Micromorphology and *in vitro* antibacterial evaluation of *Zanthoxylum* and *Hymenocardia* species

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Nature itself is the best physician.

- Hippocrates

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Resumo

As plantas desenvolveram mecanismos funcionais e metabólicos como uma estratégia de defesa para sobreviver em ambientes adversos. Terpenóides, alcalóides, flavonóides e compostos fenólicos são biosintetizados e acumulados em organelos celulares ou em estruturas secretoras. Sendo assim, as plantas são uma fonte valiosa de produtos naturais e são usadas em medicina tradicional. O estudo das plantas proporciona novas ferramentas para o tratamento várias doenças e infeções. O objetivo deste trabalho foi estudar a micromorfologia da folha, atividade antibacteriana e antifúngica, potencial sinérgico e determinação do perfil fitoquímico de três espécies nativas da Guiné-Bissau, conhecidas por possuírem propriedades etnobotânicas: *Zanthoxylum zanthoxyloides*, *Zanthoxylum leprieurii* e *Hymenocardia acida*.

O material vegetal foi recolhido na Guiné-Bissau. As folhas foram observadas em microscopia de campo claro, microscopia de fluorescência e microscopia eletrónica de varrimento. Extratos de polaridade crescente foram obtidos por extração sequencial do material vegetal e posteriormente testados contra bactérias Gram-positivas, Gram-negativas e fungos. A concentração mínima inibitória (CMI) e a concentração mínima bacteriostática (CMB) foram determinadas. O potencial sinérgico foi avaliado e o índice da concentração inibitória fracionária (ICIF) foi calculado. O perfil fitoquímico foi realizado por TLC em sílica gel.

Foi possível identificar características micromorfologicas que permitem a diferenciação das espécies de *Zanthoxylum.* Os testes histoquímicos revelaram que as diferenças entre estas são de natureza quantitativa e não qualitativa. Para *H. acida* foi detetada a presença de vários compostos, sendo que os extratos apolares são os mais ativos, capazes de inibir o crescimento de bactérias Gram-positivas. Extratos de espécies de *Zanthoxylum* revelaram atividade contra Gram-positivas e fungos, e quando combinados com antibióticos demostraram um efeito sinérgico, revertendo a atividade antibacteriana em estirpes resistentes.

Os resultados obtidos enfatizam o valor de estudos adicionais para melhor compreensão dos compostos e mecanismos que podem ser fundamentais para ultrapassar a problemática da resistência das bactérias aos antibióticos.

Palavras-chave: *Zanthoxylum zanthoxyloides*; *Zanthoxylum leprieurii*; *Hymenocardia acida*; micromorfologia; atividade antibacteriana.

Abstract

Plants developed functional and metabolic mechanisms as a defence strategy to survive. Compounds like terpenoids, alkaloids, flavonoids, and phenolics are biosynthesized and accumulated in cellular organelles or in secretory structures. Therefore, plants are a valuable source of natural products. The goal of this work was to study the micromorphology, antibacterial and antifungal activity, synergy potential, and phytochemical screening of three species from Guinea-Bissau known to have ethnobotanical properties: *Zanthoxylum zanthoxyloides*, *Zanthoxylum leprieurii*, and *Hymenocardia acida.*

Plant material was collected in Guinea-Bissau. Leaves were observed under light, fluorescence, and scanning electron microscopy. Extracts of increasing polarity were obtained by sequential extraction and were tested against Gram-positive bacteria, Gram-negative bacteria, fungi and yeast. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. Synergy potential was assessed and fractional inhibitory concentration index (FICI) was calculated. Phytochemical profile screening was carried out through TLC on silica gel.

It is possible to identify features that allow *Zanthoxylum* species differentiation. The histochemical tests revealed that the difference between *Zanthoxylum* species is rather quantitative than qualitative. *H. acida* tested positive for presence of several compounds. *H. acida* leaves extracts are mostly active against Gram-positive bacteria but no fungal growth inhibition was detected. Certain *Z. zanthoxyloides* and *Z. leprieurii* extracts revealed activity against bacteria and fungi. *Zanthoxylum* species extracts combined with antibiotics show a synergic effect, reverting the antibacterial activity in resistant strains. Terpenes, flavonoids and phenolic compounds, known to have antibacterial properties, were found for these species. The results obtained for total phenolic content when compared to the extracts that have evidenced antibacterial activity indicate that higher content may not be directly connected to the antibacterial potential.

The obtained results emphasize the worthwhile of additional studies of these species to better understand the compounds and mechanisms that may be valuable to restore antibacterial activity.

Key-words: *Zanthoxylum zanthoxyloides*; *Zanthoxylum leprieurii*; *Hymenocardia acida*; micromorphology; antibacterial activity.

Resumo Alargado

As plantas desenvolveram mecanismos funcionais e metabólicos como uma estratégia de defesa para sobreviver em ambientes adversos. Terpenóides, alcalóides, flavonóides e compostos fenólicos são biosintetizados como mecanismo adaptativo e acumulados em organelos celulares ou em estruturas secretoras. Sendo assim, as plantas são uma fonte valiosa de produtos naturais e desde há milhares de anos que são usadas em medicina tradicional. Ao longo dos anos tem-se verificado um aumento do número de estirpes patogénicas que apresentam resistência aos antibióticos, causando sérias infeções e doenças que põe em causa a saúde humana tanto em ambiente hospitalar como comunitário. Por este motivo, há uma necessidade crescente de encontrar novos agentes antibacterianos capazes de reverter essas resistências. No que diz respeito ao estudo da micromorfologia das plantas, esta análise é especialmente importante na identificação de espécies, uma vez que se revela mais precisa e menos influenciada pelo ambiente. Além disso, uma vez que o uso de plantas medicinais requer a fragmentação das mesmas, a análise micromorfológica é indispensável para a sua caracterização. O estudo destas características fornece um critério válido para a diferenciação das espécies e parâmetros de controlo de qualidade precisos e reprodutíveis. O estudo das plantas e dos seus compostos proporciona não só um conhecimento aprofundado das mesmas como também a descoberta de novas ferramentas eficazes no tratamento várias doenças e infeções, numa tentativa de ultrapassar a problemática das resistências. O objetivo deste trabalho foi estudar a micromorfologia da folha, atividade antibacteriana e atividade antifúngica, potencial sinérgico e determinação do perfil fitoquímico de três espécies nativas da Guiné-Bissau, conhecidas por possuírem propriedades etnobotânicas: *Zanthoxylum zanthoxyloides*, *Zanthoxylum leprieurii* e *Hymenocardia acida*.

O material vegetal foi recolhido na Guiné-Bissau em 2016 e 2017. Uma parte do material recolhido foi herborizado e outra parte foi sujeita a processo de fixação ainda na Guiné-Bissau. A identificação das espécies foi confirmada e os vouchers foram depositados no LISC. O material vegetal inclui raízes e ramos jovens com folhas no entanto apenas as folhas foram submetidas aos métodos usuais para estudos de microscopia em campo claro, microscopia de fluorescência e microscopia eletrónica de varrimento. Para estudos anatómicos da folha, o material vegetal foi processado com a técnica de micro-parafina. Extratos de polaridade crescente foram obtidos por extração sequencial do material vegetal seco reduzido a pó com *n*-hexano, CH_2Cl_2, EtOAc, MeOH e H_2O. Após filtração e concentração, os extratos foram armazenados a -20 ºC e posteriormente foram testados contra sete bactérias Gram-positivas: *Bacillus subtilis* (ATCC 6633), *Enterococcus hirae* (CIP 5855), *E. faecalis* (ATCC 51299), *Staphylococcus aureus* (ATCC 6536, ATCC 43866, CIP 106760) e *S. epidermidis* (ATCC 12228), três bactérias Gram-negativas: *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 9997) e *Pseudomonas aeruginosa* (ATCC 9027) e onze fungos: *Candida albicans* (ATCC 90028), *Candida dubliniensis* (FFUL6

21), *Candida glabrata* (FFUL 12B), *Candida tropicalis* (ATCC 750), *Candida guilliermondii* (FFUL 1403), *Candida kruzei* (ATCC 6258), *Candida parapsilopsis* (ATCC 90018), *Rhodotorula rubra* (FFUL 190), *Trichosporon cutaneum* (FFUL 18H), *Saccharomyces cerevisiae* (FFUL 1997) e *Cryptococcus neoformans* (FFUL 948). A concentração mínima inibitória (CMI) e a concentração mínima bacteriostática (CMB) foram determinadas. Foi considerada atividade antimicrobiana em amostras com valores de CMI < 100 μg / mL. O potencial sinérgico foi avaliado e o índice da concentração inibitória fracionária (ICIF) foi calculado. O efeito sinérgico não é considerado para FICI ≤ 0,5. O perfil fitoquímico foi realizado por TLC em silica gel.

As espécies do género *Zanthoxylum* revelaram características micromorfológicas comuns, como folhas dorsiventrais e hipoestomáticas, células de formato poliédrico com paredes retas na superfície adaxial, presença de estruturas secretoras internas e feixes vasculares colaterais. No entanto, é possível identificar particularidades que permitem a sua diferenciação. *Z. leprieurii* tem estruturas secretoras grandes, enquanto que em *Z. zanthoxyloides* são muito menores. *Z. leprieurii* possui idioblastos com cristais de oxalato de cálcio do tipo drusa que não são observados nos tecidos de *Z. zanthoxyloides*. No entanto, *Z. zanthoxyloides* revela a presença de depósitos cuticulares na superfície adaxial, mais especificamente estrias. Não foi detetado para nenhuma das espécies de *Zanthoxylum* a presença tricomas. *Z. leprieurii* apresenta um índice estomático de 7% e com estomas do tipo anomocítico, anisocítico e braquiparacítico, enquanto *Z. zanthoxyloides* possuí o tipo braquiparacítico como o mais frequente, exibindo um índice estomático de 10%. Para todas as medições anatómicas da folha (mesófilo total, cutícula superior, epiderme superior, epiderme inferior, parênquima paliçada e parênquima esponjoso) estas duas espécies são significativamente diferentes, sendo a folha de *Z. leprieurii* mais espessa. Outra diferença observa-se na nervura central, *Z. leprieurii* não apresenta uma concavidade na face superior da nervura, mas assim uma saliência. Em relação a *H. acida*, as células do tecido epidérmico têm formato poliédrico com paredes retas na epiderme adaxial e abaxial. Tricomas peltados, amarelo-acastanhados, estão presentes na superfície abaxial, constituídos por células quase isodiamétricas com inserção radial e pedúnculo pluricelular. Foram também encontrados cristais de oxalato de cálcio poliédricos perto das nervuras. O tipo de estoma, exclusivamente na face abaxial, é classificado como paracítico e o valor do índice estomático é de 13%. Esta análise contribui com importantes dados que permitem a caracterização e identificação das espécies em estudo.

Os testes histoquímicos revelaram que a diferença entre as espécies de *Zanthoxylum* é de natureza quantitativa e não qualitativa. Para *H. acida* foi detetada a presença de vários compostos e a deteção de lipídios em tricomas mostrou uma possível natureza lipídica da secreção.

Nenhuma atividade antibacteriana foi encontrada para *Bacillus subtilis* e para as bactérias Gram-negativas testadas, *Escherichia coli*, *Klebsiella pneumoniae* e *Pseudomonas aeruginosa.*

Das espécies em estudo, os extratos apolares de folhas de *H. acida* são os mais ativos, sendo capazes de inibir o crescimento de seis bactérias Gram-positivas, incluindo estirpes resistentes de *S. aureus* e estirpes de *E. faecalis* resistentes à vancomicina. Determinados extratos de *Z. zanthoxyloides* e *Z. leprieurii* revelaram atividade antibacteriana em Gram-positivas, sendo que extratos de folhas destas espécies causaram a inibição do crescimento de estirpes de *S. aureus* resistentes à meticilina (MRSA) e com resistência intermédia à vancomicina (VISA). *H. acida* não revelou qualquer atividade contra o crescimento de fungos. *Z. zanthoxyloides* revelou-se ativo contra estirpes sensíveis de *C. tropicalis* e *R. rubra*. O extrato de metanol da raiz de *Z. leprieurii* foi capaz de inibir o crescimento de *C. albicans* resistente. O extrato da raiz de *Z. zanthoxyloides* foi capaz de reverter sinergicamente a atividade antibacteriana da amoxicilina e da oxacilina contra a estirpe VISA e a combinação com oxacilina também diminuiu a CMI de forma eficaz para estirpe MRSA. Os extratos de *Z. leprieurii* combinados com oxacilina também demostraram um efeito sinérgico nas estirpes VISA e MRSA. Terpenos, flavonóides e compostos fenólicos, conhecidos por apresentarem propriedades antibacterianas, foram encontrados na maioria dos extratos de *Z. zanthoxyloides* e *Z. leprieurii*. Esses grupos químicos foram também detetados nos extratos apolares de folhas de *H. acida*, os mesmos que neste estudo apresentaram potencial antibacteriano. Os resultados obtidos para o teor total de fenóis, quando comparados aos extratos que evidenciaram atividade antibacteriana, indicam que o maior teor pode não estar diretamente ligado ao potencial antibacteriano.

Os resultados obtidos enfatizam o valor de estudos adicionais destas espécies para melhor compreensão dos compostos e mecanismos que podem ser fundamentais para ultrapassar a resistência aos antibióticos ao reverter a atividade antibacteriana dos mesmos, que atualmente representa uma grande ameaça à saúde publica.

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Abbreviations

MRSA	Methicillin-resistant *Staphylococcus aureus* strain
VISA	Vancomycin-intermediate *Staphylococcus aureus* strain
VRE	Vancomycin-resistant enterococci
WHO	World Health Organization
Ce3C	Center for Ecology, Evolution and Environmental Changes
LISC	Lisbon University Herbarium
I	Stomata Index
LM	Light Microscopy
UV	Ultraviolet
LP	Long Pass Filter
PAS	Periodic Acid-Schiff
***n*-hex**	*n-hexane*
CH_2Cl_2	Dichloromethane
AcOEt	Ethyl acetate
MeOH	Methanol
H_2O	Water
ATCC	American Type Culture Collection
CIP	Collection de l'Institut Pasteur
MSSA	Methicillin-sensitive *Staphylococcus aureus*
FFUL	Faculdade de Farmácia da Universidade de Lisboa
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
DMSO	Dimethyl sulfoxide
CLSI	Clinical and Laboratory Standards Institute
FICI	Fractional Inhibitory Concentration Index
TLC	Thin-layer Chromatography
NEU	Natural Products–polyethylene Glycol
GAE	Gallic Acid Equivalents
ANOVA	Analysis of Variance
MDR	Multidrug Resistance

1. Introduction

For the past years, the occurrence of several infectious diseases and the increasing prevalence rate of pathogens that are resistant to antibiotics have become a serious threat to human health (O'Neill, 2014; Saleem *et al.*, 2010). This growing concern leads to the need for finding new antimicrobial agents capable of reversing resistance to antibiotics (Madureira *et al.*, 2012). The use of plant medicine is not new and for thousands of years very diverse herbal formulations were created to treat several diseases (Balunas & Kinghorn, 2005; Ji *et al.*, 2009; Ríos & Recio, 2005). Currently, plants are a source of either new compounds or compounds whose molecules can be modified giving rise to new drugs. The synergistic potential between plants and antibiotics also revealed promising results to overcome antimicrobial resistance (Mundy *et al.*, 2016).

As stated by the World Health Organization (WHO, 2018), most of the life-threatening hospital-acquired infections are caused by highly resistant bacteria. Methicillin-resistant *Staphylococcus aureus* strain (MRSA), common in health facilities and the origin of severe infections, is 64% more likely to kill an infected patient than a non-resistant strain. The uncontrolled use of last resort antibiotics, like vancomycin to fight MRSA, led to the rise of new strains with a reduced sensibility to vancomycin, called vancomycin-intermediate *S. aureus* (VISA). Vancomycin-resistant enterococci (VRE), like strains of *Enterococcus faecalis,* are also considered as leading resistant pathogen in the hospital environment (Pereira *et al.*, 2016). Several previous studies proved that there is a huge potential of plant-derived compounds as antibacterial and as a promising strategy, using them for resistance-modifying of other antibiotics through synergistic behavior (Abreu *et al.*, 2012). Therefore, *in vitro* antibacterial assessment and phytochemical profile screening can be an important source of new agents against pathogens.

In plant identification, macromorphological characters are widely used due to their easy observation. In studies related to lower taxonomic levels such as genera and species, a micromorphological approach reveals to be more precise and less influenced by the environment. Also, anatomy study is an important assessment for plant correct identification, consisting in the arrangement of dermal, ground and vascular tissue systems (Oggero, Arana, & Reinoso, 2016). In addition, if we consider the use of medicinal plants, usually involves fragments or even powdering and their characterization can only be achieved by micromorphological examinations. These phytognostic characters enable a comparative study and supply a valid criterion for species differentiation, as well as the accurate and reproducible herbals quality control (Stace, 1989). That is the reason why microscopy data are part of the Pharmacopoeias plant monographs (Teixeira & Monteiro, 2017). Histochemical methods, using staining techniques, are also an important assessment to the investigation of biosynthesized compounds by plants, such as secretions accumulated in cell organelles or secretory glands. The result of these reactions is observed in

bright field and fluorescence microscopy, allowing the study of chemical composition *in situ*, identifying, at a cellular level, certain compounds (terpenoids, phenolics, alkaloids, etc.) by analyzing colorimetric reactions (Gabe, 1968; Lison, 1960).

