maior parcela da biodiversidade do mundo, além de um considerável conhecimento tradicional, o qual é passado de geração a geração, que inclui um vasto acervo de informações sobre manejo e uso da biodiversidade. As plantas são uma importante fonte de produtos naturais biologicamente ativos, muitos dos quais se constituem em modelos para síntese de substâncias com diversas finalidades. Nelas também podemos encontrar uma diversidade de estruturas e propriedades físico-químicas e biológicas (LEÃO, 2007).

A caracterização morfológica utiliza parâmetros que contemplam desde a escala macroscópica, como a espessura de estruturas, tamanho, forma, cor, até as escalas microscópicas, como forma de células, estruturas e presença de compostos químicos. Recentemente,a escala nanoscópica vem ganhando destaque em estudos biológicos, fornecendo dados sobre as propriedades físicas, tais como interacções moleculares, hidrofobicidade e propriedades mecânicas. Essas medidas fornecem uma nova visão sobre as relações estrutura-função de superfícies. Na literatura podem ser encontrados estudos de bioinspiração baseados na morfologia tanto na escala microscópica quanto nanoscópica e na química, principalmente da cutícula, tendo como objetivo identificar a natureza e os fatores que influenciam a sua permeabilidade (BHUSHAN, 2012; KOCH *et al*., 2009).

A cutícula apresenta várias funções nas plantas, tais como, reflexão da luz, proteção contra radiação UV, resistência contra tensão mecânica, proteção da integridade fisiológica e retenção de líquidos,é formada principalmente de cutina e ceras (MEUSEL *et al*., 2000; HOLMES, 2002). As ceras são importantes para a estrutura e funcionalidade da cutícula, e são encontradas integradas na cutícula (ceras intracuticulares) e sobrepostas a cutícula (ceras epicuticulares) (YEATS, 2013). As ceras epicuticulares apresentam diversas formas e na sua composição química são encontrados hidrocarbonetos, álcoois, cetonas, aldeídos e ácidos graxos e também podem estar presentes compostos cíclicos e aromáticos como flavonóides e terpenóides (TULLOCH, 1976; BACKER, 1982, BIANCHI, 1995).Além de depósitos de cera encontrados na superfície, podemos também encontrar outras estruturas, como tricomas. São eles que desempenham funções de proteção, adesão, e de síntese de produtos.

O *Anacardium occidentale* é conhecido popularmente como cajueiro. Pertence a família Anacardiaceae que reúne cerca de 70 gêneros, dentre eles o gênero *Anacardium* (MABBERLEY, 1997). O Cajueiro, cuja castanha possui grande valor no mercado internacional de alimento, possui inúmeros usos na indústria de plásticos e de resinas. É uma árvore que alcança até 15 m de altura e tem um tronco grosso e tortuoso; o fruto é do tipo aquênio reniforme pendente de um receptáculo carnoso e aromático, de grande valor na produção de sucos (FERNANDES, 1993). Na medicina tradicional, muitas partes são aproveitadas para o tratamento de doenças, tais como hipertensão, infecções bucais, gastrointestinais e urogenitais. No Brasil, as folhas são usadas para problemas gastrointestinais, genitais e de pele (ARUL, 2011).

Apesar de o cajueiro ser uma planta popular e de ampla distribuição, os estudos morfológicos e químicos das folhas são limitados. Diante da importância medicinal e comercial do cajueiro, estudos que contemplem a morfologia e propriedades físico-químicas das folhas tornam-se importantes investigações que proporcionam um maior conhecimento da espécie.

2 OBJETIVOS

2.1 GERAL

- Analisar aspectos morfológicos e as propriedades físico-químicas das folhas de *A. occidentale* L.

2.2 ESPECÍFICOS:

-Caracterizar morfologicamente as folhas através das técnicas de Microscopia Óptica, Eletrônica de Varredura e de Força Atômica;

-Investigar as propriedades físico-químicas da superfície foliar por Ângulo de Contato, Difração de Raios-X e Microscopia;

-Analisar a composição mineral do extrato bruto etanólico, infusão e folhas do *A. occidentale* por Espectroscopia de Absorção Atômica de Chama;

- Realizar um Screening Fitoquímico do extrato bruto etanólico das folhas de *A. occidentale*;

-Verificar a composição das folhas, extrato e ceras epicuticulares por FTIR;

-Analisar a composição química do extrato etanólico e ceras epicuticulares por Cromatografia Gasosa acoplada a Espectrômetro de Massas.

CAPÍTULO 1

Análise morfológica das folhas de *A. occidentale* L.

Revista: Rodriguésia

1.1 Morphological Characterization of *Anacardium occidentale* L. leaves from Amazon in Northern Brazil

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Morphological Characterization of *Anacardium occidentale* L. leaves

Caracterização morfológica das folhas de *Anacardium occidentale* L. da região Amazônica

Resumo: Em estudos morfológicos são analisados vários parâmetros, que vão desde macroescala passando pela microescala até a nanoescala, que contribuem para o estudo de taxonomia, farmacognosia e ecologia, entre outros. As folhas são os órgãos que apresentam maior interação com o meio ambiente e as suas estruturas funcionais são responsáveis pela grande variedade de superfícies. Alguns comportamentos são dados devido à adaptação celular e a presença ou ausência da cera cuticular. O objetivo deste trabalho foi a caracterização morfológica das folhas de *Anacardium occidentale* L., utilizando técnicas de microscopia. Neste estudo as folhas apresentam uma cutícula estriada e células epidérmicas sinuosas, nas faces adaxial e abaxial. A folha é do tipo hipoestomática, com estômatos paracíticos e com células subsidiárias estriadas. Também foi observada a presença de tricomas glandulares na superfície abaxial. A seção transversal mostrou uma única camada de epiderme e mesofilo compacto, com várias camadas de células do parênquima.

Palavras-chave: Anacardiaceae, Anatomia foliar, Epiderme, Microscópio Eletrônico de Varredura, Microscópio Óptico.

Morphological Characterization of *Anacardium occidentale* L. leaves from Amazon in Northern Brazil

Abstract: On morphological studies are analyzed several parameters, ranging from macroscale through the microscale to the nanoscale, which contribute to the study of taxonomy, pharmacognosy and ecology, among others. Leaves are the organs of greater interaction with the environment and their functional structures are responsible for the wide variety of surfaces. Some behaviors are given in terms of cellular adaptation and the presence or absence of cuticular wax. The aim of this work was the morphological characterization of *Anacardium occidentale* L. leaves, that is a plant of great medicinal and commercial interest, using microscopy techniques. In this study leaves presented a striated cuticle and epidermal cells on adaxial and abaxial epidermis. It is a hypostomatic type, with stomata of the paracytic sort and striated subsidiary cells. The presence of glandular trichomes on the abaxial surface was also observed. The cross section showed a single-layered epidermis and compact mesophyll, with several layers of parenchyma cells.

KEYWORDS: Anacardiaceae, Epidermis, Leaf Anatomy, Optical Microscopy, Scanning Electron Microscopy.

INTRODUCTION

On plant morphology studies are used several parameters which help in its anatomic and taxonomic characterization being many of them employed for many practical purposes such as ecology and in forensic laboratories and, also, trace the historical evolution of plants (Stern *et al.* 2003). A powerful tool for botanical studies is the microscopy, which provides images in different physical ways, through lens as in the optical microscope, by electrons, such as electron microscopes (scanning, transmission and tunneling) or by the interaction of atomic forces as the atomic force microscope. They give a detailed view of many essential structures for plants identification. Scanning electron microscopes (SEM) provide a magnification of details, overcoming the limitations of light microscopes, enabling a better qualitative detection. Besides featuring the morphological knowledge, they allow to perform analysis of certain factors that affect the plant, such as diseases and pathogenic processes (Ferreira *et al.* 2006).

Leaf, which is an extension of the stem, is one of the most studied organs of plants in botanical characterization, being highly variable in structure, and it is the part which has more interaction with the environment. Numerous studies about epidermis, that is the outermost plant layer presenting structures like stomata and trichomes (Cutter 1986), have been conducted focusing on cell differentiation and their controlling factors. Microscopic study of leaf features includes the size and shape of epidermal cells, besides morphology and distribution of stomata (Ellis 1979; Petronela & Nevana 2010; Stace 1984).

Anacardiaceae is a large family (Dicotyledonae) which comprises about 70 genus with approximately 875 species widely distributed in tropical regions. In Brazil, it can be stand out, mainly, *Anacardium, Mangifera, Spondias* and *Schinus* genus (Mabberley 1997). *Anacardium*, described by Carl Linnaeus, is distributed in several regions of the world, showing adaptation in many ecosystems. Into the different species, it is interesting to highlight the *Anacardium occidentale* Lineus, or "caju", as it is popularly called in Brazil, where its nut has great value in the international market of food, besides numerous uses in the plastics and resins industry. It is a tree reaching up to 15 m tall and has a thick and tortuous trunk; in case of the cashew tree, the fruit is the nut and the other part that is used to make juices is, in fact, called a pseudocarp or false fruit. It has an oval or pear-shaped structure that develops from the pedicel and the receptacle of the cashew flower and, it has a great value in the production of juices (Fernandes & Mesquita 1993). However, the structure and properties of their leaves are not well known.

Although the cashew is a popular plant and widely distributed in the world, morphological studies of their leaves are rarely. In this work a study of the leaf anatomy and ultrastructure of *A. occidentale* leaf surface using optical and scanning electron microscopy techniques was carried out. Several studies have reported the use of various parts of the cashew in folk medicine. Leaves are used to treat intestinal problems, sore throats, respiratory diseases, diabetes, hemorrhage, antiscorbutic, muscle weakness and urinary disorder (Kamtchouing *et al*. 1998; Taylor 2005). Pharmacognostic studies were carried out indicated the presence of flavonoids, anthocyanins, cardiac glycosides, tannins, sterols and triterpenes (Di Stasi 2002; Guerrero *et al.* 2002; Correa *et al.* 1995; Lima & Almeida 1993). The aim of the study was directed at finding microscopically characters that contribute to their taxonomy and which could assist in quality control plant etnodrugs derivate from the leaves.

MATERIALS AND METHODS

Plant Material

Leaves were collected in cashew tree at the Universidade Federal do Amapá campus, located in northern Brazil, close to French Guiana. A voucher was deposited in the Herbário Amapaense (HEMAB) located in IEPA (Instituto de Pesquisas Científicas e Tecnológicas do Estado do Amapá), under registration nº 018684. Samples of fresh leaves were cleaned with deionized water to remove any residues on the surface.

Macroscopic analysis

In the macroscopic analysis, fresh leaves were observed with the naked eye to obtain some features such as size, shape, base, apex, outline, margin, and venation of leaves.

Light Microscopy

Fresh leaf samples were cleaned with deionized water to remove any residues on the surface. For the analyses made in light microscopy, transversal sections in the midrib of the middle region of the leaf blades and longitudinal sections were made by hand. Samples were cleared with sodium hypochlorite and then stained with Alcian Blue and Safranin. Leaf tissues were observed on an Olympus XS-200 optical microscope, coupled with a digital camera to capture images.

Scanning Electron Microscopy

A section of 5 x 5 $mm^2$of the fresh leaves were fragmented using a razor blade from the plant, avoiding the midrib areas, in order to obtain a relatively consistent surface. Leaf specimens were mounted on a metal stub (10 mm in diameter) using two-sided adhesive carbon tape. Without metal coating, surfaces of leaf specimens were directly examined with an environmental scanning electron microscope (SEI-quanta 250) at an accelerating voltage of 5 kV.

RESULTS

In the observed samples, leaves show a dark green color on adaxial side and light green on abaxial surface, with the rib of the pinnate obovate shape, symmetric base and obtuse apex, entire margin and the smooth surface has a straight petiole. Leaves have a lamina with 10-20 cm long and 5-9 cm wide as shown in Figures 1a and 1b.

Leaf surfaces, examinated by light microscopy, showed an epidermal with winding cells wall on both sides (Figs. 2a, 2b). On adaxial face, epidermal cells had a polyhedral shape of irregular sizes, without the presence of stomata (Fig. 2a). However, on the abaxial surface epidermis there were a lot of stomata. The leaf was a hypostomatic type, where stomata were of the paracytic sort, with a random distribution, presenting an ellipsoidal shape (Figure 2b). On both faces can be noted the presence of glandular trichomes, but the amount on abaxial side is highest than the adaxial one, as shown in Figure 2.

SEM images showed a highly striated cuticle with depressions in some areas, and also observed the presence of wax beads (Figure 3a and 3b). It was possible to see stomata surrounded by subsidiary cells, which have a striated cuticle (Fig. 3 c and d). Moreover, it was also possible to observe the presence of a few granules of wax, but in a smaller amount than in the adaxial part. These varied in diameter (ca. 1-4 μm) and were scattered over the undulated cells along adaxial surface, and closer to the stomata on abaxial surface.

Different types of trichomes were present on both leaf surfaces. Four-branched non-glandular trichomes with one basal cell were found on the adaxial leaf surface (Fig.

4a, arrows). Unicellular glandular trichomes were found strictly on the abaxial leaf surface (Fig. 4b).

When making a cross-section of the leaf, it was observed, by light microscopy (Fig. 5), a thick cuticle and a single-layered unstratified epidermis, with a quadrangular shape on both sides. The collenchyma is different in size and distribution of the parenchyma, being viewed of 2-3 layers of lamellar collenchyma cells (Fig. 5b). Parenchyma cells, which are distributed in multiple layers, are a common type with polyhedral shape and cellular spaces of the canalmeatus type (Figs. 5b and 5c). It is possible to note the presence of calcium oxalate crystals in druze´s shape.

DISCUSSION

Cashew tree leaves showed a high stomatal density on the abaxial epidermal, with paracytic stomata, accompanied by two subsidiary striated cells in the lateral portion of the stomata and a parallel orientation in respect to the guard cells. According to Raven *et al.* (2008), plants with a large number of stomata have a higher rate of gas exchange and, it is important to note that features of the stomata, such as anatomical architecture, quantity, form and organization, are of great relevance to morphology and botanical classification. Stomata consist of a pore, an ostiole, and a pair of guard cells and these elements form the stomatal complex, which in turn are surrounded by subsidiary cells. The relevance of the position and the characteristics of these subsidiaries cells are discussed in many morphological studies as a basis for stomata classification (Carpenter 2005; Esau 1953; Pant & Mehra 1964; Stace 1965).

The morphology of the stomatal complex described here agrees with studies performed by Metcalfe & Chalk (1957) and Jaiswal *et al.* (2012), which describe the presence of paracytic stomatas linked to the subsidiary irregular cells on *Anacardium* *occidentale* leaves. The reported irregularity in the subsidiary cells is due to the presence of a striated cuticle.

Cuticle is considered the first contact barrier and has been subject of several studies focusing on its nature and the factors that influence its permeability, being a structure which incorporates several functions to the plants. The chemical composition of cuticular coating is varied, where cutin and wax are the main constituents. Cuticular waxes are those inserted into the cuticle and whereas epicuticular waxes are deposited over the cuticle and can be arranged in different size and shape (Heredia 1998). On *A. occidentale* leaves, waxes are presented in small granules shape.

In our study was also observed the presence of glandular trichomes on the abaxial leaf surface. They are capillaries present on the leaves epidermis and reproductive organs, having several characteristics and different functions, including the reflection of solar radiation. They balance the temperature of the plants, reduce water loss and promote the defense against insects too (Marin *et al.* 2010; Wagner 1991). In the literature two types of trichomes, glandular and non-glandular (also called tector), are described (Metcalfe & Chalk, 1957). Materials secreted by trichomes represent a chemical defense of the leaves, with a large number of studies evaluating mechanisms of secretion and chemical composition of the secreted material (Bosabalidis & Tseko 1982; Werker & Fahn 1981).

Metcalfe e Chalk (1957) discussed about many types of trichomes on *A. Occidentale* leaves, reporting the existence of glandular trichomes with thin layers of secretion. Jaiswal *et al*. (2012) described an uncountable number of tector trichomes on the adaxial surface of *A. occidentale* leaves, but in our case we have not been verified the presence of trichomes. Behadj *et al.* (2007) conducted a study with *Pistacia* *atlantica* leaves, from *Anacardiaceae* family, collected in different environments and concluded that the presence or absence, as well as trichome density is directly related to the type of environment and climate conditions that the plant is found.

In cross-sections, collenchyma is usually located below the epidermal and it has a distribution in strips along the central rib providing support to the plant. Parenchyma has metabolic functions, scaring and regeneration and it is located everywhere in plants (Cutter 1987).

CONCLUSION

The present work deepens today´s knowledge of the important morphological and anatomical characters through a descriptive study of leaf morphology by microscopy techniques which provided visualization of structures, improving the taxonomic data characterization of *A. occidentale*. These characters together with a chemical profile of the leaf are essential for the elaboration of a future pharmacopeia monograph of this herbal drug.

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FIGURE CAPTIONS

Figure 1 - Leaf of *A. occidentale* L. (a) Adaxial and (b) abaxial surfaces.

Figure 2 – Optical microscopy images, where arrows indicate glandular trichomes and **S** are stomata. (a) Adaxial and (b) abaxial face.

