Pharmacognostic Characterization of *Spondias mombin* L. (Anacardiaceae)

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ABSTRACT

Background: Spondias mombin L. is fruit specie found in Tropical America and the coast of Africa, popularly known as cajazeira. Several researches with extract of leaflets show a diversity of promising pharmacological activities such as antibacterial activity, antiviral against dengue virus type 2, anti diabetic, among others. Objective: This paper aims to study the anatomical characteristics, phytochemical and histochemical from leaves of this species. Materials and Methods: Cross and paradermic sections from leaves were submitted to double staining with astra blue and safranin process for the anatomical study. Anatomical analysis of the structures was performed using an optical microscope. For ultra structure analysis, the leaf fragments, after processing and gold coating, were observed under scanning electron microscopy (SEM). The phytochemical analyses were performed using thin layer chromatography and the histochemical analyses using specific reagents for each metabolite group. Results: The leaf has anomocytic and tetracytic stomata distributed on abaxial surface. The midrib has convex symmetry with collateral vascular bundles in a closed arc disposition. The phytochemical showed the presence of mono and sesquiterpenes, triterpenes and steroids, hydrolysable tannins, phenylpropanoid glycosides, cinnamic derivatives and flavonoids. The histochemical tests allow us to state that phenolic compounds are found in the palisade parenchyma cells. Lignins can be found in woody vascular bundles and in perivascular sclerenchyma fibres. The volatile oils were evidenced in the schizogenous cavity, present in the parenchyma of the midrib. Conclusion: The results allowed characterizing anatomically the leaves of this species, outlining a profile of its phytochemical constituents and, in addition, determining which anatomical structures of storage of these metabolites by the plant.

Key words: Cajá, Anacardiaceae, Leaf anatomy, Histochemistry.

INTRODUCTION

The fruit species present considerable social and economic importance due to the nutritional character of its fruit, in addition to its use in traditional medicine. Different fruit trees of great economic importance for Brazil are within *Anacardiaceae* family. In Africa and South America are found 108 species restricted to these continents,¹ while in Brazil, from 57 catalogued of this family, 14 of them are exclusive to this country.² The *Spondias* genus has a history of use that dates back at least 6500 BC in Tehuacan Valley, Mexico.³ Four species of *Spondias* are economically important in tropical America: *S. dulcis, S. mombin, S. purpurea* and *S. tuberosa.*⁴

Spondias mombin L. is a native fruit tree from tropical America having wide distribution and can be found throughout Latin America and parts of Asia and Africa. The tree can reach up to 25 meters high and has compound leaves with (sub) opposite leaflets. Its vernacular nomenclature may undergo a major change depending on the country. In Brazil, it may

be called as taperebá, cajazeira-miúda, cajá-mirim or cajá.⁴⁻⁷

Around 180 common names have been associated with *Spondias* purpurea,⁸ while 96 different common names have been described for *S. mombin*.¹⁰ In Mortom and Lim studies,^{9,10} *Spondias purpurea* and *S. tuberosa* have confusion in names.

The use of *S. mombin* is remarkable in both folk medicine and pharmaceutical industry. The aromatic and astringent bark is traditionally used for its emetic action, diarrhoea treatment, dysentery and haemorrhoids. In folk medicine, infusion of the leaves is used to treat bilious fevers, constipation and inflammation in the mouth, throat, stomach and genitals and also infectious conditions.^{7,11}

Several studies with extract of leaves show a diversity of promising pharmacological activities, among them the antiviral activity against Coxsackie and *Herpes simplex* viruses;¹² anthelmintic activity,¹³ antibacterial activity against strains of different spectra, confirmed in studies by Olugbuyiro *et al*;¹⁴ anti diabetic action,

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because of its agonist character of insulin receptors,^{15,16} activity against dengue virus type 2, shown in experiments realized by Silva *et al*¹⁷ and leishmanicide activity.¹⁸

The extract from the leaves and branches present in its composition tannins, cinnamic derivatives, coumarins, saponins, terpenes and steroids, alkaloids and reducing sugars.^{19,20} Caraballo and colleagues,²¹ reported the presence of anthraquinones, flavonoids, naphthoquinones, sesquiterpenes, quassinoids and indole and quinoline alkaloids when analyzing *S. mombin* species, where these compounds can be related to medicinal activity.