The selected species for the present study are autochthonous plants from Guinea-Bissau, an African country, where there is a wide range of plants used in herbal medicine to treat endemic diseases as result of fragile health services, easily found in local markets and pharmacies (Van Wyk, 2011). Guinea-Bissau, surrounded by Senegal, Republic of Guinea and the Atlantic Ocean, is situated in West Africa. This country is estimated to have a vascular flora with around 1507 species, 1495 of which are native. Since 1997, to protect biodiversity, a network of protected areas was established in Guinea-Bissau, a collaboration between National Institute for Biodiversity and Protected Areas and International Union for the Conservation of Nature (Catarino *et al.*, 2016).

The present work aims to characterize morphoanatomical and histochemical traits of two *Zanthoxylum* L. species and one *Hymenocardia* Wall. ex Lindl species, using different microscopy techniques, providing new important information about micromorphological characteristics, due to the lack of previous studies. The *in vitro* antibacterial and antifungal activity was also evaluated against a selected panel of microorganisms, to better understand their application in traditional medicine. Further potential synergistic effects were tested as well as the phytochemical profile.

2. State of Art

2.1. Taxonomy and botanical description of the genus *Zanthoxylum*

Rutaceae family encompasses 158 genera, widespread in tropical and temperate regions. It is an important family that comprises a large number of ornamental and food plants, such as *Citrus* L., widely cultivated all around the world. Usually found as trees or shrubs and with less frequency as annuals. The plants of this genus exhibit leaves alternate or opposite that can be simple or compound and lamina with oil dots with aromatic properties when crushed (PlantNET, 2018; The Plant List, 2013).

Zanthoxylum L. is one of the genera that pertains to Rutaceae, with around 550 species worldwide, size varying from shrubs to trees that can grow up to 20 meters. The presence of recurved spines on the trunk and branches is characteristic from the species belonging to this genus. *Zanthoxylum* genus has been formerly confused with *Fagara* L. but currently, both genera are grouped under *Zanthoxylum*. Even so, several authors still use *Fagara* (Patiño *et al.*, 2012).

Many *Zanthoxylum* species are well known for their ethnobotanical use to treat several diseases, both in humans and animals. Phytochemical studies performed for this genus revealed that the predominantly metabolites are alkaloids (mainly benzophenanthridines), lignans, coumarins, flavonoids, sterols, and terpenes (Patiño *et al*., 2012).

According to Catarino *et al.* (2016) in Guinea-Bissau there are two *Zanthoxylum* species (Table 1) with medical properties: *Zanthoxylum leprieurii* Guill. & Perr. and *Zanthoxylum zanthoxyloides* (Lam.) Zepern. & Timler. These two species have characteristic macromorphology that allows their distinction.

Z. leprieurii has a large distribution and occurs from east Senegal to Ethiopia, south to Mozambique and eastern South Africa (Figure 1A), while *Z. zanthoxyloides* occurs from east Senegal to Cameroon (Figure 1B).

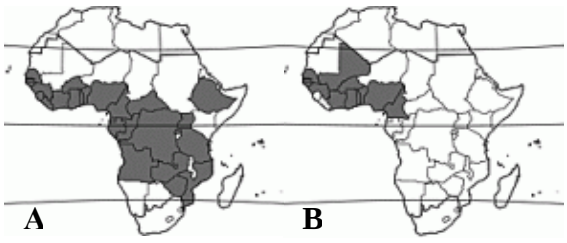


Figure 1 – Distribution in the African continent of the *Zanthoxylum* L. species under study: A – *Zanthoxylum leprieurii* Guill. & Perr.; B – *Zanthoxylum zanthoxyloides* (Lam.) Zepern. & Timler. (PROTA4U)

Table 1 – Ethnobotanical properties described for *Zanthoxylum* species (adapted from Catarino *et al.*, 2016)

Species	Parts used	Group of diseases	Vernacular names
Zanthoxylum leprieurii	Bark, Leaves, Roots	Pains; Pregnancy; childbirth; breastfeeding and diseases of the newborn; Intestinal problems; Cough and respiratory diseases; Stings; Bites and poisoning.	mádjá, mantcha, mantchu, eranha, elanha, barquelem
Zanthoxylum zanthoxyloides	Roots	Intestinal problems; Male impotence.	Not known

According to Tabuti (2011), the species *Z. leprieurii* is a tree up to 15-25 m high (Figure 2A), branches with short strong prickles 3–8 cm long, straight or occasionally curved, brown with darker tips (Figure 2B). Leaves alternate, stipules absent, imparipinnately compound with 8–24 leaflets (Figure 2C), 15–55 cm long; elliptical to oblong-elliptical, 2.5–16 cm × 1.5–7.5 cm, apex acuminate or caudate, base oblique and cuneate, margin entire towards the base, toothed elsewhere, glabrous, papery, with many minute glandular dots, pinnately veined with 8–16 pairs of lateral veins. Inflorescence is an axillary panicle with sessile flowers on the primary panicle branches, 15–45 cm long, short glandular hairs to almost glabrous. Flowers are unisexual, regular, 4-merous, small, nearly sessile; sepals slightly united at the base, broadly ovate, 0.4–0.6 mm long; petals oblong, 1.5–2 mm × 0.8–1 mm, creamy white; male flowers with 4 stamens, rudimentary pistil present (Figure 2D); female flowers upper ovary. Fruit an almost globose follicle, 4–5 mm in diameter, almost sessile, reddish, glandular pitted. Seed almost globose, 3–3.5 mm in diameter, black and shiny.

Figure 2 – *Zanthoxylum leprieurii*. A – Plant overview; B – Branch prickles; C – Compound leaf; D – Male inflorescence detail. (Photos by BT Wursten in Flora of Zimbabwe).

As described by Matu (2011) *Z. zanthoxyloides* is a shrub or small tree (Figure 3A), spiny up to 6–12 m, often with woody prickle-bearing protuberances or yellow, odorous, orange-mottled beneath. Stems are glabrous, grey, with prickles (Figure 3B). Leaves alternate, glabrous, petiole 2–5 cm long, glabrous, stipules absent imparipinnately compound with 5–11 opposite or alternate leaflets, up to 12–20 cm long; petiolules 2–5 mm long; leaflets obovate to elliptical, 5–10 cm × 2–4 cm, base cuneate to rounded, apex obtuse or rounded, sometimes apiculate or notched, with many glandular dots, smelling of pepper and lemon when crushed, rigidly papery, pinnately veined with 10–14 pairs of lateral veins, barely prominent, fusing near the margin. Inflorescence 5–25 cm long, with short branches. Flowers unisexual, regular, 5-merous, white or greenish (Figure 3C), sessile; male flowers with stamens slightly exserted; female flower with upper ovary. Fruits (Figure 3D) characterized as ovoid follicles, 5–6 mm in diameter, brown, with glandular dots, dehiscent, 1-seeded. Seed black to bluish, shiny, long persistent in the fruit.

Figure 3 – *Zanthoxylum zanthoxyloides*. A – Plant overview; B – Trunk detail with spines; C – Flowering branch; E – Immature Fruits. (Photos by Stefan Porembski and Marco Schmidt in African plants - A Photo Guide).

2.2. Taxonomy and botanical description of the genus *Hymenocardia*

Phyllanthaceae family comprises 59 genera, encompassing around 2000 species. Characterized as a pantropical family, displays high diversity in life form, varying from large forest trees, shrubs and even aquatic species. Flowers exhibit great diversity (shape, size, number of floral organs) as well as the fruits (Kathriarachchi *et al.*, 2005).

Hymenocardia Wall. ex Lindl. is a genus with six species, most are native to Africa and one is from Southeast Asia (The Plant List, 2013). This genus taxonomy showed some controversy in the past being under Euphorbiaceae family and later placed under Hymenocardiaceae (Thakur & Patil, 2011). As mentioned, now it belongs to Phyllanthaceae, one of the five segregates of *Euphorbiaceae sensu lato*, recognized at family level (Hoffmann *et al.*, 2006). *Hymenocardia acida* Tul. occurs largely in tropical Africa, from Senegal to Ethiopia and South Africa (Figure 4). This species is well known for its large range of medicinal uses, with several ethnopharmacological studies developed throughout the years (Sofidiya *et al.*, 2009).

H. acida is also found in Guinea-Bissau as a local medicinal plant with several applications (Table 2) (Catarino *et al.*, 2016).

Figure 4 – Distribution in the African continent of *Hymenocardia acida* Tul. (PROTA4U)

Table 2 – Ethnobotanical properties described for *Hymenocardia acida* (adapted from Catarino *et al*., 2016)

Species	Parts used	Group of diseases	Vernacular names
Hymenocardia acida	Bark, Leaves	Pains; Pregnancy; childbirth; breastfeeding and diseases of the newborn; Intestinal problems; Skin inflammations; wound sand burns; Stings; Bites and poisoning.	beninebahan, betenam coroncondô corocondé, netchondor, netendor, oábi (etc)

Orwa (2009) and Schmelzer (2008) described *H. acida* as a dioecious shrub or small tree up to 6–10 m tall (Figure 5A). Bark smooth, pale brown or grey, flaking off, showing a powdery reddish to orange inner bark (Figure 5B). Leaves alternate, simple and entire (Figure 5C); stipules 1–3 mm long, linear, soon falling; petiole 0.5–1.5 cm long, short-hairy; blade elliptical-ovate to oblong-oblanceolate, 2.5–9.5 cm × 1.5–5 cm, base rounded, apex rounded to obtuse, yellowish peltate secretory hairs in the lower epidermis and short-hairy over the main veins. Flowers

unisexual, petals absent, disk absent; male inflorescence (Figure 5D) dense, sessile, solitary or fascicled; female inflorescence a terminal few-flowered raceme up to 3 cm long, usually several together, or flowers solitary. Upper ovary, gland-dotted, glaucous to red. Fruit a V-shaped (Figure 5E), flattened capsule, 2–3.5 cm × 2.5–4 cm, with two apical divergent rounded to rhomboid membranous striate wings, gland-dotted or not, yellow-green at first, turning pink then reddish brown, 2-seeded, on a stipe up to 2 mm long. Dark seeds compressed circular, c. 10 mm × 5 mm.

Figure 5 – *Hymenocardia acida*. A – Plant overview; B – Trunk detail; C – Leaves; D – Male inflorescence; E – Fruits. (Photos by Emeline Assede, Paul Latham, Arne Erpenbach and Marco Schmidt *in* African plants - A Photo Guide).

2.3. Leaf micromorphology and anatomy

Both phytochemical and bioactivity studies are the most popular for many plant species, while micromorphology studies are neglected. During our bibliographic research, very few studies were found related to leaf micromorphology, including leaf anatomy, for the three species here analyzed.

For the genus *Zanthoxylum* some studies related to morphoanatomical characteristics have been published but most of them concern to other species, in different ecology context from the present study. Rakić *et al.* (2009) studied the ecophysiological, anatomical characteristics and adaptation of *Zanthoxylum acanthopodium* DC., a subtropical shrub, present in Serbia, with the goal of understanding the viability of this plant when the conditions are temperate continental climate. Igboabuchi & Ilodibia (2017) studied the species *Zanthoxylum macrophylla* L. Sarg. due to the lack of knowledge in the anatomy field, contributing with significative data for correct taxonomic and identification of plant species. Oggero *et al.* (2016) analyzed the morphology and anatomy of the leaf in *Zanthoxylum coco* Gillies ex Hook.f. & Arn. and *Zanthoxylum armatum* DC, from a xerophytic forest in Argentina, capturing interesting images of leaves transverse sections and epidermis. Ullah *et al.* (2014) examined leaves of *Z. armatum* and identified important characteristics such as vascular bundles arrangement, stomata types and stomatal index. Xochicale *et al.* (2010) analyzed twelve *Zanthoxylum* species contributing with significant information related to stomata diversity in this genus. Only one study was found on *Z. zanthoxyloides*, done by Mensah (2012) that studied the morphological and physiological responses of woody species in Ghana related to adaptative characteristics of plants under stressed environment. This study contributed with characteristics linked to the leaf anatomy.

Concerning to *H. acida*, Levin & Simpson (1994) studied the phylogenetic relationship between the genera *Didymocistus* Kuhlm and *Hymenocardia*, examining characteristics related to pollen and foliar trichomes. This work contributed with significant data for *H. acida* trichomes that can be useful to better understanding the obtained results. Solereder (1908) published a book called “Systematic Anatomy of the Dicotyledons” where some characteristics related to *H. acida* were described, such as vascular bundles and foliar trichomes. Raju & Rao (1977) also studied the stomata and epidermis characteristics variation within Euphorbiaceae family, where Phyllanthaceae were included at the time, bringing important information as comparative measure with the present study.

2.4. Antibacterial and antifungal properties

Several studies have been performed to evaluate the antibacterial and antifungal properties of the three species under analysis on the present study. *Zanthoxylum* genus has countless published works related to the chemical and biological value of various species. Adesina (2005) published a review about the several metabolites isolated from *Z. leprieurii* and *Z. zanthoxyloides*, collected in Nigeria and their chemical and biological applications. This paper gives special importance to the antibacterial properties of the acridone alkaloids present in *Z. leprieurii* and the chelerythrine, berberine and phenolic canthine-6-one isolated from *Z. zanthoxyloides*. The isolation of acridone alkaloids from *Z. leprieurii* was also studied by Ngoumfo *et al.* (2010), aiming to evaluate the *in vitro* cytotoxic activity. According to Bunalema (2017), *Z. leprieurii* acridone alkaloids also showed activity on *Mycobacterium tuberculosis* resistant strains. Queiroz *et al.* (2006) analyzed the root bark of *Z. zanthoxyloides* and several biological active compounds were isolated. They exhibited antibacterial, antifungal and acetylcholinesterase inhibition. Agyare *et al.* (2014) also tested *Z. leprieurii* extracts for antibacterial properties and performed a preliminary phytochemical screening. Mills-Robertson *et al.* (2017) carried out a study with six species against multi-drug resistant *Staphylococcus aureus* and concluded that most of the tested plants, including *Z. zanthoxyloides*, revealed antimicrobial activities. Ngane *et al.* (2000) published a study where aqueous-ethanol extract of leaves, roots and stem barks of *Z. leprieurii* and *Z. zanthoxyloides* were analyzed for antifungal properties. The results indicated that, in general, the *in vitro* growth for the tested fungi was inhibited. Some studies focus on the evaluation of antibacterial and antifungal activity of essential oils. One of those studies is the one performed by Tatsadjieu *et al.* (2003) that analyzed plants from Cameroon and conclude that *Z. zanthoxyloides* and *Z. leprieurii* essential oils have antibacterial and antifungal activity against eight microorganisms strains. Table 3 and 4 summarize all the results of the previously mentioned studies.

For *H. acida* the amount of work found is not as significant, but nevertheless, some studies were carried out with interesting results. Starks *et al.* (2014) revealed that isolated chromene stilbenoids and chromane stilbenoids from *H. acida* have moderate activity against *Staphylococcus aureus* resistant strains. Sofidiya *et al*., (2009) studied extracts from this plant leaves and concluded that there is a significant growth inhibition of Gram-positive strains tested. Similar results were obtained by a published work from Kamba & Hassan (2011) and Muanza *et al*. (1994).

No studies were found related to the species tested in the present study for synergism evaluation between plant extracts and antibiotics in resistant bacteria strains.

Table 3 – *Zanthoxylum leprieurii* summary table of research studies about antibacterial and antifungal activity.

Extract	Compound	Biological activity	
-	Acridone alkaloids	Antibacterial	Adesina (2005)
Aqueous-methanol stem bark extract	-	Antibacterial and antioxidant	Agyare *et al*. (2014)
Chloroform extract of roots and fruits	Acridone alkaloids	Cytotoxic	Ngoumfo *et al.* (2010)
Methanol extract of stem bark	Acridone alkaloids	Growth inhibition of resistant strain *Mycobacterium tuberculosis*	Bunalema *et al*. (2017)
Aqueous-ethanol extract of leaves, roots and stem barks	-	Growth inhibition of *Candida albicans* and *Cryptococcus neoformans*	Ngane *et al.* (2000)
Essential oils	-	Growth inhibition of *Staphylococcus aureus*, *Enterococcus faecalis*, and *Bacillus subtilis.*	Tatsadjieu *et al.* (2003)

Table 4 – *Zanthoxylum zanthoxyloides* summary table of research studies about antibacterial and antifungal activity.

Extract	Compound	Biological activity	References
-	Chelerythrine, berberine, phenolic canthine-6-one	Antibacterial	Adesina (2005)
Methanol extract of root bark	Fagaronine	Antifungal properties in *Cladosporium cucumerinum*	Queiroz *et al*. (2006)
Ethanol and aqueous extract of stem bark	-	Antibacterial properties against *S. aureus*	Mills-Robertson *et al*. (2017)
Aqueous-ethanol extract of leaves, roots and stem barks	-	Growth inhibition of *C. albicans* and *C. neoformans*.	Ngane *et al.* (2000)
Essential oils		Growth inhibition of *Staphylococcus aureus*, *Enterococcus faecalis*, and *Bacillus subtilis.*	Tatsadjieu *et al.* (2003)

3. Materials and Methods

3.1. Plant material

Roots and young stems of two *Zanthoxylum* species, Z. *zanthoxyloides* and Z. *leprieurii* were collected in Orango, one of the Bijagós Islands, Guinea-Bissau, in 2016 and 2017, by Luís Catarino (Ce3C) (Figure 6). Small tree branches of *H. acida,* were acquired in 2016 at a local market in Bissau. One part of the plant material was herborized and another part was subjected to a fixing process still carried out in Guinea-Bissau. The plants were identified in the Lisbon University Herbarium (LISC) where vouchers are deposited.