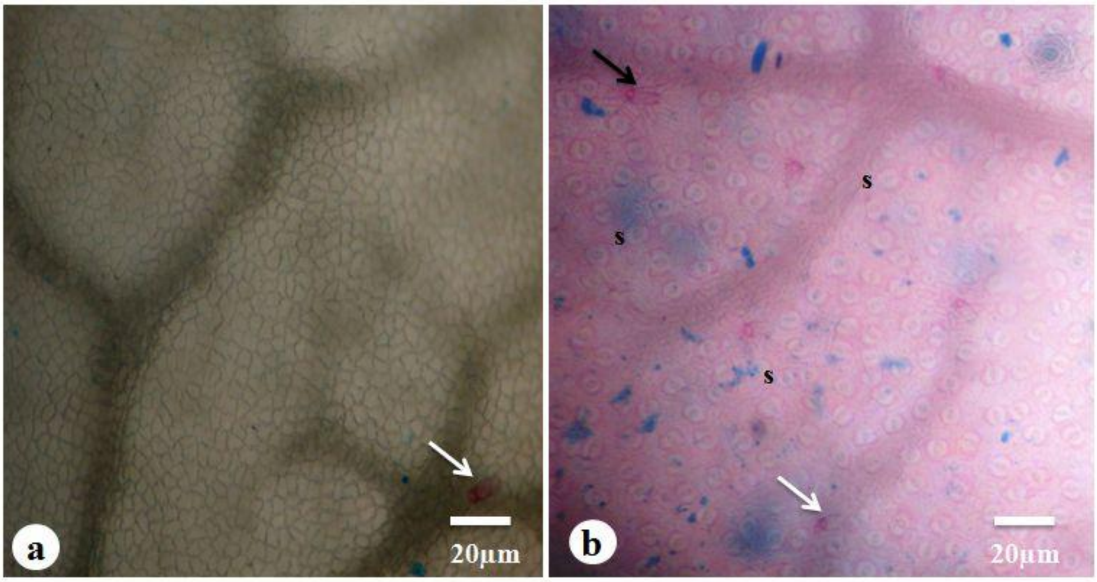


Figure 3 - Scanning electron micrograph of (a) adaxial leaf surface showing epidermis features. (b) Higher magnification where arrows indicate wax granules. (c) Abaxial leaf

surface. The stomata appearance and distribution and where arrows indicate subsidiary cells surrounded by a ridged cuticle. (d) Higher magnification of stomata.

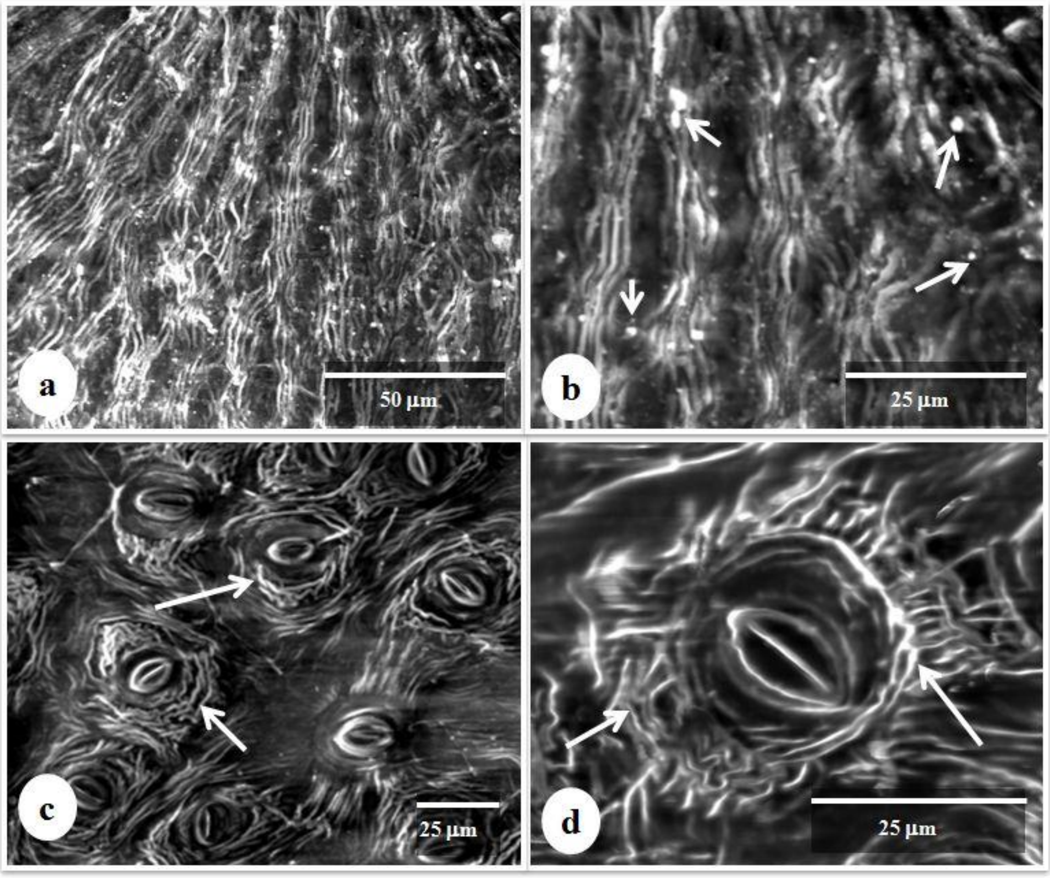


Figure 4 - Scanning electron micrographs of (a) adaxial and (b) abaxial leaf surfaces. In (a), arrows indicate a non-glandular or tector trichomes which has a basal cell and four branches. In (b), arrow shows one glandular trichomes unicellular.

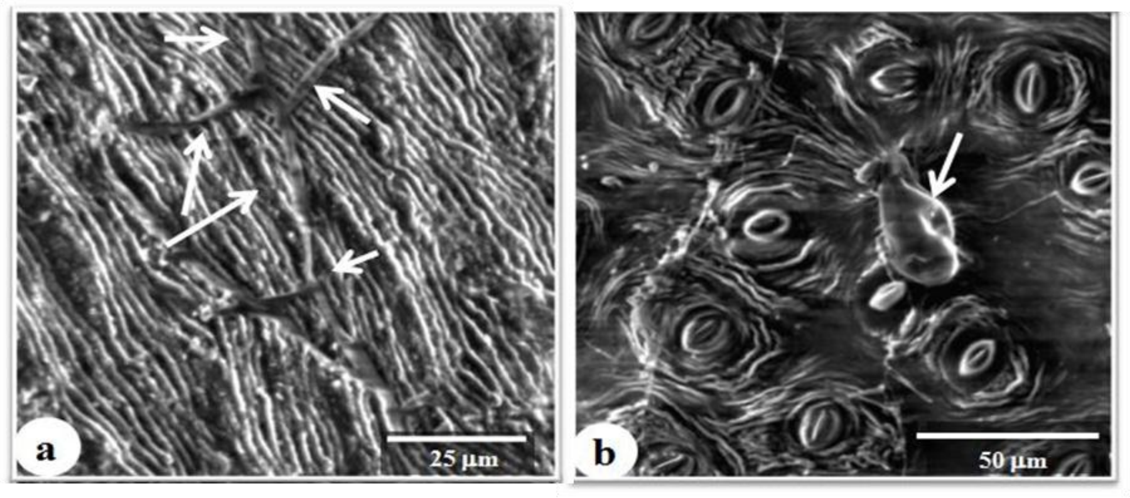
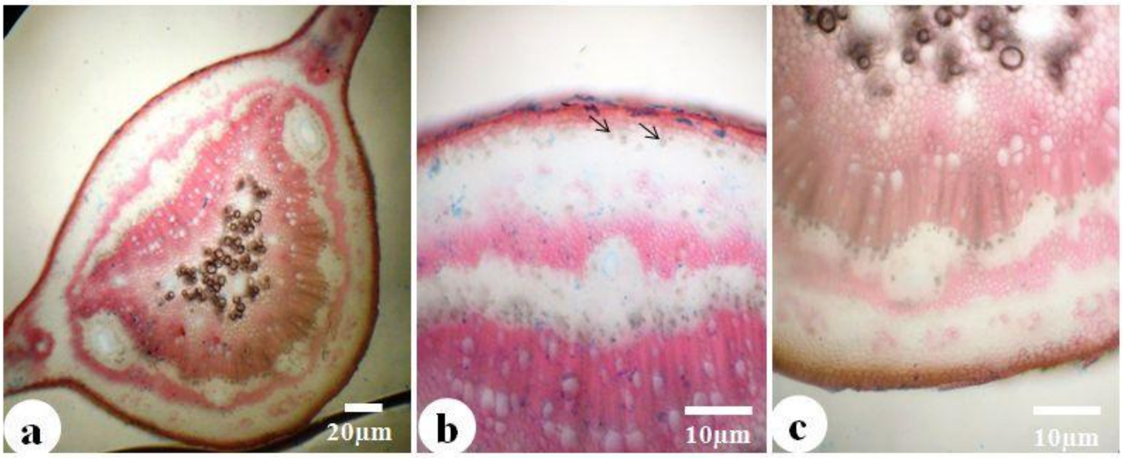


Figure 5 – Optical microscopy images of leaf midrib in cross-sections. (a) Vascular system. (b) Detail of adaxial portion, showing the calcium oxalate crystals (arrows) and (c) abaxial portion.



Revista: Acta Amazonica

1.2 Wettability and morphology of the leaf surface in cashew trees from the Amazon, Northern Brazil

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ABSTRACT

Leaf surfaces have several structures with specific functions and contribute to the relationship with the environment. Some behaviors are given in terms of cellular adaptation and the presence or absence of cuticular wax. On morphological studies are analyzed various parameters, ranging from macro scale through the micro scale to the nanometer scale, which contribute to the study of taxonomy, pharmacognosy, and ecology, among others. The aim of this paper was to analyze the effects and influences due to the presence of epicuticular waxes granules on the hydrophobicity on these surfaces. Without metal coating, surfaces of leaf specimens were directly examined with an environmental scanning electron microscope, whose images showed some characteristics (epidermis ornament, stomata type, wax, as well as trichomes). Static contact angle between water and the surface was also measured on both sides and on adaxial side was found 104,09° ± 0,95°, suggesting that it is hydrophobic. On abaxial side was 62,20° ± 1,60° showing a hydrophilic feature, probably because of the majority presence of epicuticular wax structures on the first side.The present investigation provided an important contribution to morphological and ultrastructural characterization of cashew tree leaf, that is a plant of great medicinal interest and economic importance, using microscopy techniques.

KEYWORDS*:*Hydrophobic, hydrophilic, *Anacardium occidentale* L., wax.

Wettability and morphology of the leaf surface

INTRODUCTION

Wetting (hydrophilic property) can be defined as the ability to spread a liquid on a given surface (Bhushan and Jung 2006). When a drop of water can spread easily over the surface of a material, it is said that this material is hydrophilic and it is characterized by the static contact angle (which is the angle that a liquid makes with a solid surface) measurement when $\theta < 90°$, as shown in Figure 1. This phenomenon happens when the material has high surface energy, formed by polar molecules and allows intermolecular bonds to emerge between the liquid and material. The surface atoms or molecules of liquids or solids have fewer bonds with neighboring atoms, and therefore, they have higher energy than similar atoms and molecules in the interior. This additional energy is characterized quantitatively by the surface tension or free surface energy. When a solid is in contact with liquid, the molecular attraction will reduce the energy of the system below that for the two separated surfaces. On high-energy surfaces, most liquid droplets spread out on the surface in the form of a thin liquid film, while on a low-energy surface partial wetting occurs and most droplets form a spherical cap on the surface. Therefore, when the surface of the material repels water droplets, the surface is hydrophobic and the contact angle is greater than 90°. If the contact angle is approaching 0°, it is said that the material is superhydrophilic. On the other hand, if the contact angle exceeds 150°, as in the case of water on a lotus leaf, the material is considered superhydrophobic (Figure 1A-D). The contact angle depends on several factors, such as surface energy, surface roughness and its cleanliness (Israelachvili 1992; Bhushan 2002).

Since 1970s, scanning electron microscope (SEM) studies have revealing that the hydrophobicity of the leaf surface is related to its microstructure. Three general levels of structuring are observed in leaf surfaces: the general shape of the cell, cuticular folds and epicuticular waxes. Wetting is important for many biological processes like the germination of seeds or microorganisms like fungi, reproduction of bacteria, and is essential for water uptake in soils (McHale *et al.* 2005). The plant cuticle with its integrated and exposed waxes is in general a hydrophobic material, but structural and chemical modifications induce variations in surface wetting, ranging from superhydrophilic to superhydrophobic. The sculptures of the cells, the presence of hairs and the fine structure of the surfaces, e.g., folding of the cuticle or existing epicuticular waxes (formation of three dimensional wax crystals on the plant surface), have a strong influence on surface wettability. Shapes, sizes and fine structures of cells greatly influence several functional approaches of the plant boundary layer. Then, wetting properties of surfaces have been subject to intensive studies in biology, as well as physics and chemistry. Related studies with bioinspiration (Bhushan 2012; Aizenberg 2011), hysteresis (Liu and Choi 2013), thermogenesis (Nosonovsky 2014), precipitation (Holder 2012) and external agents penetration has been well reported in the literature.

Considering the importance of detailed studies to provide a best comprehension and characterization about leaf morphology, this work examined the ultrastructural differences and compared the leaf water repellency of adaxial and abaxial sides of fresh *Anacardium occidental*e L. leaves by measuring the static contact angle and studying images from an environmental scanning electron microscope (ESEM). The hypothesis of this study was that abaxial surface would be more hidrophilic than adaxial face due to the presence of some structures, such as stomata, which would increase the roughness on the lower side. So, the purpose of this work was to characterize the leaf surfaces on microscale observing the effects and influences due to the presence of epicuticular waxes granules on the hydrophobicity on these surfaces.

MATERIALS AND METHODS

Leaves were collected in cashew tree at the Amapa Federal University campus, located in northern Brazil, close to French Guiana. A voucher was deposited in the Amapaense Herbarium (HEMAB) located in IEPA (Instituito de Pesquisa Científica e Tecnológica do Estado do Amapá), under registration nº 018684. Samples of fresh leaves were cleaned with deionized water to remove any residues on the surface.

After that, adaxial and abaxial surfaces were examined. Sections of 5 x 5 mm^2 of two fresh leaves were fragmented using a razor blade from the plant, avoiding the midrib areas, in order to obtain a relatively consistent surface. Leaf specimens were mounted on a metal stub (10 mm in diameter) using two-sided adhesive carbon tape. Without metal coating, surfaces of leaf specimens were directly examined with an environmental scanning electron microscope (SEI-quanta 250, Hitachi High Technologies America Inc, USA) at an accelerating voltage of 5 kV.

For further characterization of the surfaces, the static contact angle between water and the surface was measured. For this, it was used five different leaves to estimated the average of the contact angle on both adaxial and abaxial surfaces, getting small sections. It was done using a contact angle goniometer (model 100, Ramé-hart instrument co, Succasunna, USA) and droplets of deionized water. For these measurements, the droplet size should be smaller than the capillary length, but larger than the dimension of the structures present on the surfaces. Droplets of 2,5□L were gently deposited on the substrate using a microsyringe for measurement of the static contact angle. All measurements were made at three different points for each leaf side at 25 ± 1 ºC and 50 ± 5% RH. The measurements were reproduced within ± 2º.

RESULTS

SEM images

Scanning electron microscopy revealed a micromorphological diversity of leaf surface. There were great differences in morphology of leaf between the adaxial and abaxial surfaces. The upper side was formed by a highly undulated cuticle or ridged with depressions in some regions, and no stomata (Figure 2A). Epicuticular wax granules were observed in the upper leaf side (Figure 2B), which varied in diameter (ca. 1-4 μm) and scattered over the undulated cells along adaxial surface.

The abaxial leaf surface was rarely smooth. In almost all the sites, a large quantity of stomata was observed (Figure 2C), with a slightly ridged cuticle, which was denser around them. Stomata were ellipsoidal and were inserted in the epidermal level, with a random distribution, and therefore, the leaf was hypostomatic. The stomata were accompanied by subsidiary cells, which had a ridged cuticle (Figures 2C-D). Metcalfe and Chalk (1957) and Jaiswal (2012) had already described the presence of paracytic stomata cells that were connected to the irregular subsidiary cells in the *Anacardium occidentale* leaves. Epicuticular wax granules on abaxial surface was not observed.

Different types of trichomes were present on both leaf surfaces. Four-arm non-glandular trichomes with one basal cell were found on the adaxial leaf surface (Figure 3A, arrows). Unicellular glandular trichomes were only found on the abaxial leaf surface (Figure 3B). Metcalfe and chalk (1957) described several types of trichomes on *Anacardium occidentale* leaves, indicating the presence of glandular trichomes with thin layers of secretion and Jaiswal *et al*. (2012) observed the presence of tector trichomes on adaxial face. After analyzed different areas at the same surface and on another leaves (not shown here), it was not found the trichomes as reported before. According to Belhadj (2007), differences in the density of trichomes of the *Anacardiaceae* are due to different climatic conditions.

Static contact angle

The mean contact angle (θ) values measured and the respectively images after depositing drops of water on to the adaxial and abaxial surfaces of *A. occidentale* leaves are presented in Figure 4. The measured static contact angle of water drops placed on the fresh leaf was 104,09° ± 0,95° on adaxial side, suggesting that the cashew leaf is hydrophobic. On the other hand, the static contact angle on abaxial side was 62,20° ± 1,60° showing a hydrophilic feature.

DISCUSSION

The starting point of the investigations presented here were the observations of the wetting properties presented by cashew tree leaves. With the aim of gaining insight into epidermal structure, composition, epicuticular wax, and function in relation to water-leaf surface interactions, we analyzed the adaxial and abaxial leaf surface of *Anarcadium occidentale* L.. It was observed a different performance of the adaxial (wettable and retaining water drops) versus the abaxial (unwettable and water-repellent) leaf surfaces when in contact with water drops.

The leaf epidermis present a thin extracellular membrane, called cuticle, which is composed by cutin and epicuticular waxes, that is, in general, a hydrophobic material, whose primary function is to create a barrier against water loss. Koch *et al*. (2008) presented possible surfaces structures based on wetting behavior of plant leaves. Different nano- and/or microscale levels of plant surface sculpturing have been observed by scanning electron microscopy, generally in relation to the topography of a wide range of different structures, ranging from glands, trichomes, stomata, smooth wax or highly crystalline microcrystalline wax crystals (Koch and Barthlott 2009). Such surface features together with their chemical composition (Khayet and Fernández 2012) may lead to a high degree of roughness and hydrophobicity, controllling the wetting of these surfaces (Koch and Barthlott 2009).