For Budel *et al.*,²² the study of plants originates from the correct identification of the species and is one of the most important steps in any research to have reproducibility. Farias²³ emphasizes the importance of the standardization of morphoanatomical markers for species, because such features become essential parameters for quality control of drugs and herbal medicines to be produced. Given the wide distribution of this species and the wide variety of uses and interests, whether traditional or scientific point of view, this paper aims to study the anatomical, phytochemical and histochemical characteristics of *Spondias mombin* leaves, native from Brazil.

MATERIALS AND METHODS

Plant material

Spondias mombin L. leaves were collected from species from Recife, Brazil, throughout October 2015. The voucher specimen was deposited in the Herbário Dárdano de Andrade Lima, at Empresa Pernambucana de Pesquisas Agropecuárias (IPA), for legitimation botany, under number 90666.

Anatomical analysis

Mature leaves of *S. mombin* were collected from up to five individuals for anatomical analysis. The samples were fixed in FAA_{50} .²⁴ Cross and paradermic sections of the middle portion of the leaflets were freehand obtained, submitted to double staining process with astra blue and safranin^{24,25} and mounted in glycerin on semi-permanent slides for the anatomical study. Analysis of the structures was performed using an optical microscope (Alltion). For scanning electron microscopy (SEM), leaf blades samples were fixed in an 2.5% glutaraldehyde solution (buffered with 0,1 M sodium cacodylate) and post fixed in 2% osmium solution (buffered with 0,1 M sodium cacodylate). Afterwards, material was critical-point dried through a graded ethanol series (Bal – Tec CPD 030). Suitable portions were mounted onto SEM stubs using double-sided adhesive tape and sputter-coated with gold (Leica EM SCD 500). Both adaxial and abaxial surfaces were examined with a QUANTA 200 FEG scanning electron microscope.

Phytochemical prospection

The methanolic extract from fresh leaves was processed by infusion (0,1%) and subsequently analyzed by thin-layer chromatography (TLC) (Kieselgel 60, 0.2 mm, Merck), with different eluents systems and reagents to search alkaloids, mono- and sesquiterpenes, triterpenes and steroids, flavonoids, cinnamic derivatives and phenylpropanoid glycosides, hydrolyzable tannins and condensed tannins. Phytochemical screening of the extract was carried out using a conventional protocol.^{26,27}

Histochemical analysis

Fresh mature leaves of *S. mombin*, without fixation, were collected from up to five individuals for histochemical analysis. Free hand cross sections from the middle portion of the leaflets were subjected to the following reagents/tests: Lugol reagent²⁴ for starch, Sudan IV²⁵ for detection of epicuticle wax, acid phloroglucin³⁰ for lignins, potassium dichromate³¹ for phenolic compounds, hydrochloric acid for calcium oxalate crystals²⁴

NADI reagent²⁸ for essential oils and oil-resins, vanillin-hydrochloric acid³² for tannins and Dragendorff reagent²⁹ for alkaloids. For each procedure employed on the samples, control samples, slides without any treatment (blanks), were also mounted. Observations and image documentation were performed with a light microscope (Alltion) equipped with a digital camera.

RESULTS

Anatomical analysis

The paradermic sections show an epidermis containing cells with thick walls, winding to polygonal contour, and those cells from the adaxial surface are more elongated and bigger than the abaxial surface ones (Figure 1A,B). The foliar limb is hypostomatic with anomocytic and tetracytic stomata randomly distributed along the abaxial surface (Figure 1B). The presence of simple and short unicellular trichomes can be seen in both adaxial and abaxial surfaces. Scanning electron microscopy images show the details of these structures (Figure 1C,D).

The cross section presents the epidermis covered with an epicuticular wax layer on both sides. The mesophyll is dorsiventral with two compact palisade layers of regular cells and relatively narrow, near the midrib. There is a tendency to form a monolayer of this parenchyma near the leaf margin. Several spongy parenchyma layers of loose elongated cells with little intercellular spaces form the abaxial surface of the mesophyll (Figure 1E).

The midrib has biconvex symmetry with collateral vascular bundles in a closed arc disposition. Two small groups of accessory vascular tissue can be found laterally to the central vascular bundle and higher up, composed of xylem and phloem surrounded by a sheath of sclerenchyma fibers (Figure 1F).