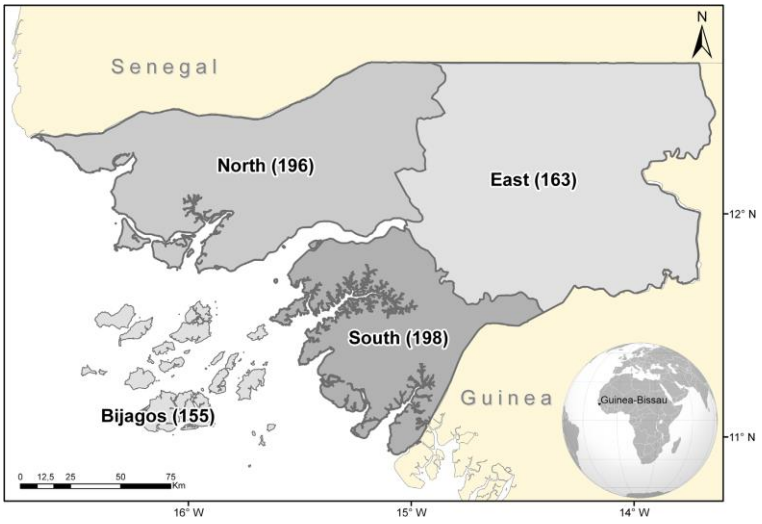


Figure 6 – Location of Guinea-Bissau, distribution of medicinal plants and collecting site. (Catarino *et al.*, 2016)

3.2. Leaves micromorphology and anatomy analysis

The collected plant material includes young roots and stems with leaves, but our morphoanatomical study focused only on leaves of the three species under study. All observations and measurements related to this study where made on random fields, always at comparable leaf situations and magnifications, using different techniques of microscopy, bright field, fluorescence and scanning electron microscopy.

The dried plant material was hydrated and clarified in a 1% sodium hypochlorite solution and washed in distilled water (Evans, 2009). Fixation was achieved in a 2.5% glutaraldehyde solution (Hayat, 1981).

Mid-lamina of the abaxial surface is considered to have less variability, thus the best area to perform the measurements (Teixeira & Monteiro, 2017). So, for leaf anatomy study, cross-sections were performed in the middle zone of the leaves. Both upper and lower epidermis were observed, and epidermal leaf surface characters were analyzed, such as cell shape and orientation

and the existence of stomata, trichomes, and secretory structures, their type, size, and orientation. Stomata type was classified according to Stace (1989). On the lower epidermis, the stomata index (I) was also calculated according to Salisbury (1927), applying the following formula:

$$I = [\text{number of stomata} / (\text{number of stomata} + \text{number of epidermal cells})] \times 100$$

The fixed plant material was processed with paraffine micro technique according to Feder & O'Brien (1968). The blocks were then sectioned in a Leitz 1512 Minot microtome, with a thickness between 10-12 μm. Some cuts were stained with acidic phloroglucinol solution to highlight cell walls with lignin. The observation focused on 20 leaf transverse sections, for each species, to address total lamina thickness, thickness of palisade and spongy tissues, upper cuticle thickness, length of glands and thickness of upper and lower epidermal cells. Light microscopy (LM) observations were made with Nikon Labophot-2 microscope and images were captured by Nikon FX-35W camera, with semi-automatic Nikon PFX adapter (Nikon®) and Cokin 80A blue filter.

For scanning electron microscopy (SEM), plant material was fixed as mentioned above, after which it was dried on a Critical Point Polaron Bio Rad E3500 and coated with a thin layer of gold on a Jeol JFC-1200. SEM observations focused on some details of the upper and lower epidermis surfaces, such as type of indumentum and stomata type and density, and on the leaves transverse sections. These observations were performed at 15kV on a Jeol JSM-5220 LV scanning electron microscope, equipped with digital image acquisition system.

Histochemical tests were carried out to aim the major chemical groups present on the leaf tissues, using hand-sectioned transverse cuts. The histochemical tests carried out during this study are listed in Table 5. Results were displayed from negative (-) to strongly positive (+++).

Further histochemical characterization was complemented with autofluorescence detected in plant tissues. The plant material was mounted in distilled water for autofluorescence observation in an epi-fluorescence microscope Olympus BX60, equipped with an HBO 50 mercury vapor lamp and two excitation filters: one for the UV band 340-380 nm and one for the blue band 450-490 nm and the corresponding two barrier filters, LP 430 nm and LP 520 nm, respectively. The fluorescence microscope is equipped with image acquisition system, IC Captur 2.4.

Table 5 – Selected histochemical tests aiming the major chemical groups present on the leaf tissues.

Major Chemical Groups		**Histochemical test**
Polyphenols		
	General	Ferric chloride and Potassium dichromate (Johansen, 1940)
	Lignin	Phloroglucinol (Evans, 2009)
	Tannins	Vanillin/HCL (Gardner, 1975)
Alkaloids		
		Wagner reagent (Iodine in potassium iodide)
Mucilages		
		Tannic acid/ferric chloride (Pizzolato & Lillie, 1973)
Pectins		
		Ruthenium Red/Copper acetate (Johansen, 1940)
Polysaccharides		
	General	PAS (Feder & O'Brien, 1968)
Lipids		
	Total	Sudan III (Johansen, 1940)
	Neutral and acidic	Nile Blue (Jensen, 1962)
	Fatty acids	Copper acetate/Rubeanic acid (Lison, 1960)
Terpenoids		
	Essential oils and resins	Nadi reagent (David & Carde, 1964)
	Steroids	Anthimonium chloride (Hardman & Sofowora, 1972)
	Terpenoids	2,4-Dinitrophenylhydrazine (Lison, 1960)

3.3. Antibacterial and antifungal *in vitro* activity

To assess the potential antibacterial and antifungal activity of our plant material, *in vitro* assays were performed with plant extracts against a panel of selected bacteria strains, susceptible and resistant to antibiotics, and fungi.

3.3.1. Preparation of plant extracts

The plant material was dried at room temperature and powdered, obtaining 20 g of *H. acida* leaves, 6 g of *Z. zanthoxyloides* leaves, 30 g of *Z. zanthoxyloides* roots, 13 g of *Z. leprieurii* leaves and 27 g of *Z. leprieurii* roots. These materials separately were subjected to extraction with ascending polarity solvents: *n*-hexane (*n-hex*), dichloromethane (CH_2Cl_2), ethyl acetate (AcOEt), methanol (MeOH), and water (H_2O). Each solvent extraction was made in 24 h periods at room temperature, with occasional shaking. After that period, the extracts were decanted and filtrated, to proceed to their concentration till dryness. This was achieved using a Rotavapor Buchi R-3 with Vacuum Pump V-710, evaporating the extracts under reduced pressures at 40-45 ºC. The dried extracts were stored at -20 ºC until use (Madureira *et al.*, 2012).

3.3.2. Microbial strains

The selection of the microorganism panel to perform the *in vitro* assays was based on the model proposed by Cos *et al.* (2006). The microorganisms used in our study are listed in Table 6 and includes a range of Gram-positive and Gram-negative bacteria, fungi, and yeast, which are known to be the cause of several human diseases. Bacteria growth medium used was Mueller-Hinton, while for fungi it was used Sabouraud.

Table 6 – Selected pathogen panel of Gram-positive bacteria, Gram-negative bacteria, fungi, and yeast.

Microorganism		**Strain**
Gram-positive bacteria		
	Bacillus subtilis	ATCC[1] 6633
	Enterococcus hirae	CIP[2] 5855
	Enterococcus faecalis	ATCC 51299
	Staphylococcus aureus	ATCC 6538 – MSSA[3]
		ATCC 43866 – MRSA[4]
		CIP 106760 – VISA[5]
	Staphylococcus epidermidis	ATCC 12228
Gram-negative bacteria		
	Escherichia coli	ATCC 8739
	Klebsiella pneumoniae	ATCC 9997
	Pseudomonas aeruginosa	ATCC 9027
Fungi and yeast		
	Candida albicans	ATCC 90028
	Candida dubliniensis	FFUL[6] 21
	Candida glabrata	FFUL 12B
	Candida tropicalis	ATCC 750
	Candida guilliermondii	FFUL 1403
	Candida kruzei	ATCC 6258
	Candida parapsilopsis	ATCC 90018
	Rhodotorula rubra	FFUL 190
	Trichosporon cutaneum	FFUL 18H
	Saccharomyces cerevisiae	FFUL 1997
	Cryptococcus neoformans	FFUL 948

[1]ATCC – American Type Culture Collection, Maryland, USA; [2]CIP – Collection de l'Institut Pasteur, Paris, France; [3]MSSA – Methicillin-sensitive *Staphylococcus aureus;*[4]MRSA – Methicillin-resistant *S. aureus;* [5]VISA – Vancomycin-intermediate *S. aureus*; [6]FFUL – Faculdade de Farmácia da Universidade de Lisboa;

3.3.3 – Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

From the extracts previously obtained, a stock solution of 5 mg/mL was made by dissolving 5 mg of each extract in 1 mL of dimethyl sulfoxide (DMSO), an amphiphilic compound that enables an easier solubilization. This solution was then diluted with culture medium to a final concentration of 1 mg/mL per extract.

These assays were performed by evaluating the minimum inhibitory concentration (MIC). MIC values were determined by the microplate broth microdilution method according to the recommendations of the Clinical and Laboratory Standards Institute. In brief, on each well of the microplate, 100 µL of Muller-Hinton broth was added plus 100 µL of each extract sample. Successive dilutions were made, obtaining final concentrations varying from 7.5–500 µg/mL. On each well, was then inoculated a 10 µL microbial suspension of the pathogen to be tested (final concentration of 10^4 cfu/mL). Each microplate also had a control row of solvent and a control row of bacteria (CLSI, 2008). The microplates were incubated at 37 ºC in dark, for 24 h. All assays were repeated three times. The inhibition of microbial growth was determined visually, at the lowest concentration where no microorganism growth was observed. Only the samples with MIC value ≤ 100 µg/mL were considered with antibacterial or antifungal activity (Cos *et al.*, 2006).

The minimum bactericidal concentration (MBC) of plant extracts against the pathogens was evaluated following the CLSI method (CLSI, 2008) with some modifications. The MIC wells of the microplate that showed no growth were sub-cultured on Muller-Hinton agar plate by collecting a loopful of broth using a sterile wire loop. The plates were then incubated for 24 h at 37 ºC. The MBC value was considered the lowest concentration of extract that didn't present any colony growth. The assessment of MBC is complementary to MIC values, allowing to determine the lowest concentration that reduces almost at 100% the viability of the initial inoculum.

3.4. Combination effects of extracts with antibiotics

To determine the combined effect of the plant extracts and antibiotics on *S. aureus* MRSA (ATCC 43866) and VISA (CIP 106760) growth, the checkerboard method was used (Hemaiswarya *et al*., 2008). The antibiotic was successively diluted on horizontal rows while the sample extract was diluted on the vertical rows of the microplate. The concentration of the antibiotic ranged from 1 to 1/2048 of the MIC and the concentration of the compounds from 1/2 to 1/64 of the MIC (Pereira *et al.*, 2016).

The antibiotics used were amoxicillin and oxacillin, with MIC values, respectively, of 62 µg/mL and 125 µg/mL for ATCC 43866 and 250 µg/mL for both for CIP 106760.

The synergic effect was determined based on the fractional inhibitory concentration index (FICI), calculated according to the formula:

$$FICI = FIC(A) + FIC(B)$$

where, FIC(A)= MIC(A in the presence of B)/MIC(A alone) and FIC(B)=MIC(B in the presence of A)/MIC(B alone) (Pereira *et al.*, 2016). When the value of FICI ≤ 0.5, synergic effect is considered. FICI values ranging between 0.5 and 4 are classified as indifference and if superior to 4 as antagonism (Hemaiswarya *et al.* 2008).

3.5. Phytochemical screening

A semi-quantitative phytochemical analysis, to detect the major compounds found in each extract, was carried out through thin layer chromatography (TLC) on silica gel plates (Madureira *et al.*, 2012). Proper mixtures of eluents were used to develop and the spots were revealed with appropriated spray-reagents made according to Wagner & Bladt (1996), anisaldehyde-sulfuric acid reagent for terpenoids, Dragendorff reagent for alkaloids, natural products–polyethylene glycol (NEU) reagent for flavonoids and Fast Blue salt reagent for phenolic compounds. Results were displayed between absent (-) and strong intensity (+++).

A Folin-Ciocalteu assay was performed according to the method described by Stamatakis *et al.* (2009) adapted for 96-wells microplates. This method allows the quantification of phenolic compounds in the samples by reduction of molybdenum atoms, present in Folin-Ciocalteu reagent, caused by electrons from phenolic compounds, when subjected to alkaline medium. From this reaction, a blue complex appears which is possible to quantify spectrophotometrically (Brito, 2016). An Epoch Microplate Spectrophotometer Biotek was used for this quantification. The obtained results are expressed in mg GAE/L, where GAE stands for gallic acid equivalents.

3.6. Statistical data analysis

Some micromorphological results were analyzed statistically using the Statistix 9 program. Data were subjected to analysis of variance on one factor ANOVA, with a significance level of 5%. Means of morphological characters were separated using Tukey Test, for pairwise comparisons, at the 5% level of significance.

4. Results and Discussion

4.1. Leaves micromorphology and anatomy analysis

4.1.1. *Zanthoxylum zanthoxyloides* and *Zanthoxylum leprieurii*

Adaxial surface is characterized for polyhedral shaped cells with straight walls for both species (Figure 7A and 7B), while abaxial surface cells are irregular yet almost polyhedral shaped cells with straight walls for *Z. zanthoxyloides* and irregular with sinuous walls for *Z. leprieurii* (Figure 7C and 7D). Both adaxial and abaxial surface present cells with non-defined orientation for the two species. The average cell dimensions for *Z. leprieurii* was 44.85 ± 5.77 x 24.05 ± 2.49 µm for adaxial surface cells and 51.35 ± 9.8 x 16.25 ± 5.77 µm for abaxial surface cells, while *Z. zanthoxyloides* adaxial surface cells measured 55.25 ± 4.44 x 25.35 ± 2.49 µm and 42.9 ± 10.06 x 18.85 ± 4.44 µm for abaxial surface cells. For both *Zanthoxylum* species, adaxial and abaxial epidermis are characterized as thick and uni-layered, no trichomes were observed but the presence of few cuticular deposits on epidermis such as striation were found, especially in *Z. zanthoxyloides* (Figure 7A and 8A).

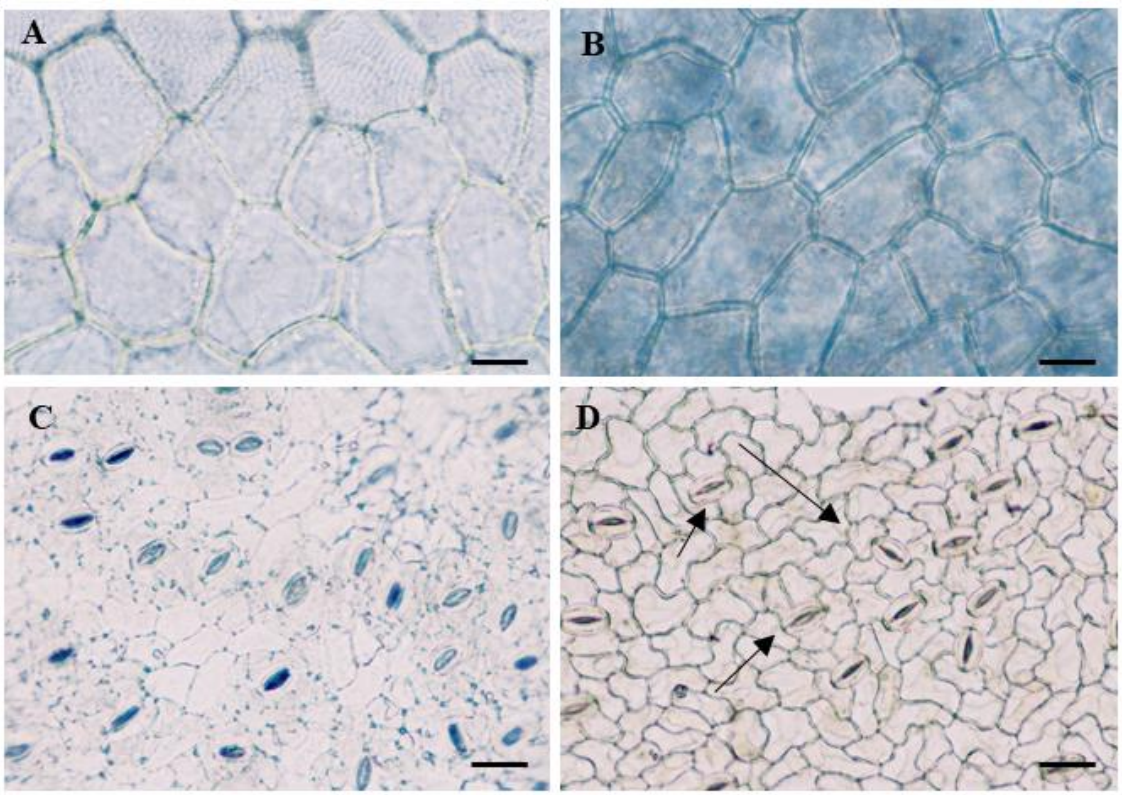


Figure 7 – Light microscopy micrographs of *Zanthoxylum zanthoxyloides* (A and C) and *Zanthoxylum leprieurii* (B and D) leaves. A – Leaf adaxial epidermal surface with visible cuticular deposits (scale bar 30 µm); B – Leaf adaxial epidermal surface (scale bar 20 µm); C – Abaxial epidermal cells and brachyparacytic stomata (scale bar 45 µm); D – Leaf abaxial epidermal surface with anisocytic (small arrow), brachyparacytic (medium arrow), and anomocytic (large arrow) stomata (scale bar 43 µm);

The observations related to dorsiventral leaf and cell shape are in agreement to Oggero *et al.* (2016) and Igboabuchi & Ilodibia (2017) for other *Zanthoxylum* species, *Z. coco*, *Z. armatum*, and *Z. macrophylla.* Oggero *et al.* (2016) also described thick epidermis with strong striations in *Z. coco* present in xerophytic forest in Argentina. Plants have the ability to react to the changing environmental conditions that result in morphological alterations, being the leaf the most sensitive organ to reflect those responses (Trewavas, 2003). Even though cuticular striation is regulated by genetic characters and is considered a good taxonomic characteristic, it can be influenced by the environmental conditions. It is known that in xerophytic habitats the cuticular striations are much more noticeable, once they promote water retention, than in woody species growing in tropical conditions, like the *Zanthoxylum* species in the present study, with little to no striation (Barthlott, 1981). According to Rollet *et al.* (1990) the presence of thick epidermis increases the reflectance and protects photosynthetic tissues from high solar radiation and the undulated and sinuous walls of the abaxial epidermis cells is related to high shadow and humidity conditions.