The surfaces of leaves vary enormously between different species, from relatively smooth surfaces of amorphous wax to highly rough surfaces where the surface of the leaf is covered by a coating of very fine epicuticular wax crystals, such as on the lotus plant or even cabbage (Jeffree 1996). In 2009, Ringelmann *et al.* have studied the adaxial and abaxial surface of perennial ryegrass (*Lolium perenne*). This is an interesting species in that the two sides show very different wettability in which the adaxial side is covered with microcrystalline wax (predominantly primary alcohols) while the abaxial side is smooth (primarily alkanes and aldehydes). A. occidentale leaf surfaces presented these features although on abaxial surface was not so smooth, due to the a huge quantity of stomata and a undulated cuticle. Barthlott and Neinhuis (1997) verified that leaves exhibiting permanent water-repellency always displayed a very conspicuous layer of epicuticular wax crystals. The waxes are complex mixtures of relatively non-polar aliphatic and cyclic compounds (Backer 1982; Bianchi 1995; Jetter *et al*. 2006).

It also is important to mention that Brewer and Smith (1997) described about leaf morphologies that reduce water capture on leaf surfaces are more common among plants in open-field habitats, where dew formation occurs at high frequency, compared with plants in the drier forest understory. The selective pressure for reducing the wettability of leaf surfaces is usually thought to be physiologically driven (Brewer *et al.* 1991, Bradley *et al.* 2003). On the other hand, plants in wet environments are thought to have been selected over time for water shedding capabilities, to prevent the blockage of stomatal pores, thereby enhancing photosynthesis rates during and immediately after fog interception. Adaptations that reduce water retention on leaves may also reduce disease incidence, but the selective advantage of these traits may vary among habitats (Huber and Gillespie 1992).

The importance of surface roughness and heterogeneity has long been recognized as a key parameter in the wettability of hydrophobic surfaces by aqueous systems (Neinhuis and Barthlott 1997). From a physical point of view the roughening, which is caused by different structural elements (trichomes, cell walls and epicuticular waxes) is not necessarily a prerequisite for an unwettable leaf. Theoretical considerations of wettability reveal that a hydrophobic leaf becomes water-repellent if air is enclosed between surface structures and an applied water droplet but, if a roughened surface loses its waxes, the water-repellent characteristic may be reversed and the leaf becomes wettable (Holloway 1970). According to Neinhuis and Barthlott (1997), the main causes of water-repellency are epicuticular waxes which have a low mechanical stability and are easily destroyed by erosion through rain or mechanical abrasion.

In case of *A. occidentale* leaf surfaces, the hypothesis of our study was that abaxial surface would be more hidrophilic than adaxial face due to the presence of some structures, such as stomata, which would increase the roughness on the lower side, and according to the results obtained here, it was correct. The cause of having the adaxial side as a hydrophobic surface is due to a combination of a great amount microcrystalline epicuticular waxes and a cuticle highly undulated with depressions in some regions. On the other hand, the abaxial side is hydrophilic due to the absence of epicuticular wax and micromorphological diversity between both sides of leaves, leading to a difference on contact angle between the surfaces of approximately 40º.

CONCLUSION

In summary, in this paper, we have studied the micromorphology of *Anacardium occidental*e L. leaf, on both adaxial and abaxial sides, by SEM illustrating distinct differences in epidermis features of the leaf and then, increasing the knowledge about this specie. Contact angle measurements were important to know how the behavior of the wettability on the leaf surface is. In conclusion, the abaxial surface is more hidrophilic than adaxial face due to the presence of some structures, such as stomata and ridge cuticle around them, increasing the roughness on the lower side and lack of epicuticular wax. influences due to the presence of granules on the hydrophobicity on these surfaces.

ACKNOWLEDGEMENTS

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FIGURE CAPTIONS

Figure 1 – (A-D) Schematic figures of four classes of surface wettability and their characteristic static contact angle. Superhydrophobic (CA > 150^0), superhydrophilic (CA < 10^0), hydrophobic (90^0< CA < 150^0) and hydrophilic (10^0< CA < 90^0) representative surfaces, respectively.

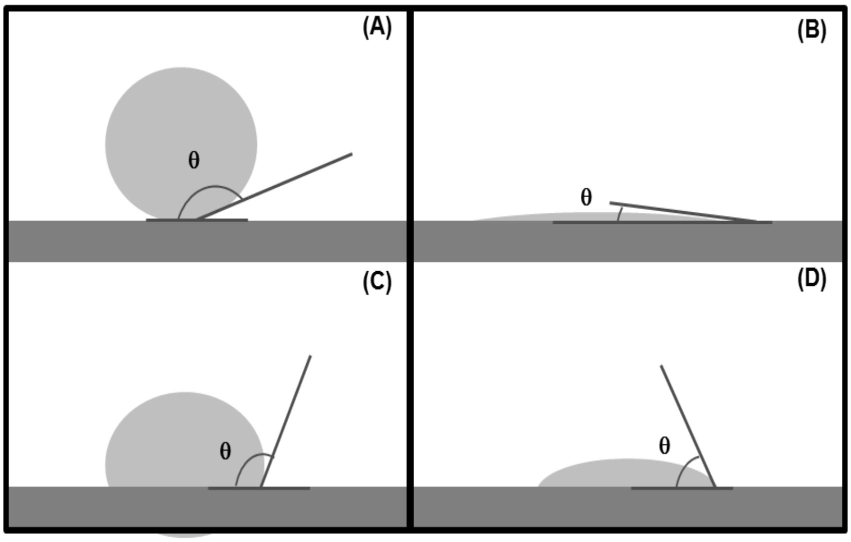


Figure 2 – Scanning electron micrograph of (A) adaxial leaf surface showing epidermis features. (B) Higher magnification where arrows indicate wax granules. (C) Abaxial leaf surface. The stomata distribution, where arrows indicate subsidiary cells surrounded by a ridged cuticle. (D) Higher magnification of stomata.

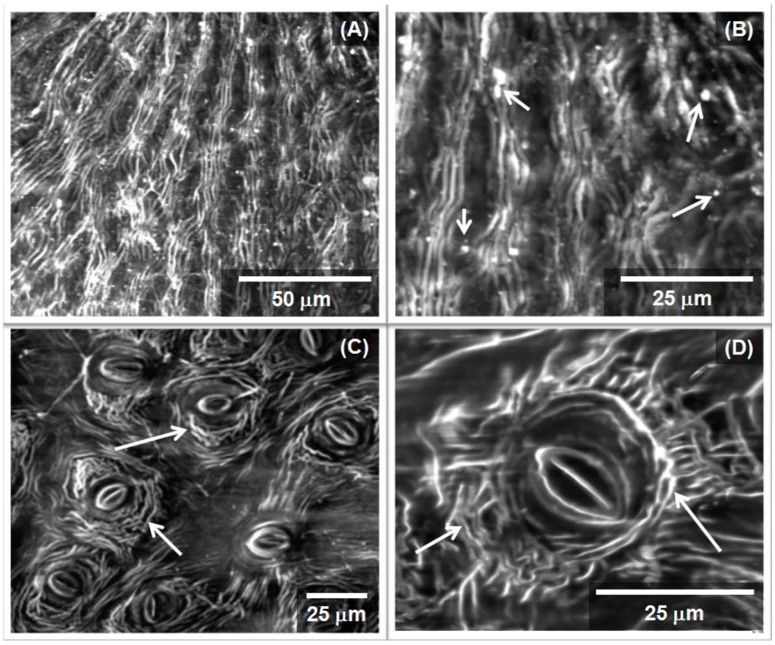


Figure 3 – Scanning electron micrographs of (A) adaxial and (B) abaxial leaf surfaces. In (A), arrows indicate a non-glandular or tector trichomes which has a basal cell and four-arms. In (B), arrow shows one glandular trichomes unicellular.

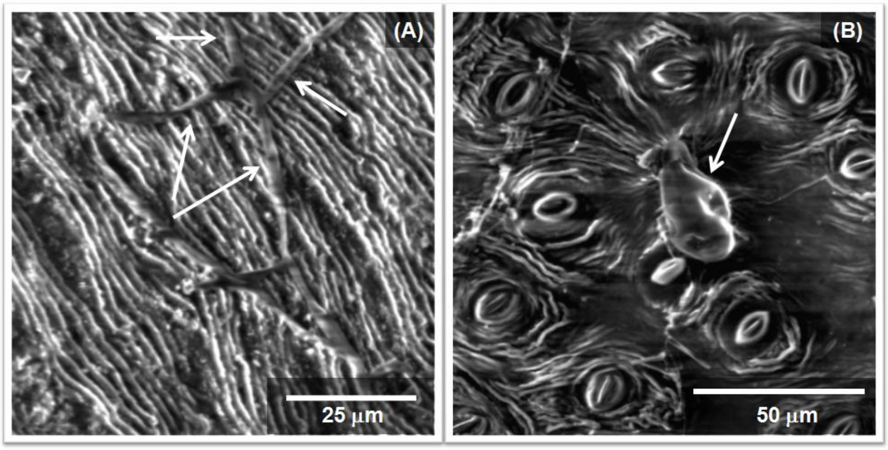
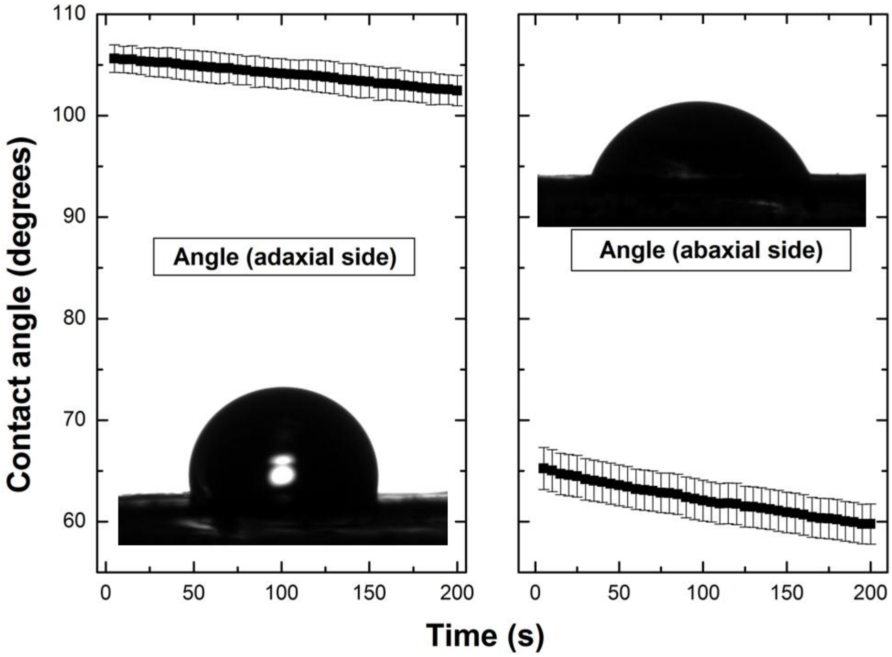


Figure 4 – Plots of static contact angle as a function of time and images of drops of water placed on adaxial and abaxial *Anacardium occidentale* leaf surfaces.



Revista: Scanning (Qualis B2 - Farmácia)

Studies on the Ultrastructure in *Anacardium occidentale* L. leaves from Amazon in Northern Brazil by Scanning Microscopy

Ultrastructure in *Anacardium occidentale* L. leaves

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Abstract: Leaves surfaces have various structures with specific functions and contribute to the relationship with the environment. On morphological studies are analyzed various parameters, ranging from macro scale through the micro scale to the nanometer scale, which contribute to the study of taxonomy, pharmacognosy, and ecology, among others. Functional structures found in leaves are responsible for the wide variety of surfaces and some behaviors are given in terms of cellular adaptation and the presence or absence of wax. This study reports the characterization of *Anacardium occidentale* L. leaf surface and the techniques used therein. A set of scanning electron microscope (SEM) and atomic force microscope (AFM) images performed on fresh leaf allowed observation of textured and heterogeneous profiles on both sides.

Keywords*: Anacardium occidentale* L., ESEM, AFM, leaf surface, stomata, adaxial, abaxial.

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1. INTRODUCTION

Leaf, which is an extension of the stem, is one of the most studied organs of plants in botanical characterization, being highly variable in structure, and they are parts that have more interaction with the environment. Numerous studies about epidermis, which is the outermost plant layer presenting structures like stomata and trichomes (Cutter, 1986), have been conducted focusing on cell differentiation and its controlling factors. Microscopic study includes the size and shape of epidermal cells, besides morphology and distribution of stomata (Ellis, 1979; Petronela, 2010; Stace, 1985).

The Anacardiaceae is a large family (Dicotyledonae) that comprises about 70 genus with approximately 875 species widely distributed in tropical regions. In Brazil, it can be stand out, mainly, *Anacardium, Mangifera, Spondias* and *Schinus*genus (Mabberley, 1997). The Anacardium genus, described by Carl Linnaeus, is distributed in several regions of the world, showing adaptation in many ecosystems. Into the different species, it is interesting to highlight the *Anacardium occidentale,* or “caju”, as it is popularly called in Brazil. It is a tree reaching up to 15 m tall and has a thick and tortuous trunk. The fruit is the nut and the other part, called a pseudocarp or false fruit, is used to make juices (Fernandes, 1993). Cashew nut has great value in the international market of food, besides numerous uses in the plastics and resins industry. However, the structure and properties of their leaves are not well known.

Scanning probe and electron microscopes are complementary high resolution techniques widely used in industrial and scientific research due to their versatile range of applications (Bhushan et al., 2006; Goodhew et al., 2001). For conventional scanning electron microscopy (SEM), which operates in high vacuum and at ambient temperature, it is a widely accepted that biological samples must be dehydrated and need a conductive coating. As a further alternative, the Environmental Scanning Electron Microscope (ESEM) can image non-conductive samples without disturbing electric charging and it is a powerful tool to analyze biological samples at ambient temperature (Shah and Beckett, 1979; Danilatos and Postle, 1982). Particularly, scanning probe microscopy (SPM) is a family consisting of a class of instruments that may generate 3D surface profiles of molecular structures with nanometer resolution. SPM family has been provided a new approach for the study of biosurfaces and/or interfaces (Takano, 1999). The number of investigations of biomaterials by atomic force microscopy (AFM) has increased rapidly in the last years. Many different approaches have been applied such as imaging of topography, force curve measurement and friction and force mapping (Hansma, 1994).

Considering the importance of detailed studies to provide a best comprehension and characterization about leaf morphology and the absence of information about cashew leaf, this work examined the ultrastructural differences comparing the adaxial and abaxial sides of fresh *Anacardium occidentale* L. leaves by studying images from an environmental scanning electron microscopy ESEM and AFM. The hypothesis of this study was both leaf surfaces would have the same epidermal structures and epicuticular waxes. So, the purpose of this work was to characterize the leaf surfaces on microscale observing the different due to the presence of some structures, such as stomata, trichomes and epicuticular waxes granules on the on these surfaces.

2. MATERIALS AND METHODS

Plant Material

Leaves were collected from a*Anacardium occidentale* L. tree that was grown into the Federal University of Amapá campus, located in northern Brazil, close to French Guiana. Sample were prepared to be deposited in the Amapaense Herbarium located in IEPA (Instituto de Pesquisas Científicas e Tecnológicas do Estado do Amapá, Brazil), for proper identification of the species. In this study, both adaxial and abaxial sides of cashew leaves were selected and thoroughly washed with running water for some minutes to remove particles and dust, and then were washed with distilled water. This step was necessary because of their biological nature, where leaves get contaminated with several particles.

Environmental SEM

Small leaf samples (adaxial and abaxial surfaces, respectively) were examined. A section of 5 x 5 mm^2, with approximately 1 mm thickness of underlying tissues, of the fresh leaves were cut using a razor blade from the plant, avoiding the midrib areas so as to give a relatively consistent surface. Leaf specimens were mounted on a metal stub (10 mm in diameter) using two-sided adhesive carbon tape. Without metal coating, surfaces of leaf specimens were directly examined with an environmental scanning electron microscope (FEI-quanta 250), suitable for biological material examination, at an accelerating voltage of 5 kV.

Atomic Force Microscopy

Fresh small samples of cashew leaves were gently fixed on a sample holder using a double-sided adhesive tape. A Nanosurf EasyScan 2 AFM apparatus (ST Instruments, Liefstal, Switzerland) was used to scan and study adhesion of surfaces at nanometer scale. This AFM apparatus was mounted on a TS-150 table from Table Stable LDT® and protected using a noise reduction hood to prevent any vibration influence during measurements. The device was used in tapping mode and equipped with a silicon tip and Al coated cantilever (Tap190AL-G from BudgetSensors™, Sofia, Bulgaria). This one has a length of 225.0 μm ± 12.00 μm, a thickness of 7.00 μm ± 1.00 μm and a width of 38.0 μm ± 9.00 μm. The spring constant is 48 N/m. The tip radius is supposed to be below 10 nm and in average around 7 nm. The tip deflection was measured through a LED system that reflects onto the cantilever by means of a detector splitted in two partitions. Scans were performed with 512 × 512 pixels at a speed of 0.7 s/line. All measurements were performed at room temperature (296 ± 1 K) and 65 ± 1% relative humidity. The feedback control was adapted to the surface in order to obtain the best possible images, and they were analyzed using the WSxM software (Horcas, 2007).

Measurements of Stomatal width and lenght

Adaxial and abaxial surfaces were examined by environmental scanning electron microscope (FEI-quanta 250). For this, it was used five different leaves to estimate the average of the width and length of the stomata on both adaxial and abaxial surfaces, according to Alvarez et al. (2009), getting small sections. They were measured from lower epidermis images of each leaf. All measurements were achieved by the software Gwyddion 2.40. All the data were analyzed by Microcal Origin 6.0.