The angular chollenchyma is followed by perivascular sclerenchyma fibers, externally surrounding the xylem and phloem (Figure 1G). There is a presence of schizogenous secretory cavity in the parenchyma region, located near the phloem (Figure 1H). The presence of idioblasts with druse-crystal forms is remarkable and are found throughout the mesophyll and midrib nearby the vascular bundles (Figure 1I).

The petiolule has convex symmetry with some undulations in a crosssectional view (Figure 1J). There are short to medium simple unicellular trichomes in both adaxial and abaxial surfaces. The epidermis has a thin cuticle that covers on both faces. A collenchymatous tissue anullar type can be seen after the epidermal monolayer, composed of short cells and slightly elongated axially. Crystalline inclusions of druses type are present with higher incidence in idioblasts located near the epidermis, on both sides. The crystals observed in petiolule are more numerous and have a greater diameter than observed in leaf blade (Figure 1K).

Vascular bundles with collateral arrangement in closed arc disposition are shown surrounded by a sclerenchymatous fiber layer. They are organized in small groups of vascular cylinders arranged concentrically. Schizogenous secretory cavities type are observed in phloematic vascular regions of each grouping, having one of the cavities with a greater diameter, located at the centre of the parenchymatous region (Figure 1L). Secretory cavity in medullary region has longitudinally elongated lumen. The lumen of the cavity is delimited by a secretory epidermis, composed of elongated cells of small dimension. A sheath is formed by two or three layers of flattened cells, arranged radially and externally the secretory epidermis (Figure 1M).

Phytochemical prospection

In the phytochemical prospection performed by TLC in leaves, mono- and sesquiterpenes, triterpenes and steroids, hydrolysable tannins, phenyl-propanoid glycosides, cinnamic derivatives and flavonoids were found. The search for alkaloids and condensed tannins was negative.

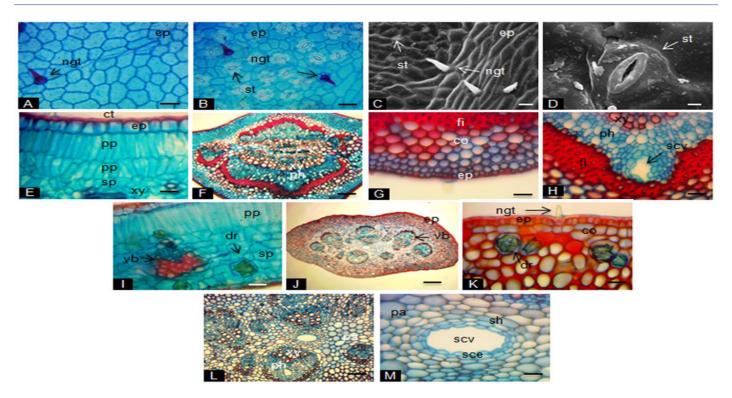


Figure 1 : Anatomical structures of Spondias mombin L. leaves.

Leaf: *Paradermic sections*: A. adaxial cells. B. abaxial cells. C, D. trichomes and stomata by SEM. Cross sections. E. palisade layer. F. midrib. G. sclerenchyma and chollenchyma. H. schizogenous secretory cavity. I. druse. Petiolule: J. biconvex symmetry. K collenchyma. L. collateral vascular bundles. M. schizogenous secretory cavity. ngt: non-glandular trichome, ct: cuticle, fi: fiber, co: collenchyma, , vb: vascular bundle, dr: druse, sce: secretory cell, sh: sheath. Bars: A,B,E,G,H,I,K,M: 25 µm. C,L,F: 100 µm. D: 10 µm.