Stomata are only presented in the abaxial surface for both species, brachyparacytic in *Z. zanthoxyloides* (Figure 7C and 8B) and anomocytic, anisocytic and brachyparacytic for *Z. leprieurii* (Figure 7D and 8C). Hypostomatic leaf is a characteristic present in other species of *Zanthoxylum* genus (Ullah *et al.*, 2014; Igboabuchi & Ilodibia, 2017; Oggero *et al.*, 2016; Rakić e*t al.*, 2009). The anomocytic stomata were the predominant type in *Z. leprieurii*, which is in agreement with a study performed in twelve species of *Zanthoxylum* genus (Xochicale *et al.*, 2010). Ullah *et al*. (2014) studied the stomata diversity in *Z. armatum* and brachyparacytic stomata were also found as one of the most frequent. Stomata size varies between the two studied species, being 30.33 ± 3.55 x 9.10 ± 1.42 µm in *Z. zanthoxyloides* and 37.27 ± 2.12 x 20.37 ± 3.04 µm in *Z. leprieurii*. *Z. zanthoxyloides* have an estimated stomata index of 10% while in *Z. leprieurii* the value was 7%. These stomata index values are similar to the values register for *Z. coco* and *Z. armatum* (Oggero *et al*., 2016; Ullah *et al.*, 2014). While stomata density can be influenced by environmental conditions, stomata index is preferred by the researchers to compare leaves of different sizes, being independent from factors like plant age, habitat and leaf surface position, therefore important diagnostic character in systematics (Rowson, 1946). Furthermore, stomata index values have great importance to evaluate leaf origin crude drugs (Evans, 2009). According to Metcalfe & Chalk (1950), the stomata size is inversely proportional to the stomata index. Although the difference between the two species on the present study is not accentuated, the obtained results are in agreement with the previous statement.

As for internal secretory structures, both species have this characteristic, but while *Z. zanthoxylum* presents reduced structures with an average diameter of 34.84±17.01 µm (Figure 8D and Figure 9A), *Z. leprieurii* presents very large inner cavities that occupy almost the entire width of the mesophyll (Figure 9B, 9C, and 9D) with average diameter of 170.63±19.45 µm. The

structures are lysigenous, present across the leaf, mostly located in spongy parenchyma. The presence of these structures is common for this genus as it was already described in other studies for *Z. coco, Z. armatum* and *Z. acanthopodium*, with the difference that they are located at leaf margin, except for *Z. coco* (Oggero *et al.*, 2016; Rakić *et al.*, 2009). As stated by Metcalfe & Chalk (1950), the secretory structures of *Zanthoxylum* have protective, defensive and ecological meaning, therefore important at metabolic and taxonomic level.

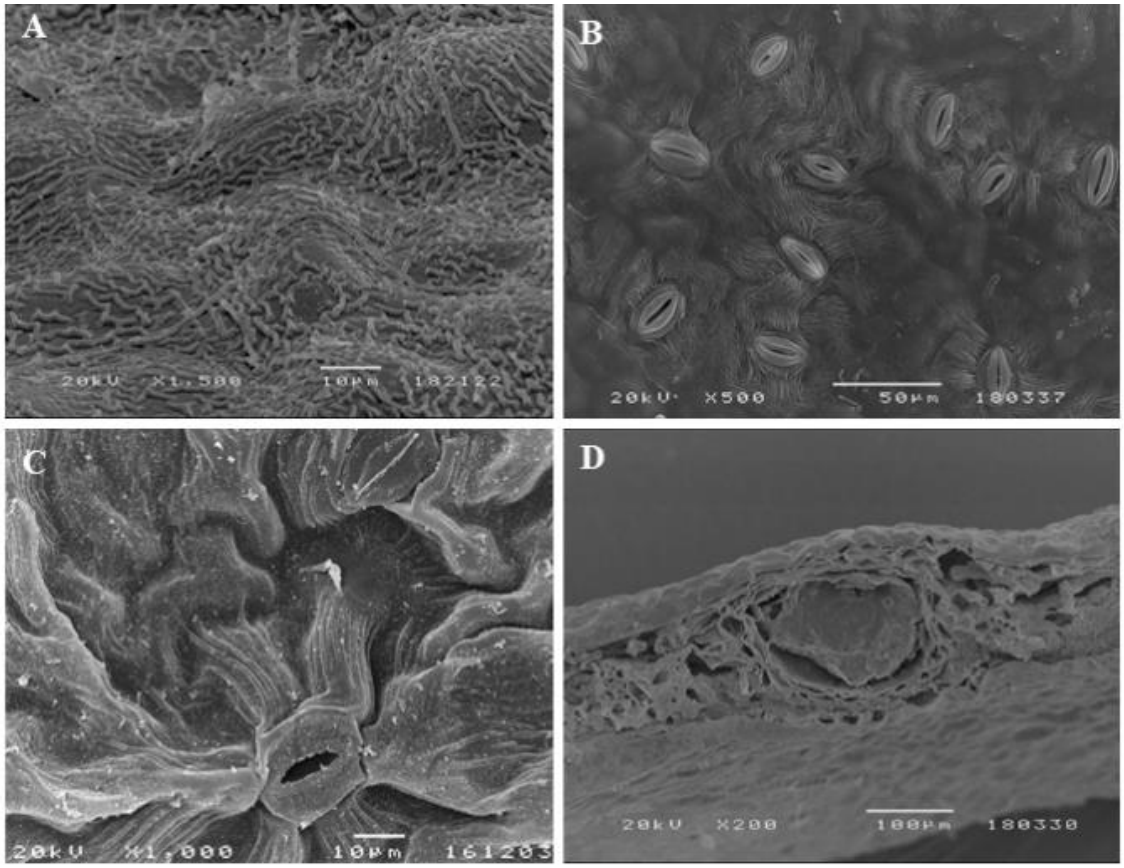


Figure 8 – SEM micrographs of *Zanthoxylum zanthoxyloides* (A, B and D) and *Zanthoxylum leprieurii* (C) leaves. A – Cuticular deposits on adaxial epidermal surface (scale bar 10 µm); B – Abaxial epidermal cells and stomata distribution (scale bar 50 µm); C – Leaf abaxial epidermal surface and stoma (scale bar 10 µm); D – Leaf cross-section with a cavity type internal secretory structure (scale bar 100 µm);

Z. leprieurii observation revealed the presence of idioblasts with druse type calcium oxalate crystals (Figure 9D). These crystals result from the transformation of oxalic acid, that is formed during the respiration process, once it becomes toxic when accumulated inside the vacuoles, becoming non-toxic as a salt. Same crystals where reported in *Z. armatum* (Ullah *et al.*, 2014). *Z. leprieurii* mesophyll is mostly spongy parenchyma, characterized for loosely arranged cells with very large intercellular spaces that resemble the characterization of aerenchyma tissue (Figure 9B). As for *Z. zanthoxyloides*, this tissue is more organized and with smaller spaces between cells, which may contribute to better tolerance of drought (Mensah, 2012) (Figure 9E). *Z. leprieurii* palisade parenchyma, for instance, is continuous and multilayered and with shorter

cells when compared to *Z. zanthoxyloides* unlayered palisade parenchyma with very elongated vertical cells (Figure 9B and 9E). Both species present collateral vascular bundles (Figure 10) where phloem almost surrounds all the xylem and vascular tissues are accompanied outside by a sclerenchymatous tissue. Igboabuchi & Ilodibia (2017) described the same vascular bundles for *Z. macrophylla* (L.) Sarg.. Also, while *Z. zanthoxyloides* follows the common rule of eudicots in *Z. leprieurii* leaves the main vein is protruding on both faces (Figure 10). Mensah (2012) studied *Z. zanthoxyloides* leaf anatomy, being one of the few anatomical studies addressed for the selected species in the present study and observed similar organization.

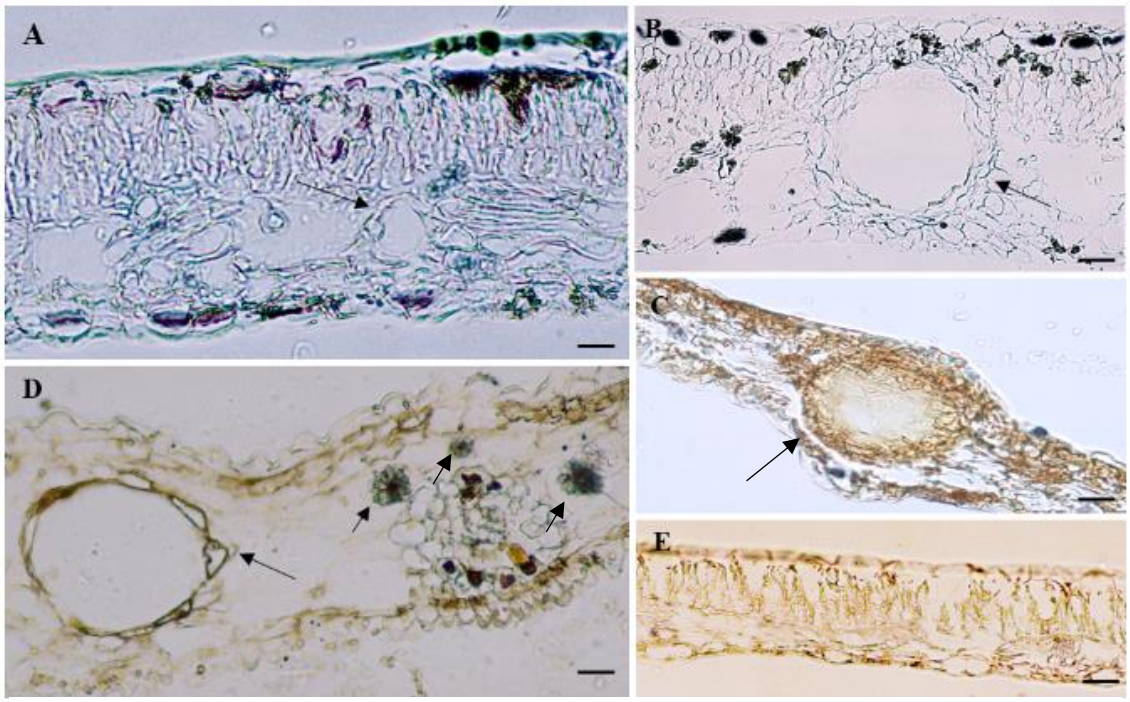


Figure 9 – Micrographs of leaf cross-section. A – *Zanthoxylum zanthoxyloides* secretory structure (arrow) (scale bar 27 µm); B – *Zanthoxylum leprieurii* parenchyma and secretory structure (arrow) (scale bar 40 µm); C – *Zanthoxylum leprieurii* large secretory structure detail (arrow) (scale bar 100 µm); D – *Zanthoxylum leprieurii* secretory structure (large arrow) and druse calcium oxalate crystal (small arrows) (scale bar 44 µm); E – *Zanthoxylum zanthoxyloides* mesophyll organization (scale bar 80 µm).

The anatomical characterization allowed to differentiate these two *Zanthoxylum* species once that for all measurements addressed, *Z. leprieurii* revealed to be thicker than *Z. zanthoxyloides* and according to the statistical analysis performed, they are significantly different (Table 7). There is a lack of anatomical studies and measurements in *Zanthoxylum* species and the present study is pioneer in addressing a complete analysis of these two species. Therefore, no comparative parameters are available except for total mesophyll thickness that was registered for *Z. acanthopodium* by Rakić *et al*. (2009), varying from 221-278 µm, which is not very dissimilar from the ones obtained in this work. The comparative morphoanatomical analysis on the present

study between two *Zanthoxylum* species provided elements for their characterization and differentiation.

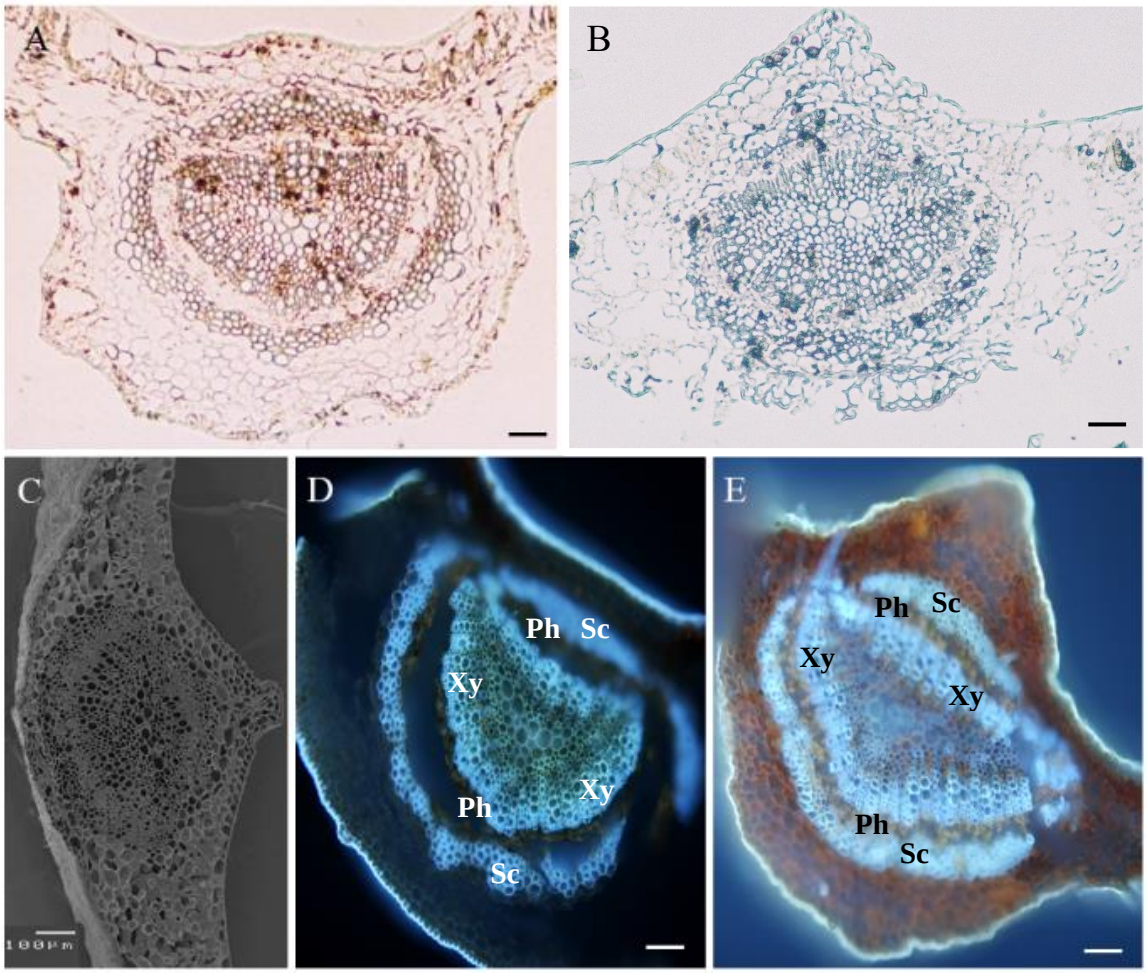


Figure 10 – Micrographs of leaf main vein cross-section. *Zanthoxylum zanthoxyloides* main vein cross-section (A, C and D) and *Zanthoxylum leprieurii* main vein cross-section (B and E). A and B images were obtained through light microscopy, C trough scanning electron microscope and D and E by fluorescence microscopy. Ph – Phloem; Sc – Sclerenchyma; Xy – Xylem (Scale bar: A and D - 180 µm; C - 100 µm; B and E - 120 µm)

Table 7 – Measurement results in the two *Zanthoxylum* species under study for total mesophyll thickness; adaxial cuticle thickness; adaxial epidermis thickness; palisade parenchyma thickness; spongy parenchyma thickness; abaxial epidermis thickness. *** – highly significative difference at $p < 0.001$ according to Tukey Test; ** – Significantly different at $p < 0.05$ according to Tukey Test.