3. RESULTS

Environmental SEM images

Scanning electron microscopy revealed a micromorphological diversity of leaf surface. There were great differences in morphology of leaf between the adaxial and abaxial surfaces. The upper side is formed by a cuticle highly undulated or ridged with depressions in some regions, and had no stomata

(Figure 1(a)). In figure 1(b) can be noted a large quantity of stomata and it has a slightly ridged cuticle that is denser around them.

Stomata are ellipsoidal and are inserted at the level of the epidermis, with a random distribution, and therefore, the leaf is hypostomatic. The stomata are accompanied by subsidiary cells, which have a ridged cuticle (figure 2) and it was not observed the presence of epicuticular wax granules around them. Stomatal density on the lower epidermis ranged from 325 to 506 mm^{-2}, and the average number or stomata founded was 426,25 ± 31,83. The stomatal width and length ranging from 6,7 to 16 μm and 8 to 23,5 μm, respectively. The average width and length were 10,60 ± 0,18 μm and 14,40 ± 0,28 μm, respectively.

Two different types of trichomes are present on the leaf surfaces. Four-branched non-glandular trichomes with one basal cell are found on the adaxial leaf surface (Figure 3a, arrows). Unicellular glandular trichome is found strictly on the abaxial leaf surface (Figure 3b).

The presence of epicuticular waxes granules on both surfaces was also studied. As shown in Figures 4(a) and 4(b), granules varied in diameter (ca. 1-4 μm) and are scattered over the undulated cells along adaxial surface, however these granules were not observed on abaxial surface.

Atomic Force Microscopy images

AFM data were collected at 3 x 3 μm, 15 x 15 μm and 25 x 25 μm scans for adaxial surface and 5 x 5 μm, 25 x 25 μm and 100 x 100 μm scans for abaxial surface show an irregular profile with bright and dark regions corresponding to high and low parts, respectively. On the upper epidermis, images present a cuticle highly undulated or ridged with depressions and wax beads around them. These wax granules were mostly globular, varied in diameter according to ESEM results, and randomly arranged on the abaxial surface, at least 180 nm high, as shown in figure 5. Comparing both leaf sides, the height profiles on the adaxial face is less than the other because of the stomata absence, as shown on 3D images of the figures 5 and 6. Figure 6(a) shows 3 different stomata, exhibiting an ellipsoidal shape, and they are inserted at the level of the epidermis according to the SEM results.

4 DISCUSSION

Carpenter (2005) reported the importance dedicated to the study of the stomata based on the anatomical architecture, quantity, shape and the organization of epidermis cells associated with guardians cells, because it provides subsidies to the taxonomy of plants (Stace, 1965). Stomata are composed of a pore, the ostiole and a pair of guardians' cell and they are surrounded by subsidiary cells that differ from the others in size, shape and orientation (Esau, 1953; Pant, 1964). The position of the subsidiary cells is an important factor to classify the type of stomata on the leaf epidermis (Carpenter, 2005). This morphology of the stomatal complex is according to Metcalfe and Chalk (1957) and Jaiswal (2012), which described the presence of paracytic stomata cells that are connected to the irregular subsidiary cells in the *Anacardium occidentale* leaves. This irregularity is due to the presence of a ridged cuticle. Cashew tree leaves used in this study were located on the Equatorial Line in northern Brazil, which is a hot and humid place and high solar intensity, presenting a high stomatal density. It can vary within leaves, plants, and individuals of a single species (Al Afas et al., 2006) and Woodward and Kelly (1995) mentioned that it can also vary due to environmental factors such as light, air humidity, water availability and atmospheric CO_2 concentration. In general, that density decreases with increase in [CO_2] and in comparison with leaves developed under low light intensity, sun leaves have higher stomatal densities (Givnish, 1988).

Trichomes are unicellular or multicellular projections from plant surfaces that originate from the epidermal cells. The materials secreted by trichomes play an important role in protecting leaves. Several studies make mention of trichomes, its structure and also the chemical composition of secretion taken by glandular trichomes, as well as their own secretion mechanisms (Bosabalidis, 1982; Werker, 1981). In the literature two types of trichomes are described: glandular and non-glandular, also called tector (Metcalfe and Chalk, 1957). The essential oils in the glandular trichomes play a role in guiding pollinators or in defense against excessive transpiration and solar radiation. The non-glandular trichomes probably collaborate together with the glandular trichomes in the mechanical defense against predators (Marin 2010; Wagner, 1991). Metcalfe and Chalk (1957) reported several types of trichomes on *Anacardium occidentale* leaves, indicating the presence of glandular trichomes with thin layers of secretion and Jaiswal et al. (2012) observed the presence of tector trichomes on adaxial face. Cashew tree leaves were collected during the rainy season presenting quite a few trichomes on the surfaces. Pérez-Estrada et al. (2000) showed leaf trichome density in *Wigandia urens* was highly variable and depended on seasonal changes in light availability and soil water content. Trichome density decreased during the rainy season and increased during the dry season. Plants growing in sun exposed areas had higher trichome densities than plants growing in shaded areas. Furthermore, trichome density decreased in response to irrigation and shade treatments. According to Belhadj (2007), differences in the density of trichomes of the *Anacardiaceae* are due to different climatic conditions.

The epidermis present a thin extracellular membrane, called cuticle, which is composed by cutin and epicuticular waxes, that is, in general, a hydrophobic material, whose primary function is to create a barrier against water loss. Koch *at al*. (2008) presented possible surfaces structures based on wetting behavior of plant leaves. The sculptures of the cells, the presence of hairs and the fine structure of the surfaces, e.g., folding of the cuticle or existing epicuticular waxes, have a strong influence on surface wettability. Beside this, structural and chemical modifications can induce variations in surface wetting, ranging from superhydrophilic to superhydrophobic. Epicuticular waxes are the most common type of cellular structuring. Several investigations by SEM showed that most of the epicuticular waxes form three-dimensional structures with large variations in their morphologies.

Since its appearance in 1986 (Binnig et al.), the AFM has proven to be a powerful tool to investigate structures of several different biological samples. It has demonstrated its ability to image under ambient conditions, rendering all such pre-treatment and damages irrelevant. It can work even on liquid surfaces (Hannig et al., 2010). AFM has the same resolution along and perpendicular to the surface and can provide three-dimensional images of the surface topography. Three dimensional epicuticular wax structures usually occur in sizes from 0.5 to 100 micrometres, whereas 2-dimensional wax films range from a few molecular layers up to 0.5 micrometres. Recent overviews about terminology and micro-morphology are given by Barthlott et al. (1998) and Jeffree (2006). Jeffree distinguishes six main morphological wax types, including a background epicuticular wax film, which covers the cuticle surface below and between the 3-D waxes. This wax film is rarely visible in SEM when leaves are investigated, but can be well visualized after mechanical separation of the waxes from the plant surface and by AFM investigation. (Koch and Ensikat 2007). In cashew leaves, the AFM examination showed a great variation between the two leaves surface in details and a 3D information. The topography images clearly exhibited numerous small circular bodies on upper cuticle. The morphology of these bodies were similar to epicuticular wax complexes that have been described (e.g. *Pistacia Vera, Vitis vinifera;* Baker, 1982).

These epicuticular waxes granules were spread over the entire adaxial surface. Similarly, Perkins et al. (2004) and Bensalem-Fnayou et al. (2009) reported that AFM topography images of *Prunus laurocerasus* and *Vitis vinifera* leaves, respectively, revealed a textured granular surface.

5 CONCLUSION

In this paper, we have studied the micromorphology of *Anacardium occidental*e L. leaf, on both adaxial and abaxial sides, by SEM and AFM, where on the upper side there was a cuticle highly striated or ridged with depressions in some regions, and had no stomata. On the other hand, on the lower side was noted a large quantity of stomata and it had a slightly ridged cuticle that was denser around them. Four-branched non-glandular trichomes with one basal cell were found on the adaxial side and a unicellular glandular trichome was found only on the abaxial leaf surface. Beside this, epicuticular waxes granules were spread over the entire adaxial surface. This work contribute to the study of taxonomy, pharmacognosy, and ecology, among others about the *A. occidentale* L. mainly because of the AFM images that not reported yet with this plant species. In conclusion, this study illustrated distinct differences in epidermis features of the cashew leaf.

6 ACKNOWLEDGEMENTS

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FIGURE CAPITIONS

Fig. 1 – Scanning electron micrographs of *Anarcadium occidentale* leaf surface showing epidermis features. (a) Adaxial and (b) abaxial side.

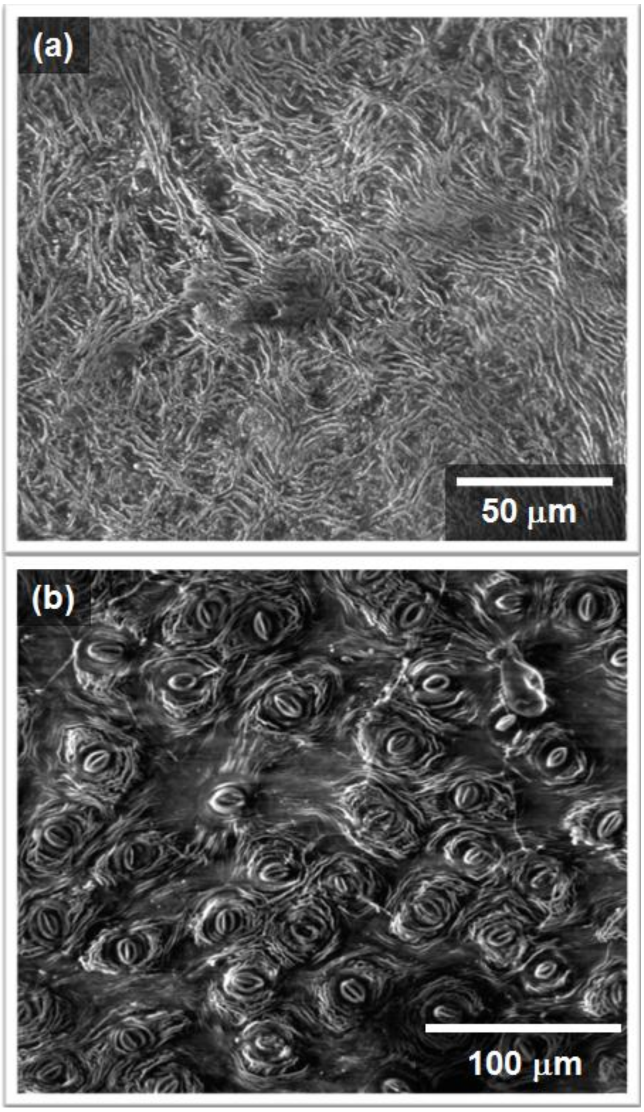


Fig. 2 - Scanning electron micrographs of lower leaf surface. (a) The stomata appearance and distribution and where arrows indicate subsidiary cells surrounded by a ridged cuticle. (b) Higher magnification of stomata.

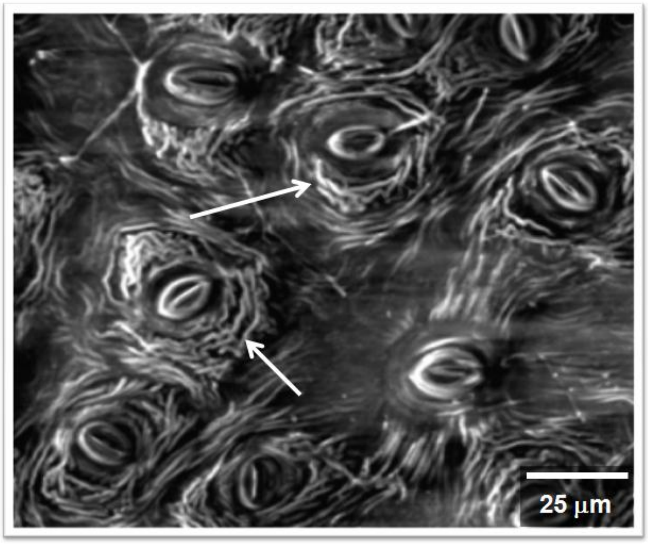


Fig. 3 - Scanning electron micrographs of (a) adaxial and (b) abaxial leaf surfaces. In (a), arrows indicate a non-glandular or tector trichomes which has a basal cell and four branches. In (b), arrow shows one glandular trichomes unicellular.

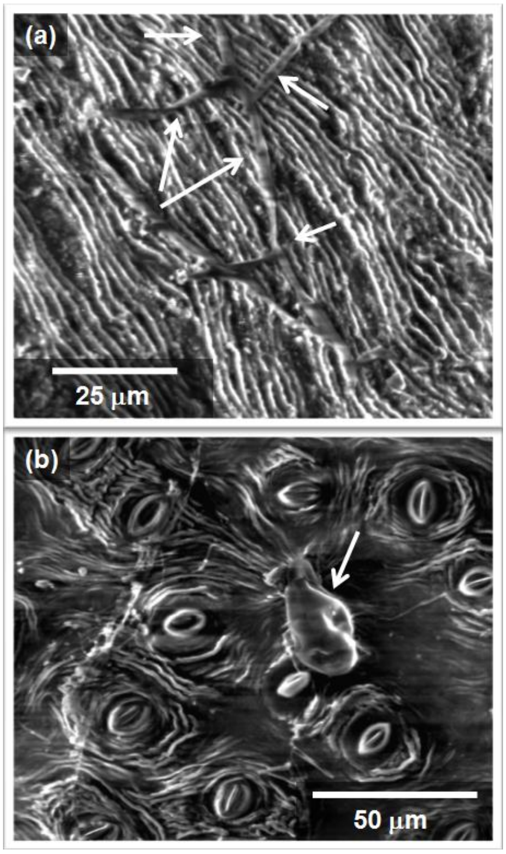


Fig. 4 - (a) Scanning electron micrograph of adaxial leaf surface. (b) Higher magnification where the arrows indicate epicuticular waxes granules.

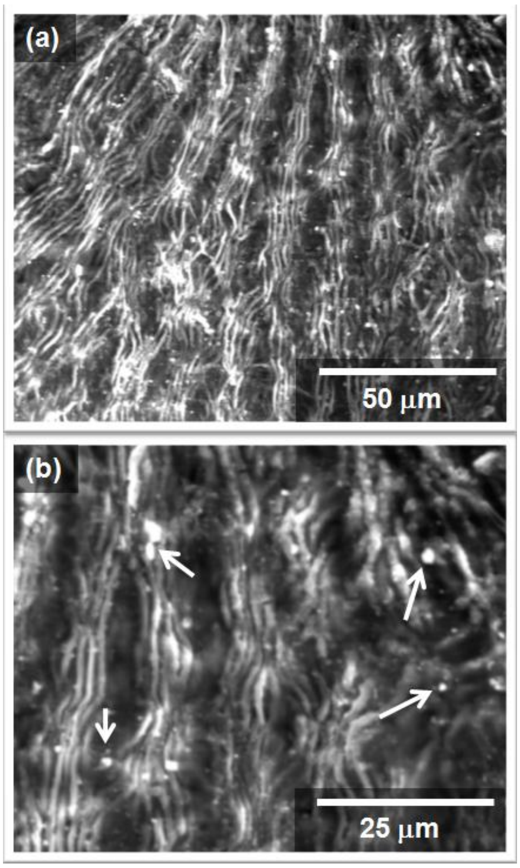


Figure 5: (a), (b) and (c) AFM micrographs of upper epidermis. 2D and 3D images showing the wax beads around a cuticle highly undulated on different magnifications. (d) AFM section of epicuticular wax granules, shown in (c).