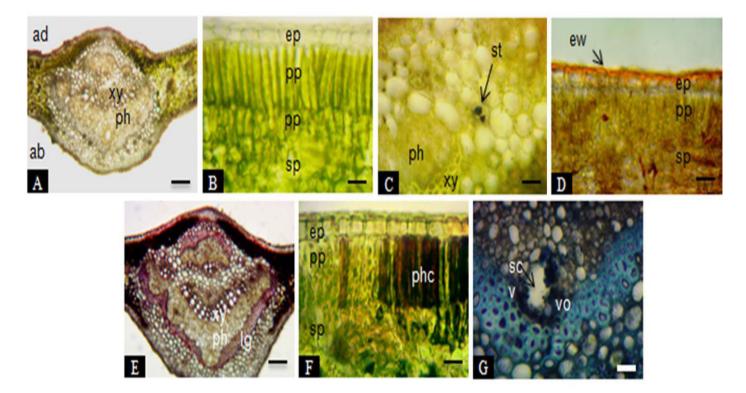


Figure 2 : Histolocalization of metabolites present in leaves of Spondias mombin L.

Control sample: A. midrib. B. mesophyll. Histochemical Tests: C. starch grains into idioblasts. D. epicuticular wax. E. lignins in sclerenchyma fibres. F. polyphenols in parenchyma cells. G. volatile oils inside secretory cavity. ad: adaxial, ab: abaxial, xy: xylem, ph: phloem, ep: epidermis, pp: palisade parenchyma, sp: spongy parenchyma, st: stomatum, ew: epicuticular wax, Ig:lignin, phc: phenolics compounds, scv: secretory cavity, vo: volatile oils. Bars: A, E. 100 µm. B,C,D,F,G. 25 µm.

Histochemical analysis

Histochemical analysis were performed comparing the control samples, cross sections of the midrib and mesophyll, with no chemical treatment (Figure 2A, B), with the sections after specific reagents. The tests show plant substances belonging to the primary metabolism, as the presence of starch granules within the parenchyma mesophyll cells (Figure 2C). After leaf treatment with Sudam IV it is observed the presence, on both sides, of a thin layer of epicuticular wax, with orange staining, constituting the protective cuticle (Figure 2D). The reaction with hydrochloric acid showed the soluble nature of the crystalline inclusions found, confirming the chemical composition of these calcium oxalate crystals. The presence of lignin can be verified by the pink structures stanning, not only in woody vascular bundles but also in the full extent of perivascular sclerenchyma fibres, both in the central vascular bundles, as accessories (Figure 2E).

The results of the phytochemical prospection served as support to direct the tests to histolocalization of compounds from plant secondary metabolism. Thus, the potassium dichromate tests, displayed the presence of phenolic compounds, distributed across the leaf, mainly in prismatic cells which compose the palisade parenchyma (Figure 2F). Leaf showed positive reactions to the NADI reagent, which blue stanning of cell contents of secretory cavity characterized the presence of volatile oils presence of tannin. The volatile oils in leaves were highlighted in this schizogenous cavity in the parenchyma of the midrib (Figure 2H). The tests used in the detection of alkaloids and condensed tanins returned negative results.

DISCUSSION/ CONCLUSION

Anatomical analysis

An adaptation of plants to high sunlight environments can be observed by decreased sinuosity of their cell walls as an alternative way to prevent excessive loss of water.²² This fact can be observed in targeted cells facing to the adaxial surface of *Spondias mombin* where the little sinuosity of their epidermal cells can be seen, a fact justified because it presents a predominant distribution in places of intense light radiation.³³ This feature can also be observed in *S. tuberosa* and *S. dulcis.*^{34,35}

The results obtained in relation to stomatal type and distribution showed a hypostomatic leaf blade. Similar characteristics as the pattern of distribution and types of stomata were observed by Silva *et al*,³⁴ when analyzing leaves of *Spondias tuberosa*. The presence of stomata anomocytic was also described by Chisom *et al*³⁶ when analysing *Anacardium occidentale* and *S. mombin* leaves. Santos *et al*³⁵ also reported a leaf blade with the same distribution pattern of *S. dulcis*. The presence of such stomata is defined by Metcalf and Chalck³⁷ as a standard for the *Anacardiaceae* family, present in *Spondias* species.

The presence of trichomes on both sides of the leaf, as observed in *S. mombin*, is very important because it creates a microenvironment rich in water vapor around the leaf, thereby helping to prevent excessive loss of water.³⁸ Similar features were found by Silva *et al*.³⁴ who reported the presence of simple tector trichomes on both sides of the leaf of *S. tuberosa*, whereas this type of trichomes is a common feature found in species of this family.³⁷ Descriptions made by Mitchell and Daly³ that realized a review of the species belonging to this genus, point to two types of trichomes, short and long thin forms. In contrast, the results of this study show the presence of uniseriate, unicellular, short trichomes.