Measurement (µm)	***Zanthoxylum zanthoxyloides***	***Zanthoxylum leprieurii***	
Total mesophyll	192.67 ± 19.44	285.33 ± 14.57	***
Adaxial cuticle	3.81 ± 1.34	7.28 ± 2.01	***
Adaxial epidermis	24.44 ± 3.23	29.29 ± 5.93	**
Palisade parenchyma	66.73 ± 10.48	97.76 ± 9.96	***
Spongy parenchyma	73.84 ± 8.61	108.68 ± 14.38	***
Abaxial epidermis	14.56 ± 1.92	17.33 ± 2.33	**

4.1.2. *Hymenocardia acida*

The study of epidermal surface allowed to observe certain characteristics of *H. acida*. Both adaxial and abaxial epidermal cells have polyhedral shape with straight cell walls, being the adaxial epidermal surface classified as glabrous (Figure 11A). The cell shape observed is according to what is expected for the Phyllanthaceae family (Raju & Rao, 1977). Average cell dimension was 39.32 ±3 .62 x 27.90 ± 6.75 µm for adaxial surface cells and 27.91 ± 5.51 x 18.68 ± 4.52 µm for abaxial surface cells.

Stomata are present in the abaxial surface, classified as paracytic, with an elliptical-round shape and variable dimensions, distributed randomly (Figure 11B, 11C, and 11D). This observation is in agreement with the family characteristics once that paracytic stomata is the most common for the Phyllanthaceae family (Raju & Rao, 1977). Stomata size for *H. acida* was 21.92 ± 2.42 x 14.99 ± 4.23 µm and the estimated stomata index is 13%.

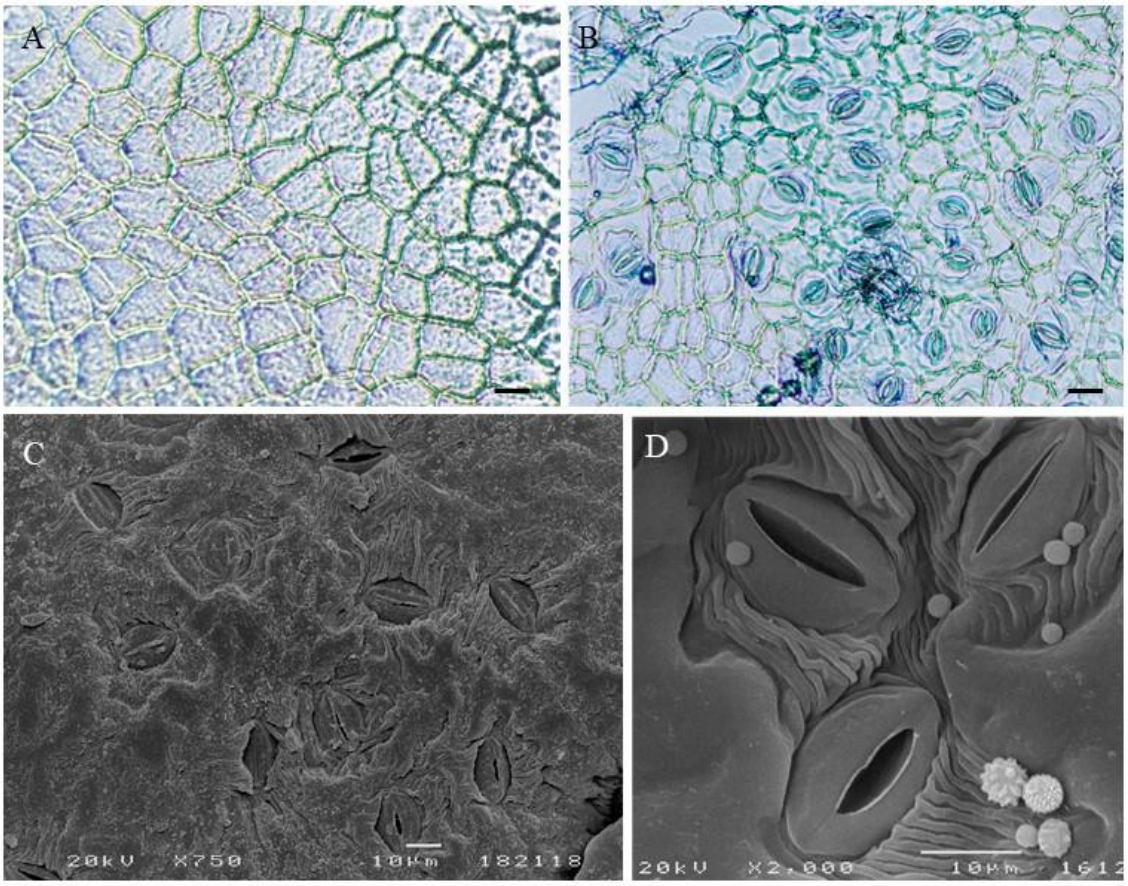


Figure 11 – Micrographs of *Hymenocardia acida* leaf. A – Leaf adaxial epidermal surface (scale bar 20 µm); B – Leaf abaxial epidermal surface with paracytic stomata (scale bar 20 µm); C – SEM micrograph of leaf abaxial epidermal surface with stomata (scale bar 10 µm); D – SEM micrograph of stomata detail (scale bar 10 µm).

H. acida is also characterized for secretory hairs, more specifically, yellow-brownish peltate trichomes on the abaxial surface. These trichomes diameter range between 115 µm for bigger ones and 53 µm for smaller, consisting of almost isodiametric cells with radial insertion and a pluricellular stalk (Figure 12 and 13A). The presence of peltate leaf trichomes was also described by Solereder (1908) and later by Levin & Simpson (1994).

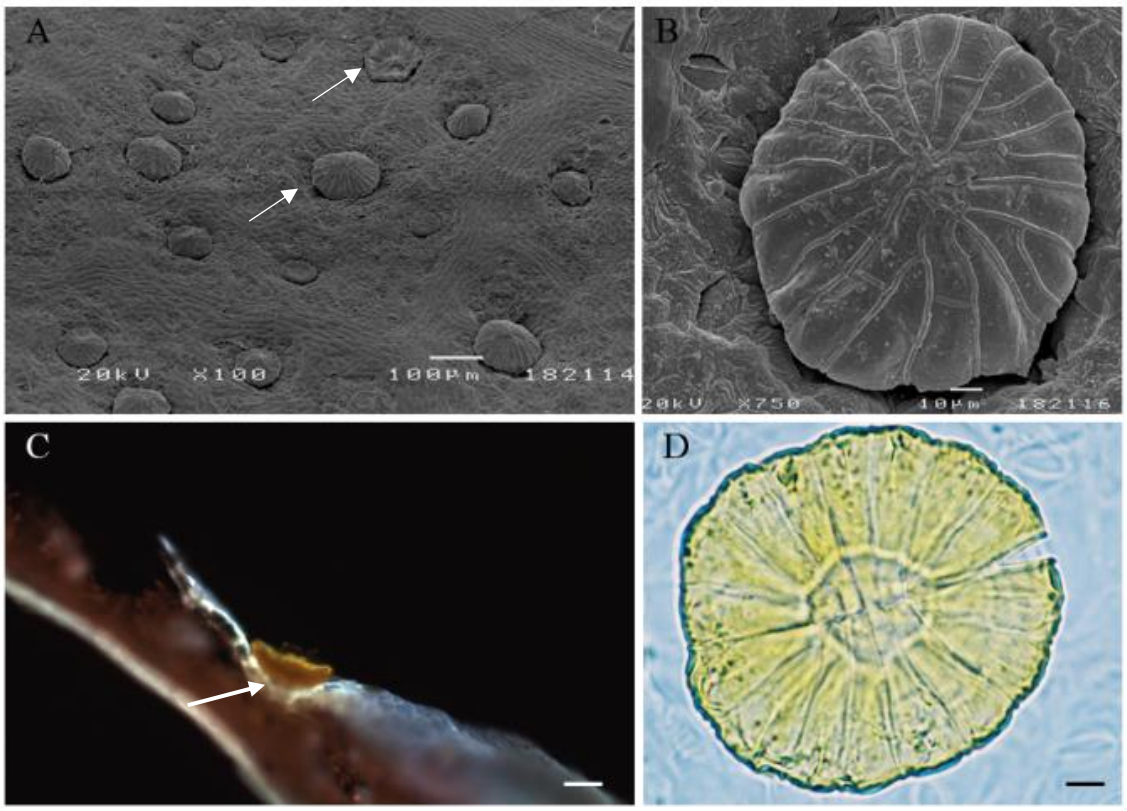


Figure 12 – Micrographs of abaxial surface of *Hymenocardia acida.* A – SEM micrograph of peltate trichomes distribution (arrow) (scale bar 100 µm); B – SEM micrograph of detailed peltate trichome (scale bar 10 µm); C – Side-view of peltate trichome in leaf transverse section (arrow) (scale bar 50 µm); D – Peltate trichome detail under LM (scale bar 11 µm).

For anatomical characterization, the measurements addressed on the leaf transverse sections are presented in Table 8. Palisade parenchyma is characterized by one layer with elongated cells and spongy parenchyma have a cell loose arrangement with somehow large intercellular spaces (Figure 13A). Among the mesophyll cells, polyhedral calcium oxalate crystals are found, most of them near the veins (Figure 13B). The leaf cross-section presents a typical dicotyledonous vascular arrangement in the main vein, with collateral vascular bundles, almost concentric, found in the petiole and leaf blade, surrounded by sclerenchymatous tissue (Figure 13C). Solereder (1908) described the same vascular bundles for Phyllantheae tribe, where *H. acida* was inserted at the time.

As it was verified on the last chapter for *Zanthoxylum* species, this is a pioneer study towards a more detailed understanding of morphoanatomical characteristics there are often left aside when compared with the studies related to chemical and medicinal properties of *H. acida.*

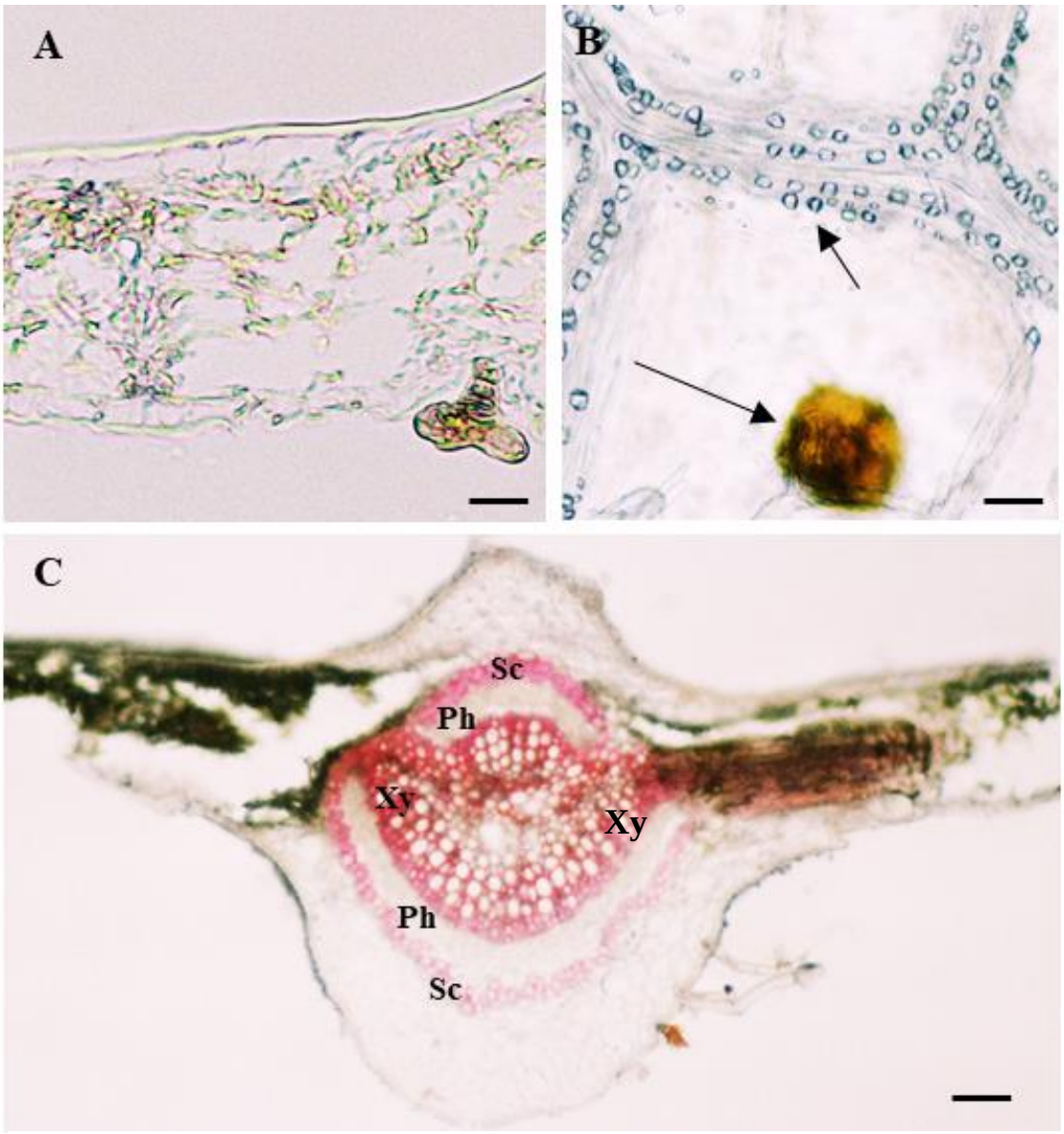


Figure 13 – Light microscopy micrographs of *Hymenocardia acida*. A – Mesophyll and peltate trichome in the abaxial surface with visible pluricellular stalk (scale bar 25 µm); B – Polyhedral calcium oxalate crystals near the veins (small arrow) and upper view of the trichome (large arrow) (scale bar 7 µm); C – Main vein cross-section with collateral vascular bundle surrounded by sclerenchymatous tissue (scale bar 50 µm). Ph – Phloem; Sc – Sclerenchyma; Xy – Xylem

Table 8 – Measurement results in Hymenocardia acida for total mesophyll thickness; adaxial cuticle thickness; adaxial epidermis thickness; palisade parenchyma thickness; spongy parenchyma thickness; abaxial epidermis thickness.

Measurement (µm)	**Hymenocardia acida**
Total Mesophyll	153.00 ± 44.26
Adaxial Cuticle	4.68 ± 1.60
Adaxial Epidermis	11.83 ± 2.46
Palisade Parenchyma	42.77 ± 12.67
Spongy Parenchyma	67.34 ± 11.18
Abaxial Epidermis	10.14 ± 2.78

4.1.3. Histochemical characterization

For histochemical tests addressed, Table 9 presents the comparative results for *Zanthoxylum* species and Table 10 display the results obtained for *H. acida.*

Table 9 – Histochemical tests performed on transverse cuts of leaf tissues of *Zanthoxylum* species*;* - negative; + slightly positive; ++ positive; +++ strongly positive.

Histochemical test (chemical group)	***Zanthoxylum leprieurii***	***Zanthoxylum zanthoxyloides***
Ferric chloride (phenolics)	+++	+++
Potassium dichromate (phenolics)	-	+
Phloroglucinol (lignin)	+++	+++
Vanillin/HCL (tannins)	-	+
Wagner reagent (alkaloids)	+	+
Tannic acid/ferric chloride (mucilages)	+	+++
Ruthenium Red/Copper acetate (pectins)	+	+++
PAS (polysaccharides)	-	++
Sudan III (total lipids)	+	+++
Nile Blue (neutral and acidic lipids)	+/++	++/+++
Copper acetate/Rubeanic acid (fatty acids)	-	-
Nadi reagent (essential oils and resins)	-	-
Anthimonium chloride (steroids)	-	+
2,4-Dinitrophenylhydrazine (terpenoids)	-	+

Table 10 – Histochemical tests performed on transverse cuts of leaf tissue from *Hymenocardia acida*; - negative; + slightly positive; ++ positive; +++ strongly positive.

Histochemical test (chemical group)	***Hymenocardia acida***
Ferric chloride (phenolics)	+++
Potassium dichromate (phenolics)	++
Phloroglucinol (lignin)	+++
Vanillin/HCL (tannins)	+++
Wagner reagent (alkaloids)	+
Tannic acid/ferric chloride (mucilages)	+++
Ruthenium Red/Copper acetate (pectins)	+++
PAS (polysaccharides)	+/+++
Sudan III (total lipids)	+++
Nile Blue (neutral and acidic lipids)	+++
Copper acetate/Rubeanic acid (fatty acids)	+++
Nadi reagent (essential oils and resins)	+
Anthimonium chloride (steroids)	+
2,4-Dinitrophenylhydrazine (terpenoids)	-

Histochemical tests are useful and allow to identify the tissue location of chemical groups and corroborate the importance of these three plants in ethnopharmacology by revealing the presence of compounds that are synthesized as metabolites due to plant necessities. However, these tests cannot be conclusive and might be erroneous as sometimes it is impossible to detect the presence of some chemical groups as they might be present in amounts below the detection limit of histochemical tests. On the other hand, some groups are chemically unstable and undergo changes and thus they may mask the presence of the unchanged chemicals.