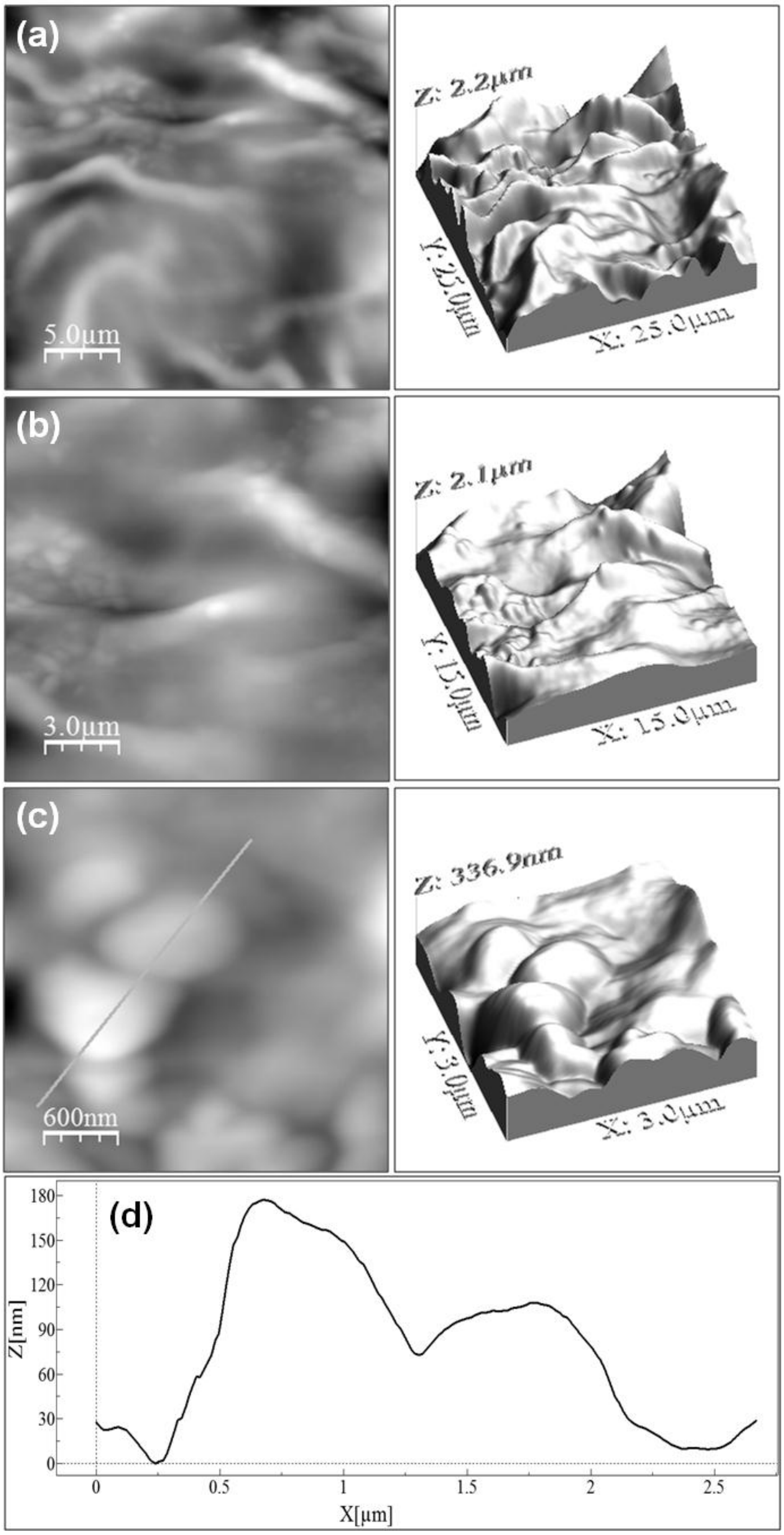
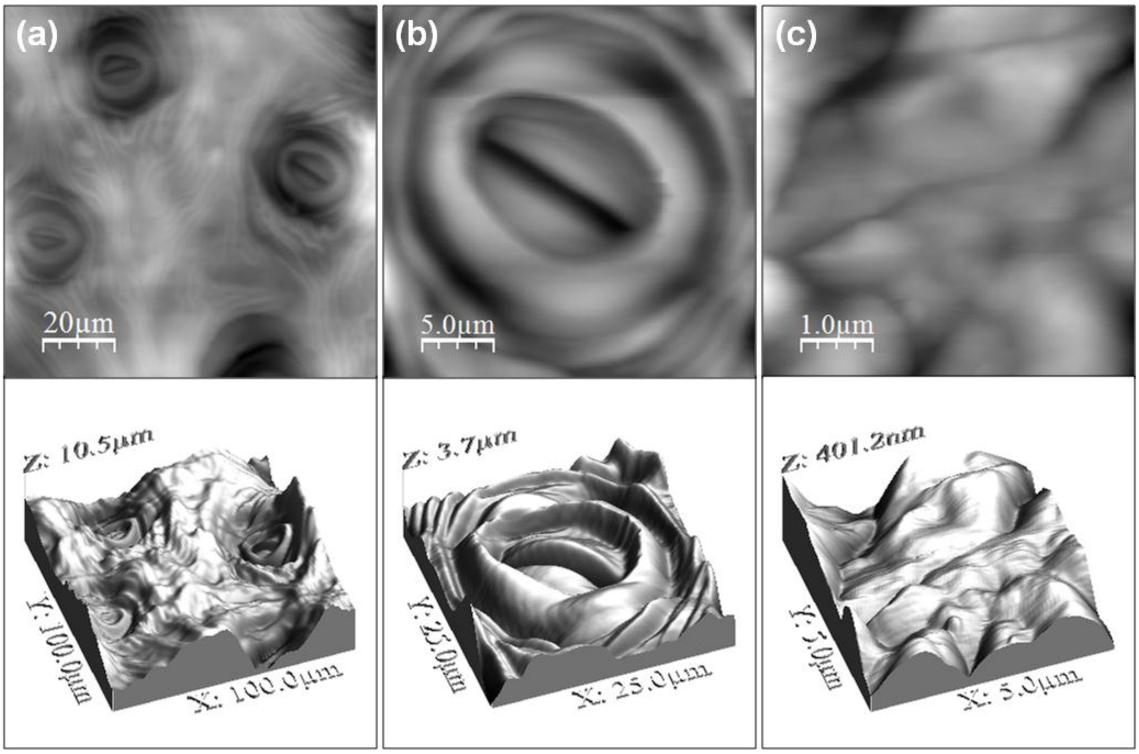


Figure 6: AFM micrographs of lower epidermis. (a) and (b) 2D and 3D images showing the stomata, and (c) a high magnification among the stomata.



CAPÍTULO 2

Análise Química das folhas de *A. occidentale L.*

Revista: Bioterra

ANÁLISE FITOQUÍMICA DAS FOLHAS DE *Anacardium occidentale* L., AMAPÁ, BRASIL.

PHYTOCHEMICAL ANALYSIS OF *Anacardium occidentale* L. LEAVES, AMAPA, BRAZIL.

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Resumo: Os estudos fitoquímicos têm o objetivo de conhecer os constituintes químicos de espécies vegetais ou avaliar a sua presença. O Brasil é o país que detém a maior parcela da biodiversidade do mundo, além de um considerável conhecimento tradicional, o qual vem sendo passado de geração em geração, constituindo assim um vasto acervo de informações sobre manejo e uso da biodiversidade. O *Anacardium occidentale*, ou cajueiro, é uma planta muito conhecida e tem grande importância econômica, mas, para fins terapêuticos poucos estudos foram realizados, limitando-se apenas a conhecimentos tradicionais. Este trabalho teve como objetivo realizar uma triagem fitoquímica das folhas do cajueiro. Os testes realizados foram positivos para alcalóides, fenóis, taninos e saponinas, compostos estes que possuem atividades biológicas.

Palavras-chave: Anacardiaceae, cajueiro, fitoquímica, metabólitos secundários.

Abstract: Phytochemical studies aim to know the chemical constituents of plant species or evaluate their presence. Brazil has the largest share of the world's biodiversity, and a considerable traditional knowledge, which has been passed from generation to generation, thus providing a vast amount of information on the management and use of biodiversity. The Anacardium occidentale, or cashew, is a well-known plant and has great economic importance, but, for therapeutic purposes, few studies have been performed, limited only to traditional knowledge. This work was to aim for a phytochemical screening of cashew leaves. The tests were positive for alkaloids, phenols, tannins and saponins, which compounds have biological activity.

Keywords: Anacardiaceae, cashew, phytochemical, secondary metabolites.

INTRODUÇÃO

As plantas representam uma importante fonte de produtos naturais biologicamente ativos e com o desenvolvimento da química farmacêutica, elas passaram a ser a fonte primária do desenvolvimento de medicamentos. O conhecimento popular data de muitos anos e é um dos recursos mais adotados atualmente na seleção de espécies para estudos fitoquímicos e farmacológicos, pois direciona os estudos partindo do uso terapêutico alegado por um determinado grupo que detenha um conhecimento empírico (LORENZI, 2008).

Estudos fitoquímicos de plantas constituem uma alternativa na procura de novos agentes terapêuticos. Em geral, os princípios ativos de plantas são produtos do metabolismo secundário, sendo produzidos e acumulados nos tecidos vegetais. São imprescindíveis para a sobrevivência, viabilizando a adaptação e respondendo as interações com o meio ambiente (MONTANARI, 2002).

A família Anacardiaceae reúne cerca de 70 gêneros, com aproximadamente 875 espécies, distribuídas em regiões tropicais. No Brasil destacam-se principalmente os gêneros *Anacardium, Mangifera, Spondias* e *Schinus* (MABBERLEY, 1997). O gênero *Anacardium,* descrito por Carl Linnaeus, possui distribuição em diversas regiões do mundo, apresentando adaptação a diversos ecossistemas.

Das variadas espécies destaca-se o *Anacardium occidentale,* conhecido popularmente como Cajueiro, cuja castanha possui grande valor no mercado internacional de alimentos, além de inúmeros usos na indústria de plásticos e de resinas. É uma árvore que alcança até 15 m de altura e tem um tronco grosso e tortuoso; o fruto é do tipo aquênio reniforme pendente de um receptáculo carnoso e aromático de grande valor na produção de sucos. Originário do Brasil, o cajueiro é muito utilizado na medicina tradicional com efeitos terapêuticos, possuindo representatividade também no comércio de alimentos (FERNANDES *et al.*, 1993). Estudos relatam a utilização do cajueiro na medicina tradicional, com efeitos terapêuticos. Na literatura, encontram-se atividades farmacológicas, como sendo uma planta antiinflamatória, antidiabética e antimicrobiana (AKINPELU, 2001; OLAJIDE, 2004; BARBOSA-FILHO *et al.*, 2005).

MATERIAL E MÉTODOS

Coleta

As folhas foram coletadas no município de Macapá-AP, em uma área no Campus Marco Zero da Universidade Federal do Amapá (UNIFAP). Uma espécime foi preparada e depositada no Herbário Amapaense - HERBAM, localizado no Iepa (Instituto de Pesquisas Científicas e Tecnológicas do Estado do Amapá), sob o registro de número 018684.

Extração

As folhas coletadas foram lavadas e secas em estufa de ar circulante à temperatura de 40ºC. Após secagem, as folhas foram trituradas em moinho elétrico. Para obter o extrato bruto das folhas, o material foi macerado em álcool etanólico (v/v) e realizado uma extração exaustiva. Posteriormente, a solução extrativa foi submetida à evaporação em evaporador rotativo até a remoção do solvente e obtenção do extrato bruto etanólico de *A. occidentale* L. (MATOS, 1997).

Testes fitoquímicos

Os testes de prospecção fitoquímica foram realizados no Laboratório de Farmacognosia da UNIFAP, segundo as técnicas adaptadas de Barbosa *et al.* (2004) e Matos (1997). Esses testes visaram a análise qualitativa dos metabólitos secundários presentes no extrato. Neste estudo, foram realizados testes para Ácidos Orgânicos, Açucares Redutores, Antraquinonas, Alcalóides, Flavonóides, Fenóis e Taninos, Esteróides e Triterpenos e Saponinas.

RESULTADOS E DISCUSSÃO

Nas análises preliminares os testes foram positivos para ácidos orgânicos, açúcares redutores, fenóis, taninos, alcalóides e saponinas, como mostra a tabela 1.

Tabela 1- Resultado da análise fitoquímica do extrato bruto das folhas de *A. occidentale*.

Metabólitos	Resultado
Ácidos orgânicos	Positivo
Açúcares redutores	Positivo
Fenóis e taninos	Positivo
Flavonóides	Negativo
Alcalóides	Positivo
Esteróides e Triterpenos	Negativo
Antraquinonas	Negativo
Saponinas	Positivo

Conforme Jaiswal (2012) nas pesquisas fitoquímicos com o extrato etanólico de folhas de *A. occidentale* L. realizou testes para ácidos orgânicos, fenóis, taninos, alcalóides, saponinas, flavonóides, esteróides, triterpenos, e constatou a presença de saponinas, flavonóides, alcalóides, fenóis e taninos. Os testes realizados neste trabalho apresentaram resultados que corroboram parcialmente com os resultados apresentados por Jaiswal (2012), devido a detecção de saponinas, alcalóides, fenóis e taninos. Estudos de prospecção fitoquímica em duas espécies da família Anacardiaceae (*Astronium franxinifolium* S. e *Muracrodruom urudeuva*), realizados por Silva *et al.* (2010), também mostraram resultados positivo para os testes de alcalóides, saponinas e taninos.

As saponinas são glicosídeos de esteróides ou de terpenos policíc1icos. São substâncias de elevada massa molecular e, de modo geral, ocorrem em misturas complexas devido à presença de estruturas com um número variado de açúcares ou ainda devido à presença de diversas agliconas. As saponinas têm sido apontadas na literatura por suas atividades hemolítica, antiviral e antiinflamatória, moluscicida, antifúngica, antibacteriana, antimicrobiana, antiparasitária, citotóxica e antitumoral, entre outras(SPARG *et al.*, 2004; SIMÕES, 2010).Com relação aos compostos fenólicos, os resultados foram negativos para flavonóides, porém positivos para fenóis e taninos. Bruneton (2001) relata que os compostos fenólicos tendem a solubilizar em água e podem estar ligados a açúcares. São compostos instáveis, facilmente oxidáveis em pH alcalino. Do ponto de vista farmacológico possuem atividade anti-séptica, anti-inflamatória e podem inibir atividade enzimática.

Os taninos têm como característica a adstringência de frutas e/ou produtos de origem vegetal. Possuem duas classificações: taninos hidrolisáveis e taninos condensados (SANTOS & MELLO, 2010). Encontram-se geralmente em plantas vascularizadas, em diferentes concentrações. Esses metabólitos são encontrados em diversos órgãos das plantas, tais como madeira, folha, fruto e casca. São substâncias solúveis em água, em alcoóis e acetona. Os taninos têm a função de proteção da planta (CUNHA & BATISTA, 2010).

Na medicina tradicional tem sido empregado no combate à diarréia, hipertensão arterial, reumatismo, hemorragias, feridas queimaduras, problemas renais e estomacais e processos inflamatórios (MARTINS, 2007). Santos e Mello (2010) discutem que as atividades farmacológicas dos taninos são devido as suas características gerais, como a habilidade de complexar com outras moléculas. Os taninos ajudam no processo de cura de feridas e inflamação por meio da formação de camada protetora formada por complexos tanino-proteínas sobre a pele ou mucosa danificada, permitindo que ocorra a reestruturação do epitélio. A ação microbiológica também é evidenciada pela presença dos taninos em plantas. Testes *in vivo* foram realizados com extratos ricos em taninos (SCALBERT, 1991; CHUNG *et al*., 1998).

Alcalóides são compostos nitrogenados farmacologicamente ativos e são encontrados predominantemente nas angiospermas. Assim como outros metabólitos secundários, os alcalóides também possuem um comprovado papel na defesa contra a invasão de microrganismos e vírus (WINK, 2008). Os alcalóides possuem ampla gama de atividades biológicas como anti-hipertensivos, antitumorais, amebicidas e anti-inflamatórias.

CONCLUSÃO

O estudo fitoquímico das folhas de *A. occidentale* L., utilizada pela medicina tradicional como anti-inflamatório e microbiano, se fundamenta com os resultados do teste que mostrou a presença de compostos que possuem estas atividades biológicas. Estudos mais aprofundados devem ser realizados para a busca de princípios ativos.

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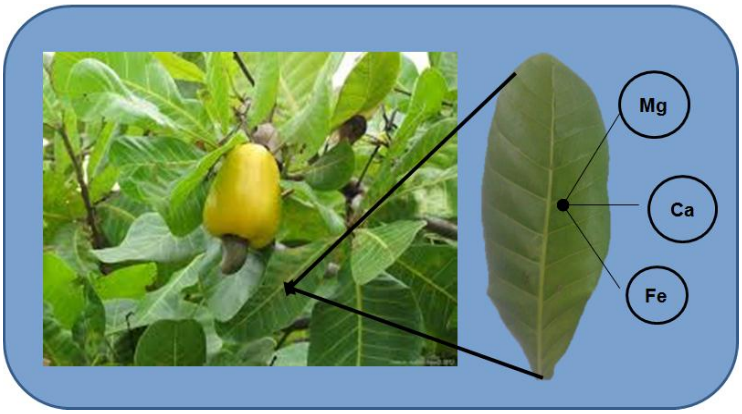
Graphical Abstract



Anacardium occidentale L is a very known plant in a whole country and it has several medicinal properties. The mineral compounds with the highest concentrations were Ca, Mg e Fe, and them have important functions on the human body.

COMPOSIÇÃO MINERAL DAS FOLHAS DE *A. occidentale L.*

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(x) Manuscrito sem material suplementar

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MINERAL COMPOSITION OF *A. occidentale* L.LEAVES

Abstract:

The present study attempts to evaluate the elements of the leaves, ethanolic extract and tea infusion leaves of *Anacardium occidentale* L. which is known as cashew in whole country. The samples were divided within two groups according to leaves used: adults or young. Determination of elements showed that element concentrations in the calcined adult leaves of *A. occidentale* were as follows: Ca = 73,74 ppm; Mg = 57,45 ppm and Fe = 22,75 ppm. The infusion showed no significant differences between adult and young, where Mg was the compound with the highest concentration, 4,35 ppm. In the extract, calcium presented a concentration of 74, 85 ppm, followed by Mg = 30,75 ppm and Fe = 21,28 ppm. All the elements were determined in Flame Atomic Absorption Spectrophotometer (FAAS).

Keywords: Anacardiaceae, tea infusion, ethanolic extract.

INTRODUÇÃO

O Brasil é o país que detém a maior parcela da biodiversidade do mundo, além de um considerável conhecimento tradicional, o qual é passado de geração a geração, que inclui um vasto acervo de informações sobre manejo e uso da biodiversidade. As plantas são uma importante fonte de produtos naturais biologicamente ativos, muitos dos quais se constituem em modelos para síntese de substâncias com diversas finalidades. Nelas também podemos encontrar uma diversidade de estruturas e propriedades físico-químicas e biológicas.[1]

O estudo da composição mineral em plantas medicinais apresenta grande relevância, devido os elementos minerais ter papéis fundamentais no organismo humano, tanto como íons dissolvidos em líquidos orgânicos, como constituintes de compostos essenciais.[2] Dentre as técnicas analíticas atualmente empregadas para a determinação de elementos traços está a Espectrometria de Absorção Atômica em Chama.

Sabe-se que os elementos minerais presentes são essenciais para o desenvolvimento humano, sendo que alguns possuem atividade preventiva de doenças. Porém, é importante ressaltar também, que níveis elevados de minerais podem ser tóxicos ao organismo.[3] Diante das possibilidades dos elementos presentes, a análise quantitativa da composição mineral das plantas é importante, tanto nos estudos básicos e aplicados sobre a planta, como para o conhecimento de seus efeitos potenciais à saúde em humana.[4]

A espécie vegetal *Anarcadium occidentale* L., conhecida popularmente como cajueiro, pertence à família Anacardiaceae. É uma planta tropical, de origem brasileira, que possui dispersão em todo o território nacional. O cajueiro é uma árvore de aparência rústica, de porte médio, troncos tortuosos e fruto reniforme. Na medicina, muitas partes são aproveitadas para o tratamento de doenças, tais como infecções urogenitais, bem como um agente anti-diabético;[5] doenças gastrointestinais, úlceras da boca, problemas de garganta,[6-8] e hipertensão. As folhas, no Brasil são usadas problemas gastrointertinais, genitais e de pele.[9]

Este estudo teve como objetivo principal caracterizar a composição mineral pela técnica de espectrometria de absorção atômica em chama das amostras de infusão e matéria seca (calcinação) de folhas adultas e jovens e do extrato etanólico de *A. occidentale.*

PARTE EXPERIMENTAL

Amostras

Planta

As folhas foram coletadas na área da Universidade Federal do Amapá. Uma amostra foi preparada e depositada no Herbário Amapaense (HERBAM), localizado no Instituto de Pesquisas Científicas e Tecnológicas do Estado do Amapá (Iepa), sob o registro de número 018684.