Anatomical analysis indicates a dorsiventral mesophyll with two layers of palisade parenchyma in *S. mombin*. Different results were obtained to species from the same genera, as *S. dulcis*³⁵ and *S. tuberosa*³⁴ for example, where the mesophyll only has an unistratified palisade parenchyma. However, the presence of a double layer parenchyma can also be observed

in other species belonging to the Anacardiaceae family, as seen in *Anacardium humile* and *Anacardium occidentale*.³⁹ The greater thickness of the exposed leaves to sunny region reflected an increase in the number of palisade layers from one to two, as observed in *S. purpurea*, resulting from the sun versus shade variables that the species is exposed.⁴⁰

Regarding the midrib plane of symmetry and vascular organization of the central rib, the results support the analyzes made by Royo *et al*,³⁹ where the existence of convex central rib with vascular bundle system side closed type was observed. Silva *et al*³⁴ also reported the presence of collateral vascular bundles in closed arc disposition, seen in *Spondias tuberosa* leaves.

Corroborating the results obtained in this work, there is a presence of angular collenchyma in *Anacardium occidenntale* and *A. humile* on the abaxial surface of the midrib.³⁹ Similar features were observed in stem of *Spondias dulcis.*⁴¹ Silva *et al.*³⁴ reported the presence of angular chollenchyma in the adaxial surface, and angular type tending to lamellar in the abaxial surface in *Spondias tuberosa* leaflets.

According to Metcalfe & Chalk³⁷ and Cronquist,⁴² the presence of secretory structures is a common feature for members of the *Anacardiaceae* family, where the presence of secreting latex resiniferous channels or ducts can be checked and usually associated with the phloem. The results corroborate the studies by Silva *et al*,⁴³ where the schizogenous resiniferous cavities were observed in cortical parenchyma of *Spondias tuberosa* leaves. Ducts associated with vascular tissue have been described in the stem⁴⁴ and the leaf of *Spondias dulcis*,⁴⁵ in the *S. pinnata* leaf⁴⁶ and in the stem of other species of *Anacardiaceae* such as *Anacardium spruceanum*,⁴⁷ *Lithraea molleoides*.⁴⁸ Schinus *terebinthifolius* and *Mangifera indica*.⁴⁴

The presence of calcium oxalate crystals was described by Cronquist⁴² as a characteristic within individuals that belong to this family. Unlike Silva and Paiva³⁴ who reported a greater incidence of crystals in the midrib region of *S. tuberosa* leaves. The presence of idioblasts containing such inclusions was observed in *S. mombin*, distributed throughout the mesophyll and midrib. According Metcalfe & Chalk³⁷ the presence of crystalline inclusions in plant leaves can be associated with an adaptive mechanism for greater capture of light and optimize the photosynthesis rate.

In studies conducted by Azevedo and contributors,⁴⁹ characteristics similar to petiolule anatomy of *S. mombin* were found in the petiole of *Schinus terebinthifolius* (*Anacardiaceae*) as vascular bundles with collateral arrangement and the presence of secretory cavities well developed, generally associated to the phloem. The lumen of the ducts located in the phloem is also oval in cross section in young stems of *Anacardium occidentale.*³⁹ These characteristics corroborate the study by Santos *et al.*⁴¹ which indicates the presence of secretory cavities in *S. dulcis* and as well as in *S. tuberosa* leaves.³⁴

Phytochemical prospection

The phytochemical profile of native species becomes an important chemotaxonomic feature to be used as a tool in the standardization of plant raw material, besides being a lever in the search for new sources of bioactive molecules.^{50,51}

According to the results of phytochemical in *Spondias mombin*, there is the presence of terpene origin of substances in the extract obtained from the leaves. Moronkola *et al.*⁵² reported more than 54 components in the essential oil composition of *S. mombin*, and caryophyllene the major compound hence corroborating the findings. In previously analyses, Lemos and contributors⁵³ also found the presence of this group of compounds in leaves of different species of this genus. Terpene source substances, such as anacardic acid and caryophyllene, are responsible for the antimicrobial activity inhibiting B-lactamase.^{54,55} An isolated

triterpene from the methanol extract of the leaves, the 3- β -urs-12-in-3-yl (9 z) hexadec-9-enoate, was assigned to hypoglycemic activity.⁵⁶