The captured images of histochemical tests results, through light microscopy, are presented in Figure 14, for the three species in study. For some tests performed, the absence of figures illustrating is explained for each situation with the corresponding result.

The evaluation of *Zanthoxylum* genus plants in the present study showed that ferric chloride test to assess phenolic content revealed strongly positive results in both species (Figure 14A – i and ii) but potassium dichromate test only showed slightly positive in *Z. zanthoxyloides*. Lignin test was strongly positive for both species once xylem is fully developed (Figure 14C – i and ii). Tannins were not detected in *Z. leprieurii* and a very faint presence was detected in *Z. zanthoxyloides* (Figure 14D – i). Alkaloids revealed a slightly positive result for both species trough Wagner reagent test (Figure 14E – i). *Z. zanthoxyloides* showed a strongly positive result for the presence of mucilages and pectins (Figure 14F – i and Figure 14G – i) while in *Z. leprieurii* was detected a slight existence of those compounds (Figure 14F – ii). Polysaccharides revealed to be present in *Z. zanthoxyloides* (Figure 14H – i) while in *Z. leprieurii* the presence of this compound was not perceived by this test. The Sudan III test for total lipids was positive for both species but showed a stronger presence in *Z. zanthoxyloides* (Figure 14I – i). For Nile Blue test, neutral lipids stain pink and acidic lipids stain blue, *Z. zanthoxyloides* revealed strongly positive for acidic lipids in parenchyma and positive for neutral lipids in cuticle (Figure 14J – i) while in *Z. leprieurii* parenchyma stained positive for acidic lipids and secretory structure slightly positive for neutral lipids (Figure 14J – ii). No fatty acids, essential oils or resins were detected for both species through the carried-out tests. The presence of steroids and terpenoids was also not detected by the assessed tests in *Z. leprieurii* but slightly positive for *Z. zanthoxyloides* (Figure 14M – i and Figure 14N – i).

As noticed, these two species showed not so much a qualitative difference but mainly quantitative distinction for the assessed compounds. While *Z. leprieurii* only had strongly positive results for phenolic compounds, *Z. zanthoxyloides* also revealed strong presence of mucilages, pectins, and lipids. Histochemical tests for the presence of tannins, polysaccharides, steroids, and terpenoids did not detect their occurrence in *Z. leprieurii* while it was positive in *Z. zanthoxyloides*. Both species present negative results for the presence of fatty acids, essential oils, and resins. One study was found that aimed a histochemical characterization of *Z. armatum* leaves, in which the

following compounds where detected: lignin, lipids, mucilage, and tannin (Alam, 2015). These results are coincident to the ones obtained for the *Zanthoxylum* species in our study.

In *H. acida*, the ferric chloride and potassium dichromate tests to detect the presence of phenolics turned out positive (Figure 14A – iii and 14B – i). For the presence of lignin and tannins, the tests showed a strongly positive result (Figure 14C – iii and 14D – ii). The assess for alkaloids exhibited a faint presence of these compounds (Figure 14E – ii). A strongly positive result was displayed for the existence of mucilages, pectins, and polysaccharides, noting that the trichome only showed slightly positive for polysaccharides (Figure 14F – iii, 14G and 14H – ii). Sudan III test detected a strong presence of total lipids (Figure 14I – ii), Nile Blue tests confirmed a strong positive for acidic lipids once it stained blue (Figure 14J – iii) and also a strongly positive result was obtained for fatty acids (Figure 14K – i). Nadi Reagent test, that stain blue for essential oils and red for resins, showed that only the cells around the trichomes were tenuous stained with these colors, indicating a slightly positive for the presence of this compounds (Figure 14L – ii and iii). Lastly, only a subtle presence of steroids was detected (Figure 14M – ii) and no terpenoids were showed through the test.

In summary, all performed tests had positive results except for the presence of terpenoids, at least for the performed histochemical test. The trichome present in this species revealed to be slightly positive to alkaloid, polysaccharides, and steroids, positive for neutral lipids and strongly positive for fatty acids. The detection of lipids on top cells of the trichomes indicates the potential lipidic nature of the secretion. As far as we know this is the first attempt to record histochemical tests performed in *Hymenocardia* genus leaves, bringing important information about their histochemical profile.

The detected alkaloids are very important for plant protection against higher animal consumption. Once these species are used as medicinal plants and alkaloids can be toxic for Human when taken in high concentration, it is need more studies to know their side effects (Robinson, 1974). Tannins, with phenolic nature, also protect the plant against herbivores, abiotic stress and show antibacterial activity(Furlan *et al.*, 2011). The presence of mucilages is very important to protect the leaf against dehydration by reducing the transpiration and for being a polysaccharide complex that increases cell water retention capacity (Fahn, 1979; Gregory & Baas, 1989). Pectin is also a polysaccharide that besides the fact that determines the water retaining ability, have an important role in plant defense against pathogens and wounding (Voragen *et al.*, 2009). The presence of these compounds is in agreement to the plant necessities of defense against stresses caused by environmental conditions as well as defense against biotic threats.

A - Ferric chloride

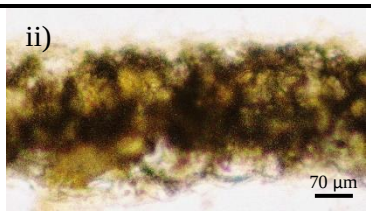
Positive: Brownish - Black

i)

Zanthoxylum zanthoxyloides

+++

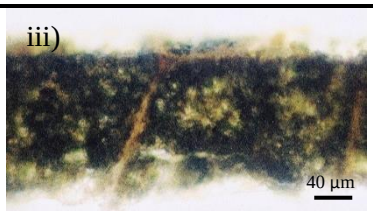
ii)



Zanthoxylum leprieurii

+++

iii)



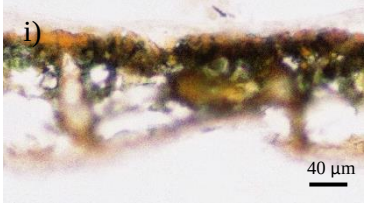
Hymenocardia acida

+++

B - Potassium dichromate

Positive: Orange - Brown

i)



Hymenocardia acida

++

C - Phloroglucinol

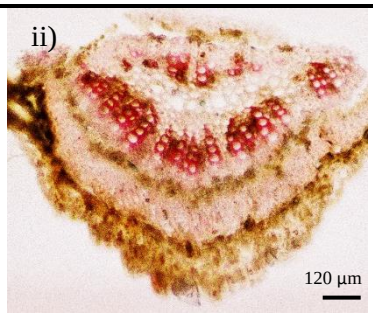
Positive: Red

i)

Zanthoxylum zanthoxyloides

+++

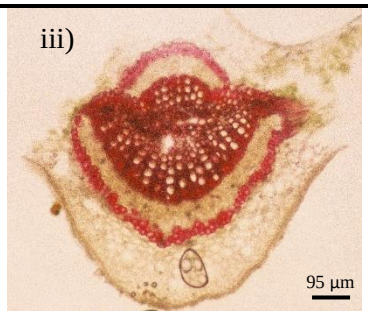
ii)



Zanthoxylum leprieurii

+++

iii)



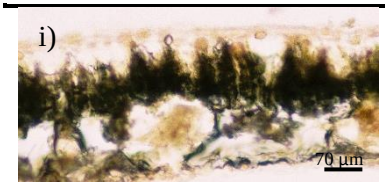
Hymenocardia acida

+++

D - Vanillin/HCL

Positive: Red

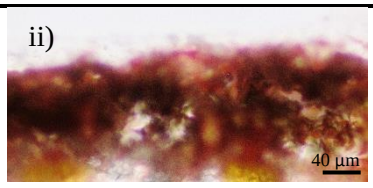
i)



Zanthoxylum zanthoxyloides

+

ii)



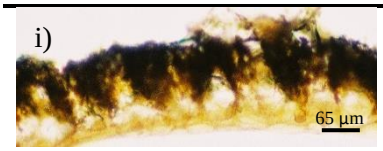
Hymenocardia acida

+++

E - Wagner reagent

Positive: Reddish Brown

i)



Zanthoxylum zanthoxyloides

+

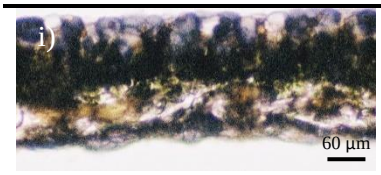
ii)

Hymenocardia acida

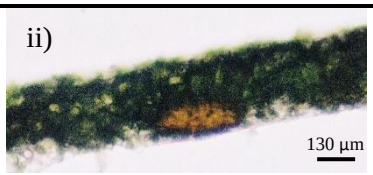
+

F - Tannic acid/ferric chloride

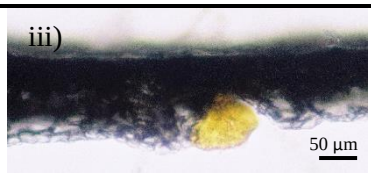
Positive: Black



Zanthoxylum zanthoxyloides
+++



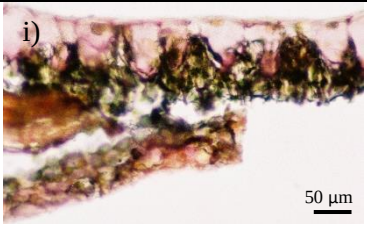
Zanthoxylum leprieurii
+



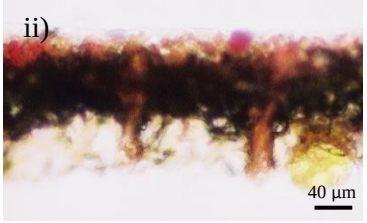
Hymenocardia acida
+++

G - Ruthenium Red/Copper acetate

Positive: Red



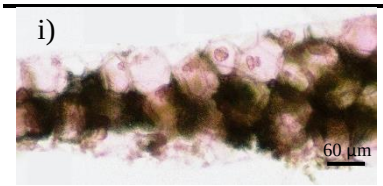
Zanthoxylum zanthoxyloides
+++



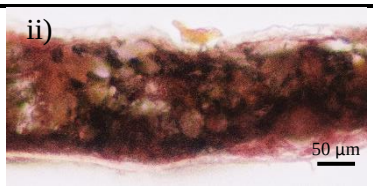
Hymenocardia acida
+++

H - PAS

Positive: Bright Pink



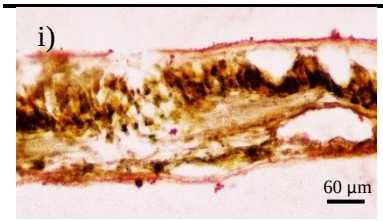
Zanthoxylum zanthoxyloides
++



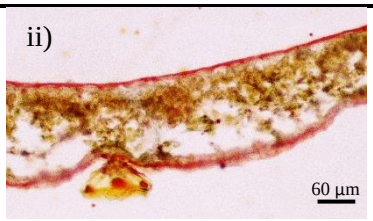
Hymenocardia acida
+++

I - Sudan III

Positive: Orange - Red



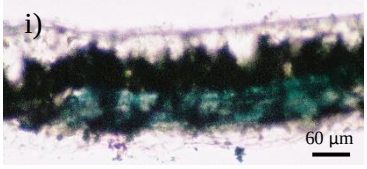
Zanthoxylum zanthoxyloides
+++



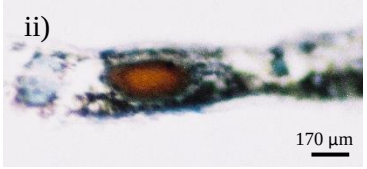
Hymenocardia acida
+++

J - Nile Blue

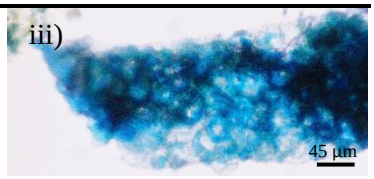
Positive: Blue and Pink



Zanthoxylum zanthoxyloides
+++ (parenchyma); ++ (cuticule)



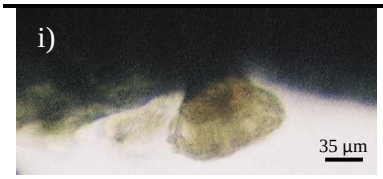
Zanthoxylum leprieurii
++ (epidermis); + (secretory structure)



Hymenocardia acida
+++

K - Copper acetate/Rubeanic acid

Positive: Dark Green

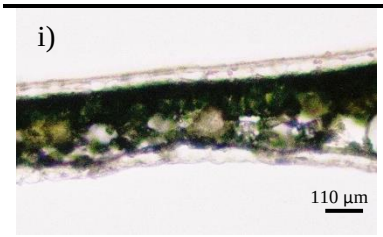


Hymenocardia acida

+++

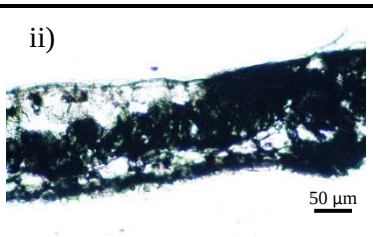
L - Nadi reagent

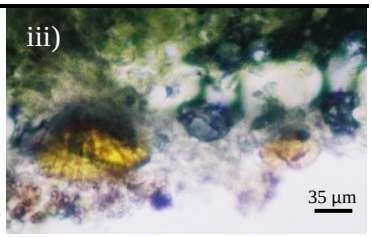
Positive: Blue and Red



Zanthoxylum leprieurii

-



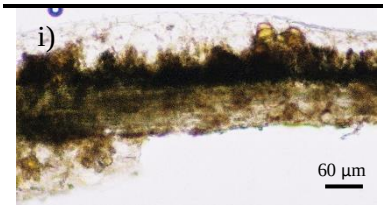


Hymenocardia acida

+

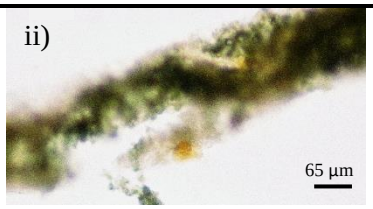
M - Anthimonium chloride

Positive: Orange - Red



Zanthoxylum zanthoxyloides

+

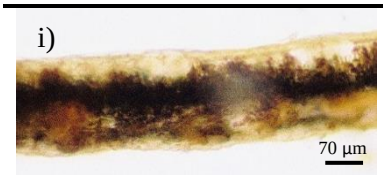


Hymenocardia acida

+

N - 2,4-Dinitrophenylhydrazine

Positive: Orange - Red



Zanthoxylum zanthoxyloides

+

Figure 14 – Light microscopy micrographs of histochemical tests results, performed for *Hymenocardia acida*, *Zanthoxylum leprieurii*, and *Zanthoxylum zanthoxyloides*. Positive identifies the expected color that indicates presence of the compound; - negative; + slightly positive; ++ positive; +++ strongly positive. Scale bar value on each image.

To complement the previous histochemical characterization, sections of the leaf were observed under UV light to detect autofluorescence in plant tissues, for the three species. This is a usual and convenient phenomenon that comes from the light absorbance (ultraviolet and visible light spectrum) by various endogenous biomolecules (chlorophyll, carotenes, lignin, etc.). The blue autofluorescence that was detected, indicates the presence of phenolic compounds in stomatal guard cell and cell walls, and the absence of these compounds on secretory structures (Figure 15).