Obtenção do extrato

O extrato etanólico bruto das folhas adultas de *A. occidentale* (EBEAo) foi obtido a partir da maceração das folhas e extraído exaustivamente com etanol por 72hs em temperatura ambiente, sendo este repetido por três vezes consecutivas. O material obtido foi levado ao rotaevaporador para a remoção do solvente.[10]

Composição mineral

Um Espectrofotômetro de Absorção Atômica com Chama (F-AAS), modelo AA-6300, do Laboratório de Absorção Atômica e Bioprospecção (LAAB) da Universidade Federal do Amapá (UNIFAP), foi utilizado para a determinação de cálcio, magnésio, ferro, manganês, zinco e cobre. Foram estabelecidos os parâmetros instrumentais juntamente com o limite de detecção (LOD) e o Limite de Quantificação (LOQ) foram calculados usando-se 10 medidas do branco analítico, conforme mostra a Tabela 1.

Os limites de detecção e de quantificação foram calculados com base na seguinte fórmula:

$$\text{LOD}= \frac{3\text{x}s}{m} \qquad \text{LOQ} = \frac{10\text{x}s}{m}$$

sendo, *s* o desvio padrão das medidas do branco analítico e *m* a inclinação da curva analítica usada na determinação dos elementos estudados.

Tabela 2. Parâmetros instrumentais usados na determinação de Ca, Cu, Fe, Mg, Mn e Zn em amostras de matéria seca, infusão e extrato etanólico das folhas de A. occidentale por FAAS.

	Comprimento de onda (nm)	Corrente de lâmpada (mA)	Resolução espectral (nm)	LOD (μg L $^{-1}$)	LOQ (μg L^{-1})
Ca	422. 7	10	0.7	14	48
Cu	324.8	6	0.7	8,1	20
Fe	248.3	12	0.2	70	234
Mg	285.2	8	0.7	1,3	4,3
Mn	279.5	10	0.2	0,9	3,1
Zn	213.9	8	0.7	140	40

Para utilização do F-AAS foram geradas curvas de calibração para cada metal analisado, a partir de soluções padrões certificadas para AAS. Os resultados foram analisados pelo cálculo da linha de regressão usando o método dos mínimos quadrados.

A determinação da composição mineral de folhas de cajueiro foi realizada em amostras do chá por infusão e por calcinação de folhas jovens e adultas *in natura*, e também no extrato etanólico das folhas adultas. Todas as medidas foram realizadas em triplicata.

Para a determinação da concentração mineral das folhas *in natura* foi utilizado o procedimento de digestão por via seca (calcinação). Pesou-se 1 g de cada amostra, folhas jovens e adultas, e foram carbonizandos-as em bico de Bunsen até completa liberação de fumos e levadas a mufla durante 8 h a 500°C. Após a calcinação, o material foi solubilizado em 3mL de uma solução de HCl 1:1 v/v, e avolumou-se para balão de 50 mL. Os teores de cinzas obtidos após a calcinação das amostras foram determinados para quantificar a fração mineral presente, pela diferença de massa obtida antes e após a calcinação.[11]

As infusões (chá) foram preparadas com amostras de folhas adultas e jovens cortada em pequenos pedaços (± 0,5 x 0,5 cm), totalizando 2 amostras. Num erlenmeyer de 150 mL pesaram-se 2,0 ± 0,05 g da amostra, e adicionaram-se 100 mL de água deionizada fervente (100 ± 5 °C) e tapou-se o erlenmeyer para diminuir as perdas de água. Após 10 min, filtrou-se a infusão. Todas as infusões foram preparadas e analisadas no mesmo dia.[12]

Para a determinação da concentração de minerais nos extratos etanólicos, pesou-se 0,01 g de EBEAo, que foram dissolvidos em etanol 95% (1ml), e avolumados para balões de 10 mL.[11]

RESULTADOS E DISCUSSÃO

As concentrações minerais de Ca, Cu, Fe, Mg, Mn, Zn determinadas na infusão, matéria seca e extrato das folhas de cajueiro são apresentadas na tabela 2.

Tabela 3 Análise Mineral de A. occidentale. Mineral analysis of A. occidentale (mg/L). FAC: Folhas Adultas Calcinadas; FJC: Folhas Jovens Calcinadas; IFA: Infusão de Folhas Adultas; IFJ: Infusão de Folhas Jovens; EBEAo: Extrato Bruto Etanólico de *A. occidentale.*

	FAC	FJC	IFA	IFJ	EBEAo
Ca	73.74±0.47	12.08±0.11	3,42±0,18	3,06±0,39	74,85±1,26
Cu	1.91±0.04	2.03±0.02	0,05±0,01	0,11±0,01	2,25±0,08
Fe	22.75±0.36	10.65±0.72	0,57±0,03	0,56±0,01	21,28±1,62

Mg	57.45± 8.04	35.24±0.13	4,35±0,07	4,35±0,01	30,75± 1,5
Mn	5.97±0.29	2.79±1.15	0,36±0,01	0,27±0,01	0,27±0,09
Zn	1.89±1.70	1.50±0.25	0,42±0,36	0,19±0,07	2,33±2,62

Comparando a matéria seca entre folhas jovens e adultas, estas apresentaram maiores concentrações de Ca (73.74), Mg (57.45) e Fe (22.75) (Tabela 2). Nas folhas jovens as maiores concentrações observadas foram de Mg (35.24), Ca (12.08) e Fe (10.65). O Zn não apresentou diferença significativa e a concentração de Cu foi maior nas folhas jovens. As folhas jovens apresentam uma maior concentração de Zn e Cu do que nas folhas adultas, fato relacionado com a biodisponibilidade de elementos nas partes das plantas e com a idade da mesma, e este efeito é dependente de muitos fatores, como a espécie, condições edáficas e climáticas, entre outros. [13]

Ao analisar os resultados obtidos por infusão, verifica-se que as concentrações foram menores quando comparadas com o método da calcinação. Entre folhas jovens e adultas não houve diferenças significativas, sendo o Mg a maior concentração encontrada em ambas, e as concentrações de Zn, Mn e Cu foram as menores (Tabela 2). Sabe-se que a quantidade de minerais extraídos na infusão das folhas não reflete o conteúdo total de minerais presentes nas plantas medicinais, pois apenas uma pequena fração é extraída no processo de infusão.

A composição mineral também foi avaliada no extrato etanólico a fim de verificar o teor de elementos que possivelmente podem estar complexados aos compostos orgânicos. No extrato as maiores concentrações foram de Ca, seguida de Mg e Fe e o Mn apresentou a menor concentração, como mostra a Tabela 2. O estudo da composição mineral de extratos mostra-se bastante relevante, pois é a partir dos extratos secos das plantas que são fabricados muitos fitoterápicos.[14,15]

Nos testes realizados o Ca, Mg e Fe foram os elementos que tiveram as maiores concentrações. Nas plantas o cálcio faz parte da composição da parede celular, regula a permeabilidade da membrana citoplasmática, neutraliza os ácidos tóxicos e aumenta a resistência a pragas e doenças. O Magnésio está relacionado com a fotossíntese, pois este elemento é integrante da molécula de clorofila e também tem papel ativo na síntese de DNA e RNA. O Ferro nas plantas tem alta capacidade redox, atuando principalmente na respiração, fotossíntese e reações enzimáticas.[16-19]

Nos organismos humanos, a função do Ca está principalmente na construção e manutenção dos ossos e dentes, além de ter uma série de papéis metabólicos. O magnésio faz parte de grande parte dos líquidos celulares e sua principal função pode ser a de estabilizar as membranas das células em todo o organismo regulando o transporte ativo de outros minerais, sendo cofator de muitas enzimas e desempenhando também um papel na transmissão e atividade neuromuscular. O Fe tem um papel no transporte respiratório de oxigênio e dióxido de carbono e é uma parte ativa das enzimas envolvidas no processo de respiração celular. O Fe também está envolvido na função imunológica e no desempenho cognitivo.[2,20]

CONCLUSÃO

Através destas análises observou-se a importância do estudo da concentração mineral nas folhas de cajueiro, pois as plantas são fontes importantes de minerais essenciais como Ca, Fe e Mg. Os minerais são importantes para o desenvolvimento da planta e fundamentais para a produção de metabólito secundários de interesse farmacológico, e também possuem funções no organismo humano.

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CHARACTERIZATION BY FOURIER TRANSFORM INFRARED SPECTROSCOPYON LEAF, EXTRACT AND EPICUTICULAR WAX OF CASHEW TREE (*Anacardium occidentale* L.) FROM AMAPA, AMAZON, BRAZIL.

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Abstract:

Objective: The present study was aimed to identify the functional groups present in the *Anarcadium occidentale* L. leaves, extract and epicuticular wax through Fourier Transform Infrared Spectroscopy (FT-IR).

Methods: The powdered leaves were extracted using the ethanol with gentle stirring for 72 h separately at room temperature and then, filtered and concentrated using vacuum distillation. Epicuticular waxes were extracted by dipping the leaf blades in chloroform under light stirring for 30 seconds.

Results: The results of *A. occidentale* L. leaves FT-IR analysis confirmed the presence of alcohols, phenols, alkanes, aldehydes and esters. The datas of the extract showed the presence of alcohols, phenols, alkanes, amides, aromatics, carboxylic acids and ethers. The wax spectrum showed the presence of alkanes, carboxylic acids, ethers, esters and aromatics.

Conclusion: Data analysis in this work indicates the presence of functional groups of medical and biological interest. These preliminary results of the present study produced the FTIR spectrum profiles of cashew leaves compounds that will be the basis for further study of chemical and pharmacological.

Keywords: Anacardiaceae; FT-IR; functional groups.

INTRODUCTION

Identification of the chemical nature of phytochemical compounds present in the medicinal plants is a slow process that relies on morphological and anatomical observations. However, it will provide some information on the different functional groups responsible for their medicinal properties.

In Fourier Transform Infrared (FT-IR) spectroscopy is a physico-chemical analytical technique that does not determine concentrations of individual metabolites but provides a snapshot of the metabolic composition of a tissue at a given time. Besides, it is a noninvasive, fast, high-resolution analytical tool for identifying types of chemical bonds in a molecule, and through the resulting spectrum which represents the molecular absorption and transmission, produces a molecular fingerprint of the sample [1]. FTIR is preferred over infrared spectral analysis due to be a non-destructive technique and it has been widely used to provide information on a range of vibrationally active functional groups (including O–H, N–H, C=O, =C–H, –CH2, –CH3, C–O–C and>P=O) in biological specimens [2].

The Anacardiaceae is a large family (Dicotyledonae) that comprises about 70 genus with approximately 875 species widely distributed in tropical regions. In Brazil, it can be stand out, mainly, *Anacardium, Mangifera, Spondias* and *Schinus* genus [3]. The Anacardium genus, described by Carl Linnaeus, is distributed in several regions of the world, showing adaptation in many ecosystems. Into the different species, it is interesting to highlight the *Anacardium occidentale*, or “caju”, as it is popularly called in Brazil. It is a tree reaching up to 15 m tall and has a thick and tortuous trunk. The fruit is the nut and the other part, called a pseudocarp or false fruit, is used to make juices. Cashew nut has great value in the international market of food, besides numerous uses in the plastics and resins industry. However, the structure and properties of their leaves are not well known. Typical of Brazil, the cashew tree is widely used in folk medicine for therapeutic purposes, and also has great representation in the food trade [4].

Pharmacognostic studies were carried out indicated the presence of flavonoids, anthocyanins, cardiac glycosides, tannins, sterols and triterpenes. Several studies have reported the use of various parts of the cashew in folk medicine. Leaves are used to treat intestinal andrespiratory diseases, sore throats, diabetes, hemorrhage, antiscorbutic, muscle weakness and urinary disorder [5,6]. Phytochemical studies showed the presence of steroids, flavonoids, catechins, phenols, tannins and saponins [5,7,8].

Taking into consideration of above benefits of FTIR analysis, the purpose of the present investigation was to characterize and identify the main functional groups present in leaves, extract and epicuticular wax of *A. occidentale* L. from Amazon in northern Brazil.

MATERIALS AND METHODS

Collection of plant

Leaves were collected in cashew into the Federal University of Amapá campus, located in northern Brazil, close to French Guiana. A sample was prepared to be deposited in the HEMAB (Herbarium Amapaense), located in IEPA (Instituto de Pesquisas Científicas e Tecnológicas do Estado do Amapá), under registration nº 018684. Fresh leaf samples were washed thoroughly in running tap water to remove soil particles and adhered debris followed by sterile distilled water. Leaves were placed on the blotting paper and spread out at room temperature in shade.

Preparation of leaf extract and epicuticular wax

To obtain the alcohol extract from the leaves, they were separated, cleaned and dried in a circulating air oven at 40° C for 72 hours. Then, they were milled and the resulting powder, stored.

The extract was prepared in the UNIFAP Pharmacognosy Lab from five hundred grams (500g) of leaf powder was weighed using an electronic weighing balance and soaked on ethyl alcohol, at a ratio of 1:4 (powder/solvent).The powdered leaves were extracted using the ethanol through maceration for 72 h, at room temperature, and that process was repeated three times, and subsequently filtered using filter paper [9]. The filtrate was concentrated using rotary evaporator and used for qualitative FTIR analysis.

After wash and dry fresh leaves material, epicuticular waxes were extracted by dipping the leaf blades in chloroform under light stirring for 30 seconds and then it was filtered [10]. The extract was concentrated using rotary evaporator and finally the organic solvent was evaporated at room temperature and the waxes were stored at room temperature until they were used in the corresponding experiment.

Fourier Transform Infrared Spectroscopy (FT-IR)

Infrared analysis was carried out in Van De Graaff Laboratory located at Pontifical Catholic University of Rio de Janeiro using a ALPHA-P/Brucker spectrometer. A small piece of leaf, a little amount of ethanolic extract and epicuticular wax were, respectively, placed directly on the crystal piece of the infrared spectrometer with constant pressure applied and data of infrared transmittance, collected over the wave number ranged from 4000 cm^{-1} to 400 cm^{-1} and computerized for analyses by using the OPUS software (version 7.0). The reference spectra were acquired from the cleaned blank crystal prior to the presentation of each sample replicate. All spectra were collected with a resolution of 4 cm^{-1} and to improve the signal-to-noise ratio, 256 scans were co-added and averaged. Samples were run in triplicate and all of them were undertaken within a day period.

RESULTS AND DISCUSSION

In FTIR spectrum, each peak was assigned a functional group and many of them give characteristic IR absorption at specific narrow frequency range. However, due to the complex interaction of atoms within the molecule, IR absorption of the functional groups may vary over a wide range. As the fresh leaves have a lot of organic compounds with many absorption bands, it becomes difficult to identifier all of those bands. Despite this, it was possible to characterize the main compound classes.

Leaves

The leaves of *A. occidentale* FTIR analysis results showed a broad band at 3370 cm^{-1}, assigned to the stretching vibration of hydroxyl groups that interact by H bonding, $\nu(O\text{-}H\cdots O)$. Two strong bands at approximately 2911 and 2852 cm^{-1} assigned to the asymmetrical and symmetrical stretching vibrations of CH_2 groups, $\nu_a(CH_2)$ and ν_s(CH2) respectively, accompanied by the corresponding $\delta(CH_2)$ bending vibrations at around 1465, 1308 and 721 cm^{-1} (Fig. 1). According to the spectrum on figure 1, it is also possible to observe the difference between adaxial and abaxial leaf surfaces in respect to the peaks intensity, mainly in 2911 and 2852 cm^{-1} bands.

Band around 1730 cm^{-1} corresponding to ν(C=O) stretching ester vibration accompanied by two bands at around 1167 and 1101 cm^{-1} attributed to asymmetrical and symmetrical C-O-C stretching ester vibrations.

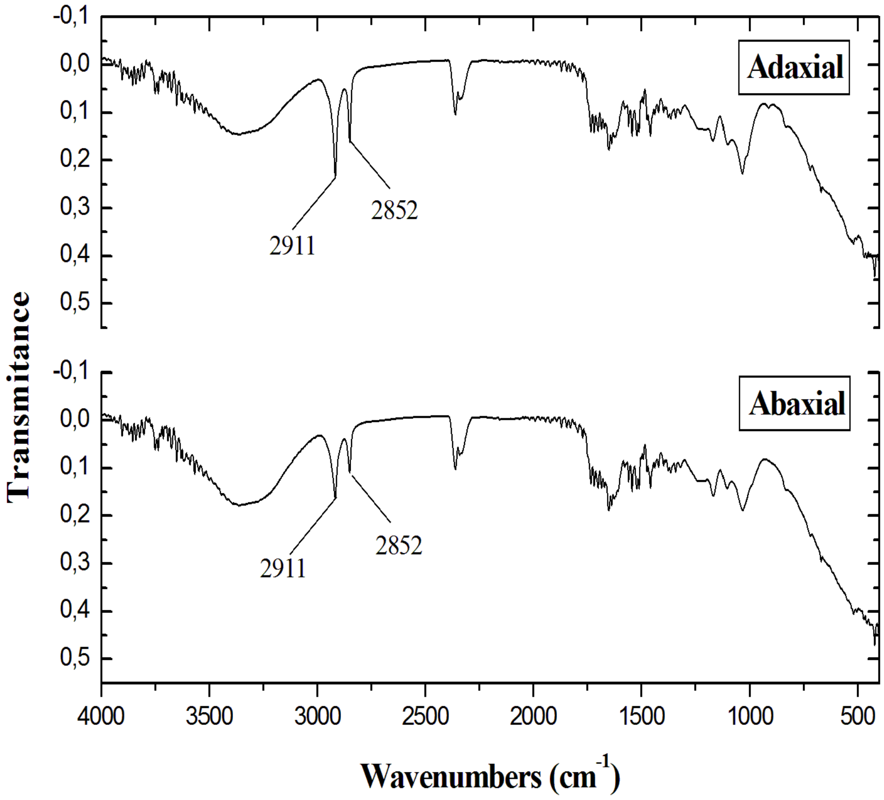

Figura 6 FTIR spectrum of *A. occidentale* leaf.