By analysing the leaves of this species, Corthout and *et al*⁵⁷ identified the presence of two ellagitannins, which was attributed to antiviral activity, effective against dengue type 2. Several other authors also corroborate the results found in the analysis performed in this study, stating the presence hydrolyzable tannins.^{19,58,59,60,61}

The tests also reveal the presence of cinnamic derivatives, flavonoids and phenylpropanoid glycosides. Studies by Corth out and colleagues⁶² demonstrate the antiviral action of the *S. mombin*, attributed to the presence of cinnamic derivatives and isolated phenylpropanoid. Preliminary data suggest that this phenolic acid in *Spondias mombin* is responsible for the antibacterial action and molluscicide of plant extract.⁶³

Although a few researches have specifically targeted the flavonoids in this species, different authors mention the presence of this group.^{21,61,63,64,65}

Histochemical Analysis

Histochemical analysis showed the presence of starch granules in the mesophyll, in the palisade parenchyma as well as in the spongy one. Starch grains are stored in the chloroplast and are important in all plants during periods when photosynthesis is not occurring, and after cellulose, the most abundant component processed by the plant cell.³⁷ A common feature of some species of this family, as *Spondias tuberosa*, which has starch grains in collenchymatic and parenchymatic tissue of mesophyll.⁴³

Fatty compounds found in the cuticle, facing to both leaf surfaces, and the presence of lignin were observed in the region of vascular bundles and sclerenchyma midrib. Reis *et al*⁶⁶ showed the presence of lignins in the wall of xylem cells in histochemical assays of *Anacardium occidentale*. Almeida⁶⁷ also reports the presence of lignin in xylem fibres of midrib and fibres in the mesophyll of leaflets *Schinus terebinthifolius* Raddi.

The histochemical tests revealed phenolic compounds contained in the palisade parenchyma in leaflet mesophyll region, within some of the epidermal cells and parenchyma of the midrib likewise observed in species of the same family, such as *Spondias tuberosa* Arruda and *Schinus terebinthifolius* in work carried out by Almeida,⁶⁷ Kuklinski⁶⁸ and Cunha *et al.*⁶⁹

Reiterating the results obtained on chemical analysis, reactions with the reagent NADI revealed the presence of essential oils in the secretory cavities of the parenchymal midrib. The presence of essential oils in *Anacardiaceae* species was reported, with emphasis on their antimicrobial properties.⁷⁰ When conducting studies of *S. mombin* leaves, Moronkola et al.⁵² found the presence of different components in the essential oil composition, and β -caryophyllene was the major compound.

Crystalline inclusions of druses were observed in the perivascular regions, near the sclerenchyma. The reaction with hydrochloric acid showed the soluble nature of these inclusions, confirming the chemical composition of these calcium oxalate crystals. The presence of these inclusions, and the form they take in plants, can help in the characterization of taxonomic groups, making it relevant in the identification of herbal drugs.³⁷ Mitchell and Daly³ report the presence of crystals in *Spondias* genera. In addition to calcium deposit, idioblasts can be formed by the accumulation of non usable substances by the plant, as protection or even as a mechanical support.^{37,71} According to Cronquist⁴² and Smith et al,^{34,43} calcium oxalate crystals in the form of druses can be observed in xylem tissue, spongy mesophyll and more frequently in the midrib, leaves in *S. tuberosa*.

As can be seen, *Spondias mombin* L. is a species that has different popular uses and proven pharmacological activities. The results allowed to characterize anatomically the leaves of this species, outline a profile of its phytochemical constituents and, in addition, determine which anatomical structures of storage of these metabolites by the plant. The lack of studies

to provide data about the pharmacognostic characteristics of the leaves of this species proves the importance of these results. Regarding both histochemical and phytochemical similarities, as well as the anatomical characteristics of the plant parts analysed, it is suggested that *S. mombin* is a promising native plant drug, whereas should be considered most depth pharmacological studies.

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CONFLICT OF INTERET

The authors have no conflict of interest to declare.

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