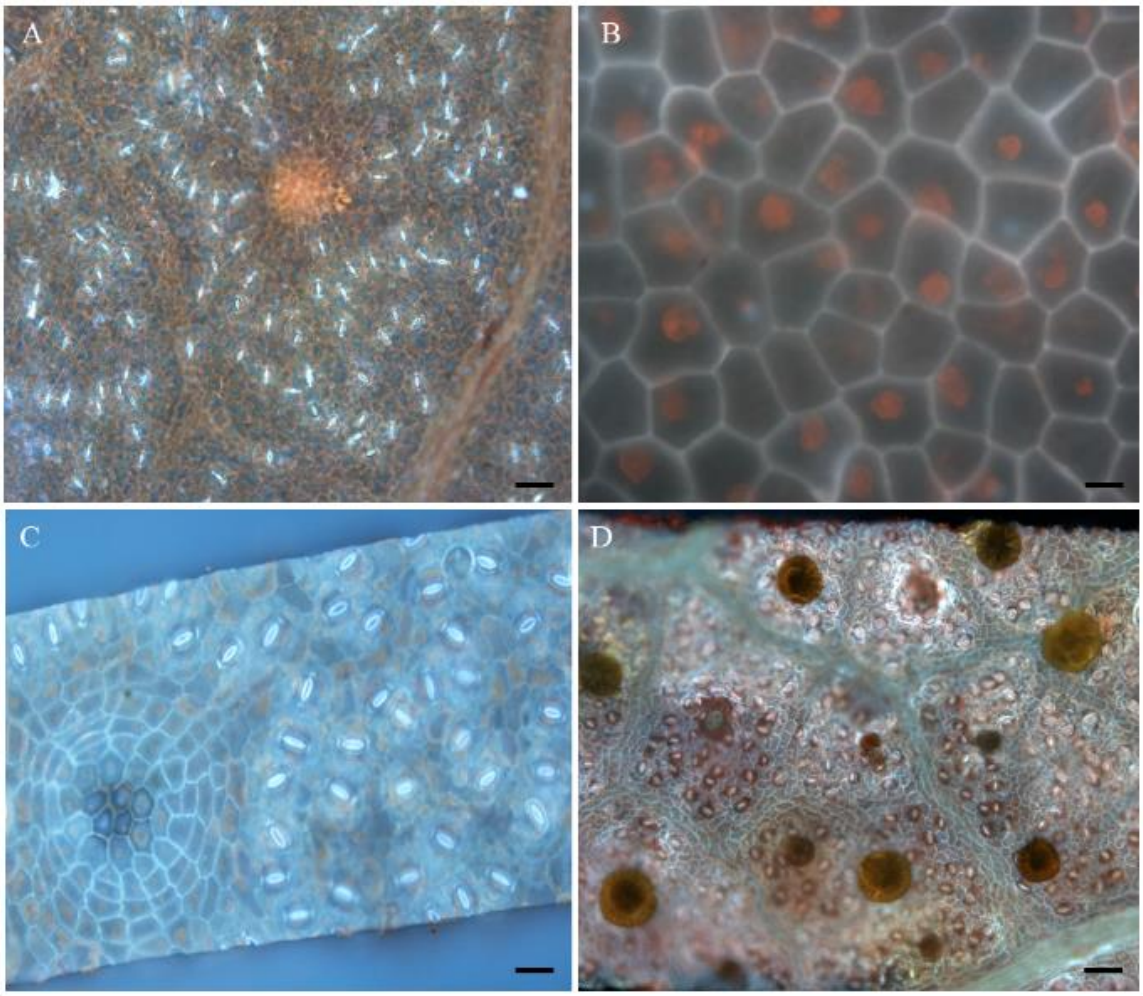


Figure 15 – Fluorescence microscopy micrographs of *Zanthoxylum leprieurii* (A), *Zanthoxylum zanthoxyloides* (B and C) and *Hymenocardia acida* (D). A – Abaxial surface with visible stomata and secretory structure (scale bar 93 µm); B – Adaxial surface cells and secretory structures (scale bar 17 µm); C –Abaxial surface with visible stomata and secretory structure (scale bar 53µm); D – Abaxial surface with visible stomata and peltate trichomes (scale bar 77 µm).

4.2. Antibacterial and antifungal *in vitro* activity

4.2.1. Extraction yield

The plant extracts were prepared with solvents of increasing polarity according to the methodology presented in subchapter 3.3.1. The extraction yields were calculated and registered in Table 11. These values are in the expected range for plant material extraction with this method (Jorge, 2014; Madureira *et al*., 2012).

Table 11 – Extraction yield of plant material with ascending polarity solvents.

		***H. acida* leaf**	***ZZ* leaf**	***ZZ* root**	***ZL* leaf**	***ZL* root**
Extraction yield (% w/w)	***n*-hex**	0.95	1.67	0.80	1.00	0.37
	CH_2Cl_2	1.40	1.83	1.27	1.23	0.52
	AcOEt	0.55	0.50	0.80	0.85	0.22
	MeOH	1.15	1.67	0.73	1.92	2.30
	H_2O	4.15	4.50	0.90	5.08	1.85

4.2.2. Minimum inhibitory concentration and minimum bactericidal concentration

The results obtained for the antimicrobial activity assessment of the plant extracts, namely the determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) are displayed in Table 12. Figure 16 and 17 illustrate two inoculated microplates with methicillin-resistant *Staphylococcus aureus.* In Figure 16 positive results were observed and Figure 17 no growth inhibition was observed for MIC <250 μg/mL. No antibacterial activity was found for *Bacillus subtilis* and Gram-negative bacteria: *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The presence of a highly hydrophobic outer membrane in Gram-negative bacteria can be the explanation for the absence of antibacterial activity once it works as permeability barrier (Stavri *et al.*, 2007).

From the three analyzed plant species, *H. acida* leaves extracts revealed to be the most active, being able to inhibit the growth of all other six Gram-positive bacteria. This plant apolar extracts (*n*-hex, CH_2Cl_2, and AcOEt) were more effective against *Enterococcus hirae,* MIC of 15 μg/mL, an enterococci species rarely found in human clinical samples, being more frequent in animal infections (Salem-Bekhit *et al*., 2012). The same extracts also showed positive results with MIC of 30 μg/mL for *Enterococcus faecalis,* a vancomycin-resistant strain which prevalence is dominant in hospital patients and responsible for the majority of infections caused by enterococci (Salem-Bekhit *et al*., 2012). All *H. acida* extracts revealed antimicrobial activity against *Staphylococcus epidermidis,* a microorganism that used to be only regarded as a harmless commensal bacteria on human epithelial microflora but now recognized as important

opportunistic pathogen with the ability of forming biofilms that enhance antibiotic and host mechanism defence resistance, such as methicillin and other acquired resistance (Otto, 2009).

S. aureus is one of the major pathogens worldwide, being one of the main agents in acquired infections not only in hospital environment but also in the community. They are related with a large range of clinic manifestations with different levels of severity, triggering lethal infections, by synthesizing a large variety of toxins and enzymes. *S. aureus* strains are especially dangerous due the increasing antibiotic resistance over the years, such as vancomycin-intermediate resistance (VISA) and methicillin resistance (MRSA) (Appelbaum, 2007). All *H. acida* extracts, with exception of the H_2O extracts, revealed to be active for both sensitive and resistant *S. aureus* strains. When comparing the obtained MIC for methicillin-resistant and methicillin-sensitive strains (MSSA), overall the extracts showed higher activity for MRSA (30 μg/mL) then MSSA (60 μg/mL). This may be explained by the extracts action on resistance mechanisms of the antibiotic-resistant pathogen, such as MDR efflux pumps (Simões *et al.*, 2009).

The results obtained for *H. acida* seem to be in agreement with previous studies that were carried out to assess antimicrobial properties of leaves extracts and isolated compounds. Muanza *et al.* (1994) described antibacterial activity from *H. acida* leaves *n*-hex, AcOEt, MeOH and H_2O extracts against *S. aureus.* Sofidiya *et al.* (2009) also referred activity of MeOH extracts against *S. aureus* and *S. epidermidis* and same conclusions were achieved by Kamba & Hassan (2011). The results of the current study support the traditional use of *H. acida* leaves in Guinea-Bissau to treat intestinal problems, wounds and skin inflammation since *S. aureus* might be in the origin of those pathologies. Therefore, this plant has potential to be a possible source for new compounds and drug development to overcome the antibiotic resistance problem in nowadays.

Z. zanthoxyloides and *Z. leprieurii* extracts didn't show any activity against *E. faecalis* but *Z. zanthoxyloides* leaves *n*-hex extract, *Z. leprieurii* leaves CH_2Cl_2 and AcOEt extracts and *Z. leprieurii* roots AcOEt extract exhibited inhibitory activity against *E. hirae* with an obtained MIC of 60 μg/mL. Likewise, *Z. leprieurii* CH_2Cl_2 and AcOEt leaves extracts revealed antimicrobial activity against *Staphylococcus epidermidis.* Furthermore, methicillin-resistant and methicillin-sensitive *S. aureus* growth was inhibited by *Z. zanthoxyloides* and *Z. leprieurii* apolar extracts with a MIC of 60 μg/mL and VISA strain was inhibited by *Z. leprieurii* CH_2Cl_2 leaves extract.

The assessed antimicrobial activity of *Z. zanthoxyloides* against methicillin-resistant *S. aureus* in the present study was previously stated by Mills-Robertson *et al.* (2017) and Tatsadjieu *et al.* (2003). Similarly, the antibacterial activity of *Z. leprieurii* extracts against methicillin and vancomycin-resistant strains was mentioned by Agyare *et al.* (2014) and Tatsadjieu *et al.* (2003). The results sustain the ethnopharmacology properties described for these *Zanthoxylum* species, once they are mostly used for intestinal problems and wound care.

Table 12 – Antimicrobial activity (MIC and MBC, µg/mL) of *Hymenocardia acida*, *Zanthoxylum zanthoxyloides*, and *Zanthoxylum leprieurii* extracts.

	S. aureus						***S. epidermidis***		***E. faecalis***		***E.hirae***	
	MSSA		MRSA		VISA				VRE			
Plant extracts	ATCC 6538		ATCC 43866		CIP 106760		ATCC 12228		ATCC 51299		CIP 5855	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
***H. acida* (leaves)**												
n-hex	**60**	60	**30**	125	**60**	nd	**60**	60	**30**	125	**15**	60
CH_2Cl_2	**60**	125	**30**	125	**60**	60	**30**	125	**30**	125	**15**	60
AcOEt	**30**	60	**30**	125	**60**	60	**30**	60	**30**	125	**15**	30
MeOH	**60**	60	**30**	125	**60**	250	**30**	-	500	-	500	-
H_2O	250	-	125	-	250	-	**60**	60	500	-	250	-
***Z. zanthoxyloides* (leaves)**												
n-hex	250	-	125	-	500	-	500	-	500	-	**60**	60
CH_2Cl_2	500	-	500	-	500	-	500	-	500	-	500	-
AcOEt	125	-	**60**	nd	250	-	250	-	500	-	125	-
MeOH	250	-	500	-	500	-	500	-	500	-	500	-
H_2O	250	-	500	-	500	-	500	-	500	-	500	-
***Z. zanthoxyloides* (roots)**												
n-hex	**60**	125	500	-	500	-	500	-	500	-	125	-
CH_2Cl_2	250	-	500	-	500	-	500	-	500	-	125	-
AcOEt	500	-	500	-	500	-	500	-	500	-	250	-
MeOH	500	-	500	-	500	-	125	-	500	-	250	-
H_2O	125	-	500	-	500	-	500	-	500	-	250	-
***Z. leprieurii* (leaves)**												
n-hex	125	-	**60**	60	125	-	500	-	500	-	250	-
CH_2Cl_2	125	-	**60**	nd	**60**	125	**15**	125	250	-	**60**	125
AcOEt	**60**	250	125	-	125	-	**60**	125	500	-	**60**	125
MeOH	500	-	250	-	500	-	500	-	500	-	125	-
H_2O	250	-	250	-	500	-	500	-	500	-	125	-
***Z. leprieurii* (roots)**												
n-hex	125	-	250	-	500	-	250	-	500	-	250	-
CH_2Cl_2	125	-	500	-	500	-	250	-	250	-	125	-
AcOEt	**60**	125	250	-	250	-	250	-	250	-	**60**	125
MeOH	125	-	250	-	500	-	250	-	500	-	250	-
H_2O	125	-	500	-	500	-	500	-	500	-	500	-

MSSA – methicillin-sensitive *Staphylococcus aureus*; MRSA – methicillin-resistant *Staphylococcus aureus*; VISA – Vancomycin-intermediate *Staphylococcus aureus*; VRE – Vancomycin-resistant Enterococcus; nd – non-defined; (-) not tested.

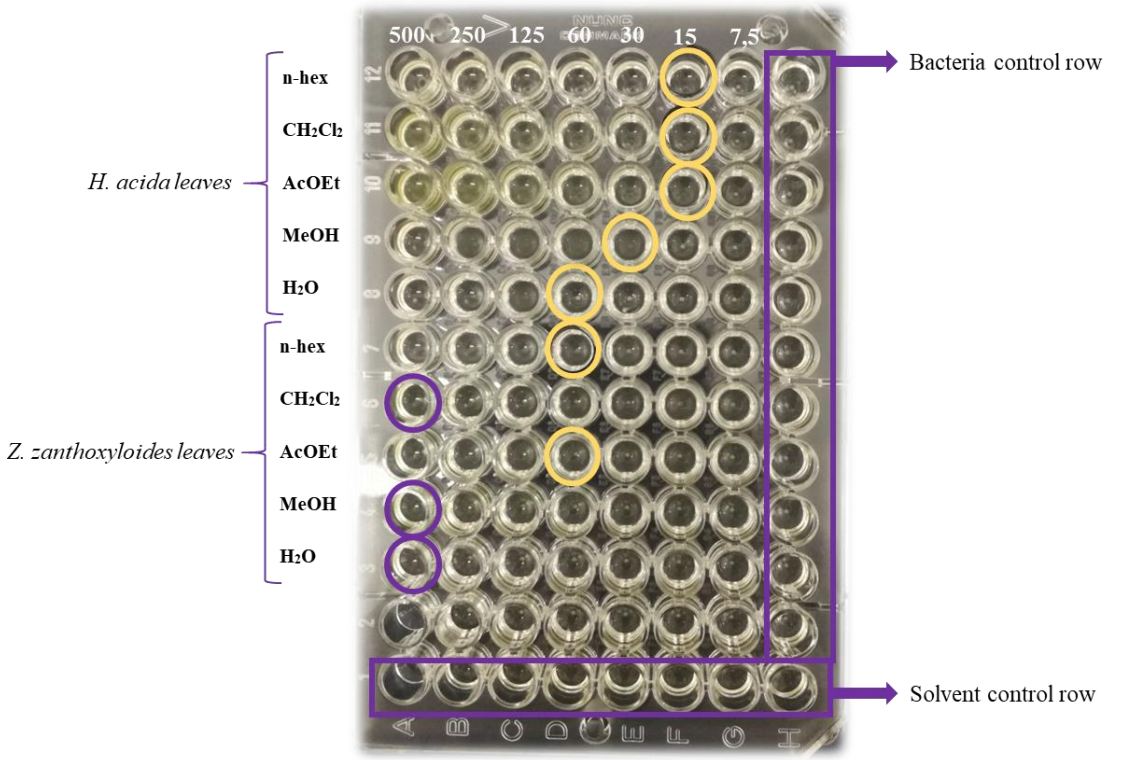


Figure 17 – Inoculated microplate model with methicillin-resistant *Staphylococcus aureus* where positive results (yellow circle) were observed.

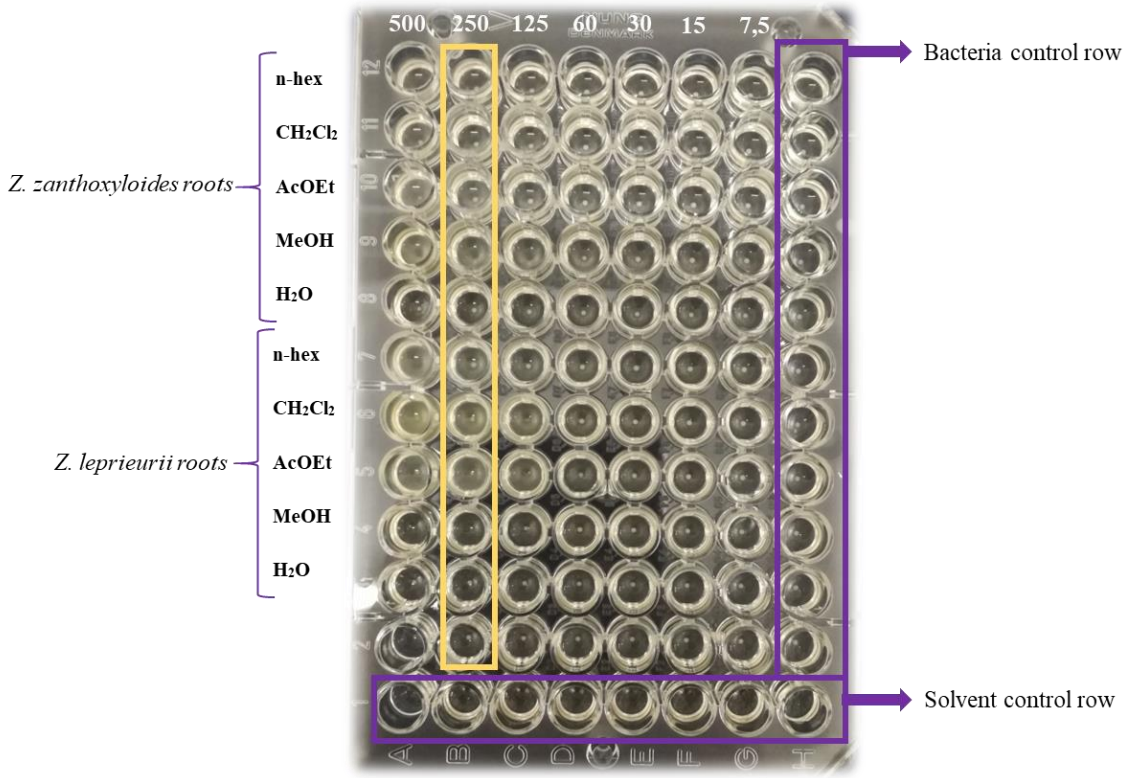


Figure 16 – Inoculated microplate model with methicillin-resistant *Staphylococcus aureus* where no growth inhibition was observed for MIC <250 µg/mL.