Extract

The data on the peak values and the probable functional groups (obtained by FTIR analysis) present in the leaf extracts of *A. occidentale*are presented in tables 2 and figure 2. A characteristic band at 3280 cm^{-1} indicates the presence of hydroxyl (-OH) group. Another characteristic absorption bands were exhibited at 2918 cm^{-1} (for C-H stretching), at 1698 cm^{-1} (for amides) and at 1608 cm^{-1} (for aromatics). Besides, absorption bands were observed at 1443 cm^{-1} (for C-H bending) for C-H group, at 1330 e 1204 cm^{-1} (for carboxylic acids C-O) and at 1028 cm^{-1} (for ethers).

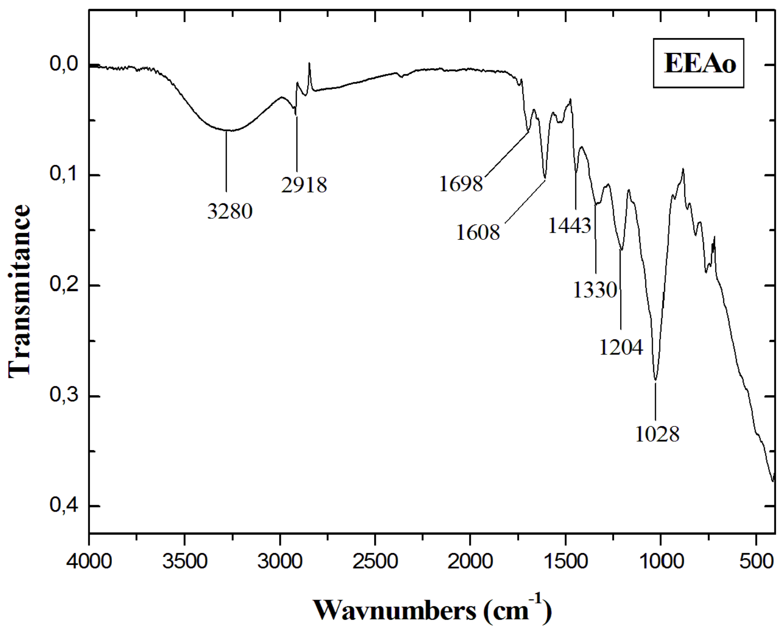

Figura 7 FTIR spectrum of ethanolic extracts of the leaves *A. occidentale*.

Wax

The FTIR analysis results of *A. occidentale* epicuticular wax validated the presence of alkanes, aromatics, carboxylic acids, alcohols, ethers and esters (Figure 3). Bands were also seen at 1737 cm^{-1} and 1165 cm^{-1}, attributed to the (C=C) stretching of carbonyl and to the C-O stretching.

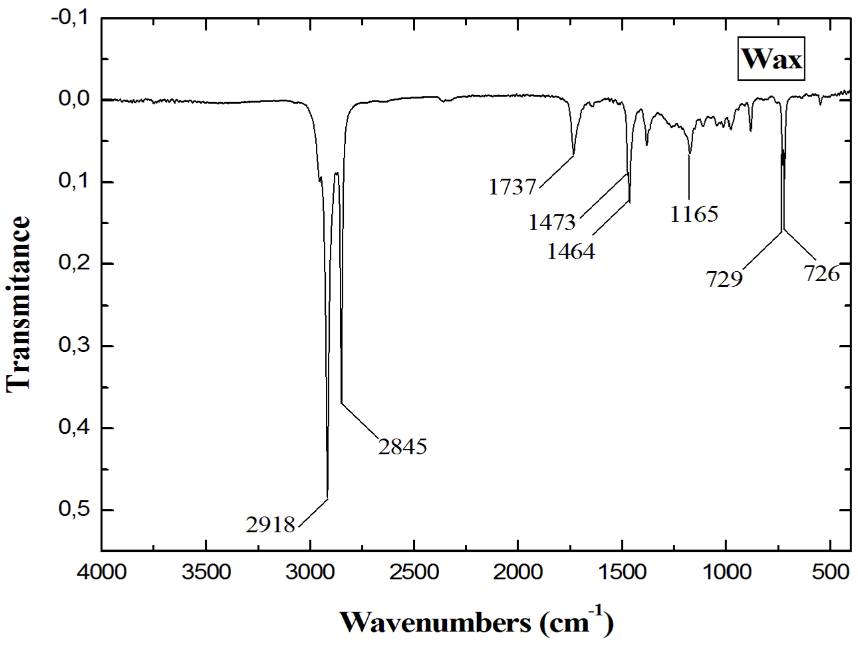

Figura 8 FTIR spectrum waxes *A. occidentale*.

DISCUSSION

The bands of the leaves were ascribed to the aliphatic material present in the plant cuticle: cutin, waxes and cutan [11-14]. A comparison with adaxial and abaxial *Acer rubrum* leaf surfaces was done and it was attributed the spectra intensity peaks difference to the wax compound concentration and also structures, as trichomes [13]. Bands in the 1600-1500 cm^{-1} spectral range with variable intensity depending on the species and they correspond to the phenolic compounds or cutan [11]. In general, the identified bands are associated to aliphatic and ester groups of cutin. These assignations have been also used in the study of non- isolated plant cuticles of seeds [15-19].

Epicuticular waxes can be divided into crystalline and amorphous domains. Crystalline regions are arranged in an ordered structure of the aliphatic chains of the waxes, while amorphous zones are formed by chain ends, functional groups, short-chain aliphatics and non-aliphatic compounds [20].

The bands proved the presence of nonacosane, a majority compound found in some wax plants. Spectra features of nonacosane presents stretching of methylene (CH_2) in the bands around 2914 cm^{-1} and 2846 cm^{-1} and bending at 1472 and 1462 cm^{-1} and methylene *doublet* at 729 and 719 cm^{-1} [13,24,25].

CONCLUSION

The characterization of leaf, extract leaves and epicuticular wax of *A. occidentale* has provided significant and valuable information about chemical nature and arrangement of the different leaf components. The results of the present work are a new basis for further work using more advanced techniques so as to the structural elucidation and identification of active compounds of pharmacological interest.

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Epicuticular wax and leaf of *Anacardium occidentale* L.: ultrastructure and crystalline feature in leaves from Amazon in Northern Brazil

ABSTRACT: Leaves surfaces have various structures with specific functions and contribute to the relationship with the environment. On morphological studies are analyzed several parameters, ranging from macroscale through the microscale to the nanoscale, which contribute to the study of taxonomy, pharmacognosy and ecology, among others. Taking into account the absence of works performed with *Anacardium occidentale* L. leaf, also known as cajueiro, this study reports results about the ultrastructural features of adaxial and abaxial sides using a set of environment scanning electron microscope (ESEM) images, highlighting the presence of epicuticular waxes only over the upper epidermis and analyzing its crystallinity by X-rays diffraction (XRD) measurements.

KEYWORDS: Cashew, XRD, ESEM, epidermis, adaxial, abaxial.

Cera epicuticular e folha de *Anacardium occidentale* L.: ultraestrutura e característica cristalina em folhas da Amazônia na Região Norte do Brasil

RESUMO: A superfície das plantas possui diversas estruturas com funções específicas e que contribuem para a relação delas com o meio ambiente. Em estudos morfológicos são analisados diversos parâmetros variando da escala macro, passando pela micro e atualmente chegando à escala nanométrica, contribuindo assim com o estudo da taxonomia, farmacognosia, ecologia, entre outras. Considerando a ausência de trabalhos realizados com a folha de *Anacardium occidental*e L., conhecido como cajueiro, este artigo apresenta resultados sobre a ultraestrutura dos lados adaxial e abaxial da folha com imagens realizadas em um microscópio eletrônico de varredura ambiental, destacando a presença de ceras epicuticulares apenas na epiderme superior e analisando sua cristalinidade através de medidas de difração de raios-x.

PALAVRAS-CHAVE: Caju, DRX, MEV, epiderme, adaxial, abaxial.

INTRODUCTION

After a million years of land plants evolution led to a large diversity of functional biological surface structures.In this way, plants have an interface, consisting of a extracellular membranes of polymeric cutin matrix and soluble cuticular lipids, that is able to share between primary plant tissues and the atmosphere. This interface, so-called cuticle, has been of great interest to many researchers for more than a century (MEUSEL et al., 2000), due to its functionality and properties such as light reflection or wettability, a barrier against UV radiation and bacterial and fungal attacks, and so on (MEUSEL et al., 2000; HOLMES; KEILLER, 2002).

The micro- and nanostructures of plant surfaces have a great influence on their attributes as interfaces. Even in a cursory look at different plant surfaces they show different optical appearances, which arise from the surface structures in the micro- and nanoscale dimension.(KOCH et al., 2009). The plant cuticle is technically a composite material mainly built up by a network of cutin and hydrophobic waxes. Based on structural characteristics, the cuticle can be divided into the cuticle proper and the thicker underlying cuticle. In both layers, the cuticle network is formed by cutin, a polyester like biopolymer, composed of hydroxyl and hydroxyepoxy fatty acids, and sometimes also by cutan, which consists of polymethylene chains, or by another polymer called lignin (JEFFREE et al., 2006; KOCH et al., 2009).

The waxes are important to the structure and functional of cuticle and are either deposited within the cutin matrix (intracuticular wax) or accumulate on its surface as epicuticular wax crystals, or films (YEATS; ROSE, 2013; JEFFREEet al., 2006). In most cases, the majority of compounds comprising the cuticular wax are derived from very-long-chain fatty acids, including alkanes, aldehydes, primary and secondary alcohols, ketones, and esters. In some species, various lipophilic secondary metabolites, such as pentacyclictriterpenoids, flavonoids, and tocopherols, can also be substantial components (TULLOCH, 1976; BACKER et al., 1982; BIANCHI, 1995).

Epicuticular waxes create a macroscopic effect as reduction wettability, leading to self-cleaning leaves surfaces (NEINHUIS et al., 1997). They also reduce the uptake of molecules from the environment, which might become a crucial factor in agriculture, when the uptake of nutrients or fungicides is desired (KOCH et al., 2009). With respect to epicuticular waxes classification, the most common wax morphologies are thin films and several three-dimensional structures such as massive crusts, granules, plates, platelets, filaments, rods, and tubules with a hollow centre (KOCH et al., 2009). According to Barthlott et al. (1998), the environment conditions could influence on the amount and how they would be distributed along the leaf surface.

X-Ray Diffraction (XRD) is one of several fundamental techniques used for materials characterization as well as Differential Scanning Calorimetry (DSC) (DASSANAYAKE et al., 2009), Fourier Transform Infrared Spectroscopy (*FTIR*) and Scanning Electron Microscopy (SEM) (SIGOLI et al., 2000). When the SEM arose, new possibilities to study plant structures, ranging from the micro- up to nanoscale, were established. For a conventional SEM, which works in high vacuum and a room temperature, it is widely accepted that biological samples must be dehydrated and require a deposition of conductive material on the surface. As an alternative, ESEM can make images on non-conducting samples, being a powerful tool to analyze biological material ate a room temperature (SHAH; BECKETT, 1979; DANILATOS; POSTLE, 1982). On the other hand, XRD enables the determination of composite structural patterns and then, it is possible to characterize them on factors such as crystallinity, amorphicity, residual stress, texture, and furthermore is possible to get analysis of the tension behavior as a function of temperature (GOLDSMITH et al., 2000). XRD investigations in waxes exhibit a relationship between chemical composition and morphology with a diversity of molecular order and crystalline structure (ENSIKAT et al., 2006; KOCH; ENSIKAT, 2008).

Considering the importance of detailed studies to provide a best comprehension and characterization about leaf morphology, this work examined the ultrastructural differences comparing adaxial and abaxial sides of fresh *Anacardium occidental*e L. leaves by images from an environmental scanning electron microscope (ESEM). The purpose of this work is also to characterize the cristallinity of reconstituted epicuticular waxes onto a glass substrate surface.

MATERIALS AND METHODS

Plant material

Leaves of *Anacardium occidental*e L. were collected in Macapá, into the Universidade Federal do Amapá campus, dried and indentified as voucher specimen Nº

018684 and preserved in the Herbário Amapaense (HEMAB) of the Instituto de Estudos e Pesquisas do Estado do Amapá (IEPA).

Isolation and purification of wax

This process consisted of collecting the leaves, followed by washing and drying at room temperature. For the extraction of epicuticular wax single carefully handled leaves were immersed and gently shaken for 30s in $CHCl_3$, at room temperature (Hamilton, 1996). The resulting solution of waxes were dried and filtered and finally, using a rotary evaporation, removed the excess solvent.

Enviromental Scanning Electron Microscopy

Fresh leaf pieces of 5 x 5 mm^2 were cut using a razor blade from the plant, avoiding the midrib areas so as to give a relatively consistent surface. Adaxial and abaxial surfaces were examined. Leaf specimens were mounted on a metal stub (10 mm in diameter) using two-sided adhesive carbon tape. Without metal coating, surfaces of leaf specimens were directly examined with an environmental scanning electron microscope (SEI-quanta 250) at an accelerating voltage of 5 kV.

X-ray diffraction

In order to evaluate the presence of crystalline compounds in the epicuticular wax it used a diffractometer model Rigaku Miniflex II using CuK_{α} (□=1,5404 Å) radiation. The diffractogram was recorded between 2 and 50º (2□) in 0,02º steps, counting 4 s per point at 30 kV and at 15 mA. The samples were deposited on glass substrate.

RESULTS

The study of the macroscopic characters of fresh leaves reveals, on the upper side a dark green in color and on the lower one a light green, lamina 15-18 cm long and 8-10 cm wide, with obovate shape, pinnately veined, symmetric base and obtuse apex, entire margin and the smooth surface has a straight petiole, as depicted in figure 1.

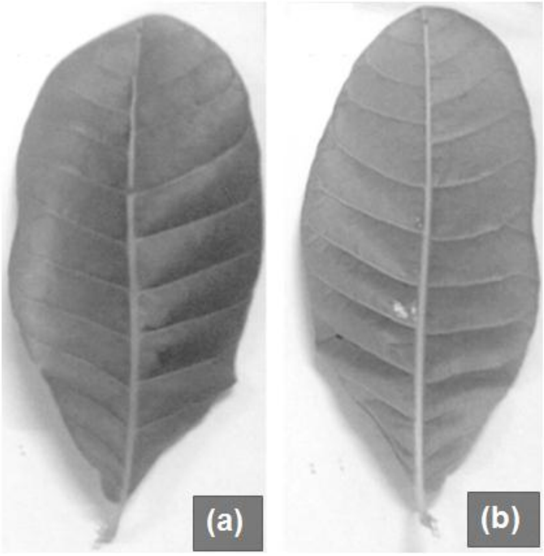

Figure9 -Leaf of A. occidentale. (a) Adaxial and (b) abaxial sides.

Scanning electron microscopy revealed a micromorphological diversity of leaf surface. There were great differences in morphology of leaf between the adaxial and abaxial surfaces. The upper side is formed by a cuticle highly undulated or ridged with depressions in some regions, and had no stomata (Figure 2a). In Figure 2(b) can be noted the presence of epicuticular wax granules that varied in diameter (ca. 1-4 □m) and are scattered over the undulated cells along adaxial surface.

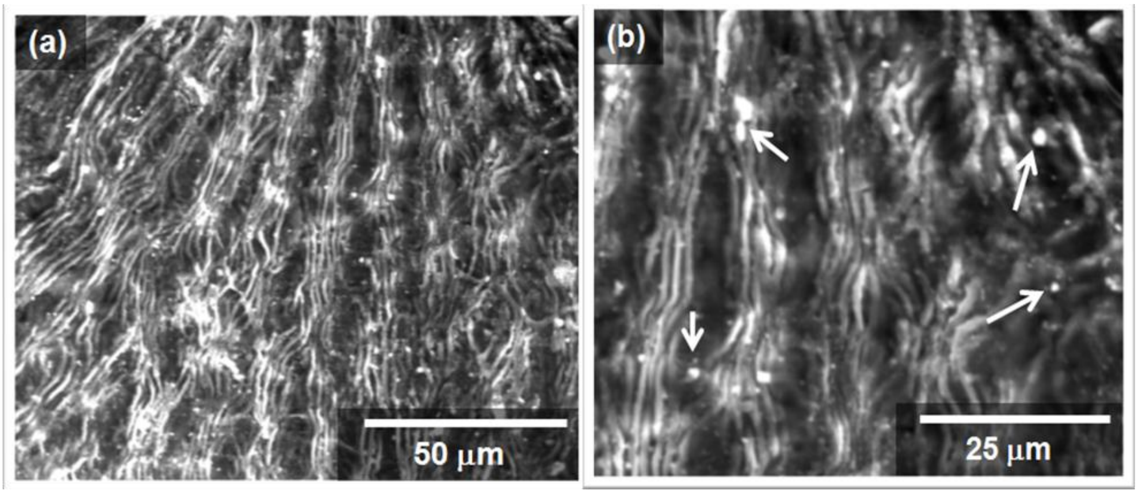

Figure 2 – Scanning electron micrograph of (a) adaxial leaf surface showing epidermis features. (b) Higher magnification where arrows indicate wax granules.

After analyzing the other side, it was noted a large quantity of stomata (Figure 3a), and they have a slightly ridged cuticle that is denser around them. Stomata are ellipsoidal and are inserted at the level of the epidermis, with a random distribution, and therefore, the leaf is hypostomatic. The stomata are accompanied by subsidiary cells, which have a ridged cuticle (Figures 3a, b) and it was not observed the presence of epicuticular wax granules on the this surface.

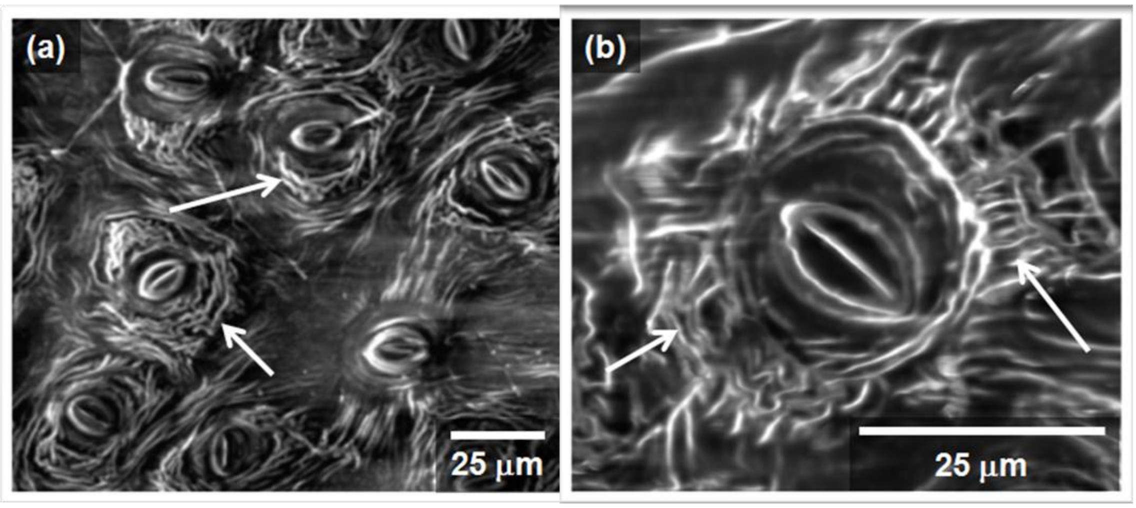

Figure 3 – Scanning electron micrograph of (a) Abaxial leaf surface. The stomata appearance and distribution and where arrows indicate subsidiary cells surrounded by a ridged cuticle. (b) Higher magnification of stomata.

The X-ray pattern of the reconstituted epicuticular wax is shown in figure 4. The X-ray diffractogram presents only diffraction peaks from lattice planes of the crystalline wax. The amorphous band, a less ordered component of the wax, contributed only to a broad band characteristic of diffraction from non-crystalline material, ranging from 10 to 40º.

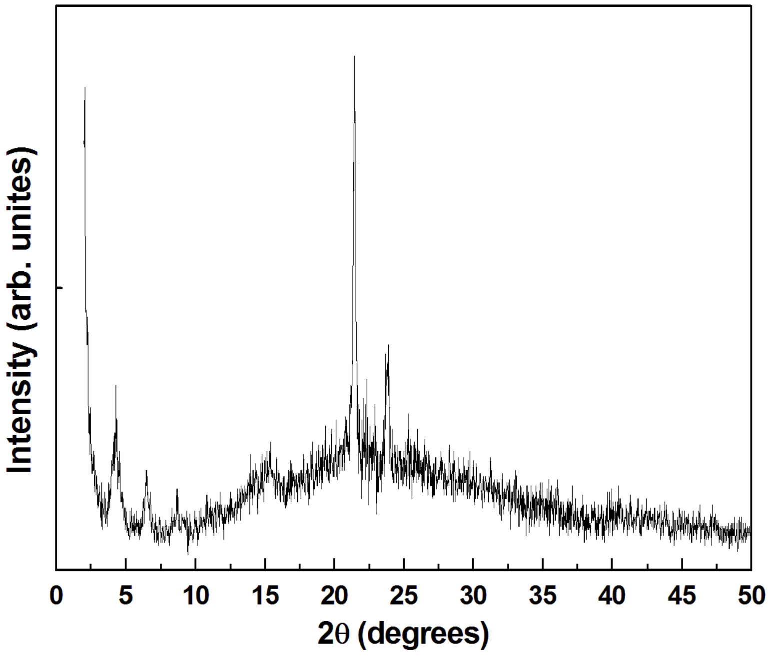

Figure 4- X-ray diffraction pattern of epicuticular wax of cashew.

DISCUSSION

Carpenter (2005) reported the importance dedicated to the study of the stomata based on the anatomical architecture, quantity, shape and the organization of epidermis cells associated with guardians cells, because it provides subsidies to the taxonomy of plants (STACE,1965). Stomata are composed of a pore, the ostium and a pair of guardians' cell and they are surrounded by subsidiary cells that differ from the others in size, shape and orientation (ESAU, 1953; PANT, 1964). The position of the subsidiary cells is an important factor to classify the type of stomata on the leaf epidermis (CARPENTER, 2005). This morphology of the stomatal complex is according to Metcalfe e Chalk (1957) and Jaiswal (2012), which described the presence of paracytic stomata cells that are connected to the irregular subsidiary cells in the *Anacardium occidentale* leaves. This irregularity is due to the presence of a ridged cuticle.

The epidermis present a thin extracellular membrane, called cuticle, which is composed by cutin and epicuticular waxes, that is, in general, a hydrophobic material, whose primary function is to create a barrier against water loss. KOCK et al. (2008) presented possible surfaces structures based on wetting behavior of plant leaves. The sculptures of the cells, the presence of hairs and the fine structure of the surfaces, e.g., folding of the cuticle or existing epicuticular waxes, have a strong influence on surface wettability. Beside this, structural and chemical modifications can induce variations in surface wetting, ranging from superhydrophilic to superhydrophobic (HOLLOWAY, 1982; BUKOVAC, 1995; KOCH, 2008).

The crystalline order can be analyzed in detail by X-ray diffraction (XRD) and electron diffraction (ED). XRD has frequently been used in the early studies of waxes as a tool for the identification of compounds isolated from natural waxes. The XRD powder diffraction diagrams contain information about lattice parameters, the chain length of the molecules and the position of oxygen-containing functional groups (MALKIN, 1952; MAZLIAK, 1968). Previous XRD studies of plant waxes were performed on solvent-extracted, recrystallized waxes, but crystal structure analyses of the natural, mechanically isolated epicuticular waxes are published rarely (MEUSEL et al., 2000).

The two narrow peaks of high intensity, superimposed on the broad peak in figure 3, can be attributed to 010 and 100 reflections, according to Casado et al. (1999) for the reconstituted cuticular waxes of grape berry cuticle.In that study, a comparison of the n-hexacosanol peaks diffractions was done with grape berry cuticle and epicuticular wax results. These peaks appeared on a very broad band of high intensity at ~21°, and were mainly attributed to the amorphous network that constitutes the cutin of the plant cuticle.

CONCLUSION

In this work it was studied the *Anacardium occidental*e L. leaf micro morphology on both adaxial and abaxial sides by ESEM, showing ultrastructural details that not found on previous studies, enhancing distinct differences on leaf epidermis. This research also analyzed the reconstituted epicuticular waxes deposited on a glass substrate by X-ray diffraction, presenting a crystalline order.

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Chemistry of Natural Compounds (Qualis B2 - Farmácia)

Chemical constituents of *Anacardium occidentale* L. leaves from the Amazon, Northern Brazil

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Abstract: Brazil is the country with the largest share of the world's biodiversity, besides to a considerable traditional knowledge which includes a vast amount of information on the management and use of biodiversity. *Anacardium occidentale* is a well-known plant in Brazil, and has great economic importance. Gas chromatographic analyses of the ethanolic extract revealed the presence of the n-alkanes, fatty acids, phytosterols and terpenes.

Anacardiaceae is a large family (Dicotyledonae) which comprises about 70 genus with approximately 875 species widely distributed in tropical regions. In Brazil, it can be stand out, mainly, *Anacardium, Mangifera, Spondias* and *Schinus* genus [1]. The Anacardium genus, described by Carl Linnaeus, is distributed in several regions of the world, showing adaptation in many ecosystems. Into the different species, it is interesting to highlight the *Anacardium occidentale* Lineus, or “caju”, as it is popularly called in Brazil, where its nut has great value in the international market of food, besides numerous uses in the plastics and resins industry [2].

Several studies have reported the use of various parts of the cashew in traditional medicine. Leaves are used to treat intestinal problems, sore throats, respiratory diseases, diabetes, hemorrhage, antiscorbutic, muscle weakness and urinary disorder [3,4]. Pharmacognostic studies were carried out indicated the presence of flavonoids, anthocyanins, cardiac glycosides, tannins, sterols and triterpenes [5-8]. This study aimed to analyze the chemical constituents of the ethanolic extract from the leaves of *A. occidentale* L. using gas chromatography/mass spectrometry.

GG analyses of ethanolic extract revealed the presence of the n-alkanes, fatty acids, phytosterolsand triterpenoids. Identified compounds and their respective percentages were shown in Table 1. The dominant compounds were a-tocoferol (21%), sitosterol (17,13%), dammaradienol (12%) and phytol (8,70%), showing that terpenes were the most prominent compounds in the ethanolic extract leaves. The presence of the terpenes and sterols were well detected in cashew tree leaves, and have contributed to the chemical knowledge of the plant [9]. Tocopherols are well-know due to their high antioxidant activity [10,11]. Sitosterols have been reported to anti-inflammatory [12], antidiabetic [13], antimicrobial and antioxidant [14] activities. In general, the triterpenoids coumpounds have anti-inflammatory activity and cytoprotective effects. Phytol, besides have property significantly antimicrobial [15], is also precursor for Vitamin E [16]. Studies performed with *A. occidentale* leaves have showed hipoglycemic effect [17,18], antioxidant [19] and antimicrobial [19,20].

EXPERIMENTAL

Collection and processing of plant material. Fresh leaves were collected in May 2014 at Federal University of Amapá campus (0°0’22,9212”, S 51°5.5896' W, altitude 6.0 m). Voucher specimens have been in the Amapaense Herbarium (HEMAB) located in IEPA (State of Amapá Institute of Scientific and Technological Research), under registration number 018684.

Extraction of plant material. This process consisted of collecting the leaves, followed by washing and drying at room temperature. To obtain the alcohol extract from the leaves, they were separated, cleaned and dried in a circulating air oven at 40° C for 72 hours. Then, they were milled and the resulting powder, stored.

The extract was prepared from five hundred grams (500g) of leaf powder was weighed using an electronic weighing balance and soaked on ethyl alcohol, at a ratio of 1:4 (powder/solvent). The powdered leaves were extracted using the ethanol through maceration for 72 h, at room temperature, and that process was repeated three times, and subsequently filtered using filter paper [21]. The filtrate was concentrated using rotary evaporator and used for CG analysis.

GC-MS Analysis and identification of Constituents. The wax was analysis using a HP6890 gas chromatograph interfaced with an HP5873, Mass Selective Detector (ionization energy 70eV), equipped with a DB-5MS capillary column (30 m×0.25 mm thickness; 0.25 μm film coating). The column temperature was programmed to 70-305ºC at5°C/min using helium as the carrier gas (1.0 mL/min). The injector and detector temperatures were 230°C and 280°C respectively. Identification of the components was done on basis of retention index, comparison with reference spectra (Wiley, NIST databases) and MS literature data [22].

TABLE 4Chemical Composition of *A. occidentale* L.

Retention time (min.)	**Compounds**	**%**
19,434	2,4-di-ter-butylphenol	0,49
28,886	Pinane;	0,39
29,291	Palmitic acid;	1,66
29,981	Acido palmítico ethylester	1,19
32,163	Phytol	8,70
32,616	9,12,15-Octadecatrien-1-ol;	0,47
33,076	Stearicacid;	0,40
33,187	7,10,13-Hexadecatrienoic acid, methylester	0,65
43.385	Squalene	1,16
45.580	Nonacosane	4,34
47.557	α-Tocopherol;	21,16
49.984	Sitosterol	17,13
50.630	a-Amyrin	2,84
51.305	Dammaradienol;	12,0

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Revista: Chemistry of Compounds (Qualis B2 - Farmácia)

Chemical constituents of epicuticular wax from the leaves of *Anacardium occidentale* L.

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The cuticle incorporates several functions for plant life and and it is formed of cutin and waxes. The waxes occur of all land plants and their proprieties, structure and chemistry are much studied. This study aimed aimed toanalyze the chemical constituents of the epicuticular wax from the leaves of *A.occidentale* L. The results of the present study show principal wax contituents are *n*-alkanes and triterpenes.

Keywords: cashew, CG-MS, alkenes, triterpenes.

The plants havea layer that allows interaction between the external and internal environment, this layer is called the cuticle formed principally of cutin and waxes . The waxes are important to the structure and functional of cuticle and are either deposited within the cutin matrix (intracuticular wax), or accumulate on its surface as crystals or films [1,2]. In most cases, the majority of compounds comprising the cuticular wax are derived from very-long-chain fatty acids, including alkanes, aldehydes, primary and secondary alcohols, ketones, and esters. In some species, various lipophilic secondary metabolites, such as triterpenoids, flavonoids, and tocopherols, can also be substantial components [3-5].

The *Anacardium occidentale* Lineus, or “caju”, as it is popularly called in Brazil, where its nut has great value in the international market of food, besides numerous uses in the plastics and resins industry [6]. This study aimed to analyze the chemical constituents of the epicuticular wax from the leaves of A. occidentale L. using gas chromatography/masss pectrometry.

Gas cromatografic analyses of the epicuticular waxes revealed the presence of the 10 constituents, totalizando 93.39% of the composition. The identified compounds and their respective percentages are shown in Table1. Accordind to CG-MS analyses, odd-numbered *n*-alkanes were the principal wax contituents, accounting for83.52% of the identified compounds. The majority of these alkanes are hentriacontane (36.74) and nonacosane (33.48). It is also verified the presence of triterpenes Moretenol (11.49%), α-amyrin (2.73) and Cycloartenol (1,13) .

In studies with epicuticular waxes present high proportions of n-alkanes C27 to C33 [7].The tripernoid have been detectados in the cuticular waxes of many plant species, and their derivatives too, with small amounts ranging from less than50% of the total wax mixture [8,9].

EXPERIMENTAL

Collection and processing of plant material. Leaves were collected in may/2014at Federal University of Amapa campus (S 0°0’22,9212” W 51°’5.5896 altidude 6.0 m). A sample was prepared to be deposited in the Amapaense Herbarium, HEMAB, located in IEPA (State of Amapa Institute of Scientific and Technological Research), under registration number 018684.

Extraction of plant material. For extraction of the waxes, after washed and drought, the leaves whole were place in contact with Chloroform and agitated slightly by 30 seconds [10]. The materials obtained were later kept for evaporation to remove the excess solvents.

GC-MS Analysis and identification of Constituents. The analysis was performed using a HP6890gas chromatograph interfaced with an HP5873, Mass Selective Detector (ionization energy 70eV), equipped with a DB-5MS capillary column (30m×0.25mmthickness, film thickness 0.25mM), using helium as the carrier gas (1.0ml min^{-1}). The injector and detector temperatures were 230°C and 280°C respectively. The programming of the column was 70 to 305ºC, 5°C/minute. The identification of the components was based on comparing the retention rates, fragmentation pattern analysis observed in the mass spectrum and the literature [11] and the device library information, Wiley.

TABLE 1 Constituents of the epicuticular wax

Retention Times (min.)	**Compound**	**%**
41.790	Heptacosane	0,32
43.207	Octacosane	0,47
44.640	Nonacosano	33,48
45.916	Triacontane	2,27
47.267	Hentriacontano	36,74
48.455	Dotriacontano	1,27
49.694	Tritriacontane	3,49
50.637	α-Amyrin;	2,73
50.882	Cycloartenol	1,13
51,335	Moretenol	11,49

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4 CONSIDERAÇÕES FINAIS

Os dados morfológicos estão de acordo com estudos realizados. A caracterização da superfície foliar é descrita pela primeira vez e apresentaram uma cutícula altamente estriada com alguns grânulos de cera. A presença de uma cutícula estriada e os estômatos presentes apenas na face abaxial são características de plantas xerófitas. A arquitetura estomática juntamente com as estrias da cutícula, também são descritas em outras espécies da família Anacardiaceae, tais como para *Schinus sp.* e *Pistacia sp,* sendo consideradas fatores de diferenciação entre as espécies. Em vista da importância morfológica, ainda são necessários estudos mais satisfatórios que decorrem de aspectos ecológicos e fisiológicos, tais como sazonalidade e senescência da espécie.

As análises químicas do extrato bruto etanólico mostraram principalmente a presença de terpenos e esteróides. Estudos mostram a eficácia desses compostos em atividades antioxidantes, antimicrobianas, anti-inflamatórios, entre outras. As folhas de cajueiro são utilizadas na medicinal tradicional para tratamentos de infecções. Estudos realizados mostram dados satisfatórios para atividades antioxidante, antimicrobiana e anti-inflamatória das folhas de *A. occidentale.*

As análises morfológicas e químicas das ceras de *A. occidentale* são descritas pela primeira vez para a espécie. Os grânulos de cera apresentados corroboram com estudos realizados nas folhas de *Pistacia vera* (Anacardiaceae). Os aspectos químicos estão de acordo com outros estudos de ceras que apresentam os alcanos como compostos majoritários e alguns terpenóides também podem ser encontrados.

Este trabalho contribuiu com dados morfológicos e químicos das folhas de *A. occidentale*, visto a importância medicinal e comercial da espécie.

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