The results for antifungal activity assessment are displayed in Table 13. No antifungal activity was found for *H. acida* leaves extracts and *Z. zanthoxyloides* leaves extracts on the tested fungus. *Z. zanthoxyloides* extracts revealed to be the most active against *Candida tropicalis* sensitive strain and *Rhodotorula rubra.* The first considered as a predominant photogenic species from *Candida* genus, whose infections have been increasing dramatically (Kothavade *et al.*, 2010) and *R. rubra* used to be considered as non-pathogenic, have now arise as opportunistic pathogen capable of cause infections in vulnerable patients (Wirth & Goldani, 2012). *C. albicans* is an important human yeast pathogen that is usually related with genital infections and oral thrush (candidiasis) but in recent years is also the origin of dangerous and fatal infections (McCullough *et al.*, 1996). Only the MeOH root extract from *Z. leprieurii* has activity against *C. albicans* resistant strain. The results obtained are supported by a previous study carried out by Ngane *et al.* (2000), where no activity was found against *C. albicans* for *Z. zanthoxyloides* roots and *Z. leprieurii leaves* while Z. *leprieurii* roots revealed to inhibit the growth. *Z. leprieurii* leaves extracts (CH_2Cl_2 and AcOEt) were active against *Saccharomyces cerevisiae*. This microorganism found in normal flora of vagina, respiratory tract and gastrointestinal tract also shows pathogenic action by causing invasive fungal infections (Pfaller *et al.*, 2009).

For the following microorganisms, no antifungal activity was found: *Candida dubliniensis, C. glabrata, C. guilliermondii, C. kruzei, C. parapsilopsis,* and *Trichosporon cutaneum.*

Table 13 – Antifungal activity (MIC µg/mL) of Z*anthoxylum zanthoxyloides* and *Zanthoxylum leprieurii* extracts.

	MIC (µg/mL)				
	C. albicans	***C. tropicalis***	***C. neoformans***	***R. rubra***	***S. cerevisiae***
Plant extracts	ATCC 90028	ATCC 750	FFUL 948	FFUL 190	FFUL 1997
***Z. zanthoxyloides* (roots)**					
n-hex	>500	15	500	60	500
CH_2Cl_2	>500	15	125	60	500
AcOEt	>500	15	125	60	500
MeOH	>500	15	500	60	500
H_2O	>500	250	500	250	500
***Z. leprieurii* (leaves)**					
n-hex	>500	250	500	500	500
CH_2Cl_2	>500	125	500	125	60
AcOEt	>500	125	500	60	60
MeOH	>500	250	500	500	500
H_2O	>500	250	500	500	500
***Z. leprieurii* (roots)**					
n-hex	>500	125	500	250	500
CH_2Cl_2	>500	30	500	60	500
AcOEt	>500	500	500	60	500
MeOH	60	30	500	500	500
H_2O	>500	500	500	500	500
Fluconazole	125	15	60	125	250

4.2.3. Combination effects of extracts with antibiotics

To evaluate interactions between the extracts and two antibiotics, amoxicillin and oxacillin, a checkerboard assay was performed against vancomycin-resistant (VISA) (CIP 106760) and methicillin-resistant (MRSA) (ATCC 43866) *S. aureus* strains. The obtained results for active extracts by using fractional inhibitory concentration index (FICI) are displayed in Table 14 and 15. FICI values inferior to 0.5 show a synergic interaction between the antibiotic and plant extracts (Hemaiswarya *et al.*, 2008). This evaluation is pioneer once it's the first time that synergic potential is assessed for the three species in the present study.

Table 14 – Minimum inhibitory concentration (MIC) and fractional inhibitory concentration index (FICI) values of amoxicillin combination with extracts for vancomycin-resistant (CIP 106760) and methicillin-resistant (ATCC43866) *Staphylococcus aureus* strains.

S. aureus **strains**	**Plant extract**	**MIC (µg/mL)**		**FIC**	**FICI**	**Output**
		Alone	Combination			
CIP 106760	*H. acida* (leaves) ***n*-hex**	60	30	0,50	0,63	Indifference
	H. acida (leaves) **CH_2Cl_2**	60	30	0,50	0,56	Indifference
	Z. zanthoxyloides (roots) **MeOH**	500	60	0,12	**0,18**	**Synergism**
	Z. leprieurii (roots) **AcOEt**	250	125	0,50	0,75	Indifference
ATCC 43866	*Z. zanthoxyloides* (roots) **H_2O**	500	250	0,50	1,00	Indifference
	Z. leprieurii (leaves) **CH_2Cl_2**	60	30	0,50	0,56	Indifference
	Z. leprieurii (roots) **MeOH**	250	125	0,50	**0,50**	**Synergism**
	Z. leprieurii (roots) **H_2O**	500	250	0,50	1,00	Indifference

Table 15 – Minimum inhibitory concentration (MIC) and fractional inhibitory concentration index (FICI) values of oxacillin combination with extracts for vancomycin-resistant (CIP 106760) and methicillin-resistant (ATCC 43866) *Staphylococcus aureus* strains.

S. aureus **strains**	**Plant extract**	**MIC (µg/mL)**		**FIC**	**FICI**	**Output**
		Alone	Combination			
CIP 106760	*H. acida* (leaves) ***n*-hex**	60	30	0,50	0,98	Indifference
	H. acida (leaves) **CH_2Cl_2**	60	30	0,50	0,74	Indifference
	H. acida (leaves) **H_2O**	250	125	0,50	0,56	Indifference
	Z. zanthoxyloides (roots) **MeOH**	500	125	0,25	**0,25**	**Synergism**
	Z. leprieurii (leaves) ***n*-hex**	125	60	0,48	0,96	Indifference
	Z. leprieurii (leaves) **AcOEt**	125	30	0,24	**0,24**	**Synergism**
ATCC 43866	*Z. zanthoxyloides* (roots) **H_2O**	500	125	0,25	**0,25**	**Synergism**
	Z. leprieurii (leaves) **CH_2Cl_2**	60	30	0,50	0,98	Indifference
	Z. leprieurii (leaves) **AcOEt**	125	60	0,48	0,74	Indifference
	Z. leprieurii (roots) **MeOH**	250	60	0,24	**0,24**	**Synergism**

Z. zanthoxyloides roots MeOH extract, which alone did not show significant antibacterial activity, when combined with amoxicillin at a concentration of 60 μg/mL was able to revert synergistically the antibacterial activity of this antibiotic for VISA CIP 106760 strain (FICI=0.18). The same extract also restored the antibacterial activity of oxacillin at a concentration of 125 μg/mL (FICI=0.25). *Z. zanthoxyloides* roots H_2O extract combination with oxacillin decreased the MIC value from 500 μg/mL to 125 μg/mL against MRSA ATCC 43866 strain (FICI=0.25). For oxacillin, *Z. leprieurii* leaves AcOEt extract reduced the MIC value from 125 μg/mL to 30 μg/mL against VISA CIP 106760 strain (FICI=0.24). *Z. leprieurii* roots MeOH extract decreased MIC value against MRSA ATCC 43866 strain when combined with amoxicillin (FICI=0.50) and oxacillin (FICI=0.24).

As mentioned before, due to the crescent problematic of drug-resistant bacteria it is urging to discover new compounds and new pathways to control and revert antibiotic resistance, in order to regulate the increasing bacterial derived diseases worldwide. Therefore, the study of plant compounds and their synergic interaction is a fundamental step to overcome the community health problems resulting of bacterial infections of nowadays. The synergic approach of combination between antibiotic and extract that, when used alone, doesn't show activity but when co-administrated is able to restore the antibiotic activity is a promising strategy. The explanation of this successful interaction might be the inhibition of bacterial resistance mechanisms or enhancement of antibiotic pharmacokinetics (Wagner & Ulrich-Merzenich, 2009). Bacteria resistance to β-lactam antibiotics, such as amoxicillin and oxacillin, is due to the production of β-lactamase, an enzyme that inactivates the antibiotic or modifies the target. Therefore, the combination of these antibiotics with β-lactamase inhibitors is a successful strategy to restore their efficacy (Yang *et al.*, 2018). For this reason, there is necessity of further study of the extracts that showed synergism potential to identify and describe their compounds to a better understanding of the origin and mechanism behind this interaction.

4.2.4. Phytochemical screening

The obtained results for the phytochemical screening of the extracts are presented in Table 16. The leaves extract of both *Zanthoxylum* species didn't show alkaloids, being only present in small quantities on root extracts. Terpenes, flavonoids and phenolic compounds were found in the majority of *Z. zanthoxyloides* and *Z. leprieurii* leaves and roots extracts.

Alkaloids are one of the most important compounds in *Zanthoxylum* genus, present in all plant organs and most abundant in root bark (Agyare *et al*., 2014; Dieguez-Hurtado *et al*., 2003). The alkaloids present *Z. zanthoxyloides* root bark extracts where identified as having antitumoral, analgesic and antibacterial activity and the ones in *Z. leprieurii* were found active against lung

carcinoma cells and also showed antibacterial activity (Adesina, 2005; Bunalema *et al.*, 2017; Ngoumfo *et al*., 2010; Patiño *et al.*, 2012;).

Terpenes, the largest class of natural products, are extensively used in cosmetic and food industry but also reveal to be important for medical purposes, such as antimalarial and anticancer drug (Wang *et al.*, 2005). The presence of this chemical group in *Z. Zanthoxyloides* and *Z. leprieurii* was also described by Mills-Robertson *et al*. (2017), Misra *et al*. (2013) and Ngane *et al.* (2000).

Flavonoid is a phenolic secondary metabolite, very represented in *Zanthoxylum* genus, with an important role in antioxidant action. This chemical group is biological active with several interesting properties from medicinal point of view such as anti-inflammatory, antibacterial, antiviral, antiallergic and antitumor and was also found in *Z. zanthoxyloides* and *Z. leprieurii* by previous studies (Andersson *et al.*, 1996; Mills-Robertson *et al.*, 2017; Ngane *et al.*, 2000).

The phytochemical screening of *Hymenocardia acida* leaves revealed that terpenes and flavonoids were the major secondary metabolites present in the apolar extracts being alkaloids and phenolics compounds slightly detected. The presence of this chemical groups was previously mention by Kamba & Hassan (2011) and Sofidiya *et al.* (2009). As mentioned before, these compounds are known to have medical properties such as antibacterial activity and their presence can be an explanation for the previous obtained results for antimicrobial potential.

Besides the phytochemical screening detection of phenolic compounds, the total phenolic content for the extracts that exhibit antimicrobial properties in the present study was determined by the Folin-Ciocalteu method and the results are presented in Table 17. Phenols are a large group encompassing flavonoids, stilbenes, lignans, lignin, and tannins, acting as plant defence mechanism and also carrying important properties for pharmacology such as anti-inflammatory, anti-septic and most important, antioxidant activity (Johnson *et al*., 2008). Although some activity was assessed in plants with phenolic content, there is no certain evidence of their efficacy (Rauha *et al*., 2000). The highest value of total phenolic content was registered for *Z. zanthoxyloides* root *n*-hexane extract, an extract that did not present a significative antibacterial activity in the present study. For *Z. leprieurii* leaves extracts, that showed a considerable activity against the tested bacteria, the total phenolic content also revealed high values.

Lastly, for *H. acida,* with exception for methanol extract, all extracts presented high quantity of total phenolic compounds. The presence of phenolic compounds in *H. acida* was previously described by Sofidiya *et al.*, (2009). Plants with higher total phenolic content have the potential to exhibit greater antioxidant activity but it may not be directly connected to the antibacterial activity potential as showed in the present study. Also, from the chemical perspective of the phenolic compounds, their molecular structure can have an influence in the antioxidant and antibacterial activity (Johnson *et al*., 2008).

Table 16 – Phytochemical screening of *Hymenocardia acida*, *Zanthoxylum zanthoxyloides* and *Zanthoxylum leprieurii*; − absent; + slight intensity; ++ moderate intensity; and +++ strong intensity.

Species	Part	Extract	Alkaloids	Terpenes	Flavonoids	Phenolics
Hymenocardia acida	**leaves**	*n*-hex	-	+++	++	-
		CH_2Cl_2	-	+++	+++	+
		AcOEt	-	+++	+++	++
		MeOH	+	+	++	++
		H_2O	+	+	++	+
Zanthoxylum zanthoxyloides	**leaves**	*n*-hex	-	+++	++	-
		CH_2Cl_2	-	++	+++	-
		AcOEt	-	++	+++	-
		MeOH	-	-	+	++
		H_2O	-	-	+	++
	roots	*n*-hex	+	++	-	-
		CH_2Cl_2	+	++	-	+
		AcOEt	+	++	-	+
		MeOH	+	+	+	+++
		H_2O	+	-	+	+++
Zanthoxylum leprieurii	**leaves**	*n*-hex	-	+++	++	-
		CH_2Cl_2	-	++	++	-
		AcOEt	-	++	+++	++
		MeOH	-	-	+++	++
		H_2O	-	-	+	+
	roots	*n*-hex	+	++	+++	-
		CH_2Cl_2	+	++	+++	+
		AcOEt	++	++	+++	+
		MeOH	++	-	++	++
		H_2O	+	-	+	+

Table 17 – Total phenolic compounds (mg GAE/L) in *Hymenocardia acida*, *Zanthoxylum zanthoxyloides* and *Zanthoxylum leprieurii* extracts with antibacterial activity.

Species	Part	Extract	Total phenolic compounds mg GAEL
Hymenocardia acida	**leaves**	*n*-hex	179,18 ± 5,88
		CH_2Cl_2	90.56 ± 0.22
		AcOEt	98.63 ± 5.35
		MeOH	49.97 ± 4.56
		H_2O	87.85 ± 7.22
Zanthoxylum zanthoxyloides	**roots**	*n*-hex	235.71 ± 15.24
		MeOH	83.31 ± 9.36
		H_2O	90.02 ± 3.79
Zanthoxylum leprieurii	**leaves**	CH_2Cl_2	114.22 ± 14.85
		AcOEt	152.70 ± 12.84
	roots	AcOEt	161.03 ± 12.37
		MeOH	86.71 ± 2.41
		H_2O	65.72 ± 4.36

5. Conclusion

The present study investigates the leaves micromorphology, anatomy and histochemical traits in *Zanthoxylum zanthoxyloides*, *Zanthoxylum leprieurii*, and *Hymenocardia acida*. *Z. zanthoxyloides* and *Z. leprieurii* share common characteristics, however, some identified features allow their differentiation. It is possible to conclude that the performed assessments give important elements for these species characterization. Regarding *H. acida*, this study contributed with crucial data for characterization and identification at a micromorphological level, filling the information gap found related to this species. To our knowledge, the anatomical characterization measurements performed are pioneer for this species. The assessed histochemical tests reveal that the difference between *Zanthoxylum* species is mostly quantitative and not qualitative, with compounds like phenolics, lignin, alkaloids, mucilages, pectins and lipids being detected. *H. acida* tested positive for presence of several compounds and the detection of lipids in trichomes show a possible lipidic nature of the secretion. This identification of synthesized chemicals by plants provides a possible valorization of the described ethnobotanical properties.

The antibacterial and antifungal potential was evaluated against a selected panel of pathogens. No antibacterial activity was found for *Bacillus subtilis* and Gram-negative bacteria. From the plant species in study, *H. acida* leaves apolar extracts are the most active, being able to inhibit the growth of six Gram-positive bacteria, including resistant strains of *S. aureus*, an important pathogen in hospital environment and community, being one of the major infectious agents worldwide. *H. acida* leaf extracts also show activity against a vancomycin-resistant strain of *E. faecalis*, a prevalent pathogen in hospital patients. These results provide a scientific validation of the ethnobotanical properties described for *H. acida* leaves in Guinea-Bissau to treat intestinal problems, wounds and skin inflammation since *S. aureus* is regularly found as the cause of those kinds of pathology. Certain *Z. zanthoxyloides* and *Z. leprieurii* extracts revealed activity against Gram-positive bacteria, to point out the growth inhibition caused by leaf extracts in methicillin-resistant and vancomycin-intermediate strains of *S. aureus.* The obtained data can corroborate the traditional use of these species for intestinal problems and wound care. It is possible to conclude that the three assessed plants have potential antibacterial properties and the present study can be a foundation to further explore their properties as a source of new compounds and drug development. *H. acida* did not reveal any activity against fungal growth. *Z. zanthoxyloides* revealed to be active against *C. tropicalis* and *R. rubra* sensitive strains. Furthermore, *Z. leprieurii* root methanol extract was able to inhibit the growth of *C. albicans* resistant strain, remarked as an important yeast pathogen that can be the origin of severe infections.

To our knowledge, this work contributes with the first assessment of potential synergistic effects between the studied species and antibiotics in resistant strains. *Z. zanthoxyloides* root extract was able to revert synergistically the antibacterial activity of amoxicillin and oxacillin

against vancomycin-intermediate *S. aureus* strain and the combination with oxacillin also decreased MIC effectively for methicillin-resistant *S. aureus. Z. leprieurii* extracts combined with oxacillin had a synergic effect on both VISA and MRSA strains. The relevance of the obtained results is highlighted due to the necessity of controlling the increasing number of antibiotic-resistant bacteria, being a great step to overcome the health problem of nowadays.

A preliminary phytochemical profile screening was performed, not only to understand the main compounds present in the extracts but also as an attempt to unveil the origin of their activity in pathogens. Terpenes, flavonoids and phenolic compounds, known to have antibacterial properties, were found in the majority of *Z. zanthoxyloides* and *Z. leprieurii* leaves and roots extracts. The results obtained for total phenolic content when compared to the extracts that have evidenced antibacterial activity indicate that higher content may not be directly connected to the antibacterial potential.

Overall, the carried-out study and the obtained results emphasize the worthwhile of additional studies of these species to better understand their efficacy and safety in traditional medicine and, therefore, what compounds and mechanisms may be valuable to restore antibacterial activity.

6. References

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