Leaf anatomical characteristics of *Avicennia* L. and some selected taxa in Acanthaceae

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Abstract: The proposed placement of *Avicennia* L. into Acanthaceae had been made based only on molecular data. The main objective of this study is to investigate whether anatomical leaf characteristics of the *Avicennia* species support this placement. This study also aims to determine the leaf anatomical characteristics and their taxonomic value for some selected taxa ofAcanthaceae. Seven species were studied, namely *A. alba* Blume, *A. officinalis* L., *A. lanata* Ridley, *Justicia gendarussa* Burm. f., *J. betonica* L., *Strobilanthes crispa* Blume and *Asystasia gangetica* subsp. *micrantha* (L.) T. Anderson. Methods used were sectioning using sliding microtome, leaf clearing, epidermal peels, and observation under a light microscope. Findings have shown great differences between *Avicennia*species andother studied taxa. There are eleven outstanding leaf anatomical variations which may be used to distinguish all *Avicennia* species from the others, such as the presence and absence of brachysclereids, hypodermal layers, cystoliths and the presence of denture on the abaxial side of leaf margin, type of trichomes and stomata, areolar venation, marginal outline, shape of vascular bundles in the midrib, and the pattern of anticlinal walls on the adaxial and abaxial epidermis cells. The presence of mucilaginous idioblasts cells is common in all species studied. This study has shown that the genus *Avicennia* does not share similar leaf anatomical characteristics with the other taxa in Acanthaceae. As a conclusion, the placement of the genus *Avicennia* into the Acanthaceae has not been supported by leaf anatomical characteristics; therefore, it is suggested that this placement be revised.

Keywords: Acanthaceae, *Avicennia*, Leaf anatomy, Leaf micromorphology

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INTRODUCTION

The genus *Avicennia* was once placed under the family Verbenaceae, before being classified into its own family, Avicenniaceae. A number of studies conducted by Mc Dade et al. (2005, 2008) have suggested that *Avicennia* has a sister relationship within the Acanthaceae, thus placing this genus under the family Acanthaceae and no longer as its own family, Avinenniaceae. However, according to Borg (2008), the placement of *Avicennia* in the Acanthaceae is questionable, as there is not enough evidence to support the relationship of *Avicennia* with other acanthaceous lineages. In addition, clear morphological support has not been provided to augment their study (Borg and Schonenberger, 2011).

Molecular studies are indeed being regarded as a new tool in plant taxonomy study. McDade and Moody (1999) emphasized the needs to do systematic studies by using molecular data to trace the phylogeny relationship and to determine the lineages among the members of the family. However, many taxonomists had agreed that morphology data and few characters system, such as pollen characters, can help in delimitating the relationship between taxa. Study of anatomy, pollen structures, flower morphology and micromorphology are needed, along with other molecular data. Therefore, this study has been conducted specifically to determine whether leaf anatomical characteristics door do not support this new replacement for *Avicennia* based on molecular database.

MATERIALS AND METHODS

Seven taxa were selected from different genera in Acanthaceae. Sampling of leaves and plant specimens was done at various localities in Peninsular Malaysia (Table 1). Leaf specimens were compressed and dried in the oven at 55ºC. Then, the voucher specimens were deposited in the Universiti Kebangsaan Malaysia Herbarium (UKMB) for future reference. The leaf parts taken for analysis were mature leaves and were fixed in AA solution (Acid acetic: Alcohol in a ratio of 1:3).Parts of petioles, midribs, leaf lamina and marginal were sectioned in a range of thickness (15–40 μm) using a sliding microtome and stained in Safranin and Alcian blue. Slides were mounted in Euparal after dehydration. Images were captured using a video camera (JVC) attached to a Leica Diaplan Microscope using Analysis Docu Software. Permanent slides were prepared, observed under a light microscope with an attached digital camera, and processed using Cell^B software. Descriptions of leaf anatomical characteristics were documented per Metcalfe and Chalk (1979) and Noraini (2006).

RESULTS AND DISCUSSION

Findings in this study have shown that it was difficult to find common leaf anatomical characteristics present in all taxa studied. There was only one common character shared by all taxa studied, which was the presence of mucilaginous idioblast cells (Figure 1) either in the petioles, midribs or lamina. The substance within the secretory cells and distribution of the cells are typically unique for a particular species or taxa (Jabeen et al., 1993). However, cell inclusions are different between species or taxa. For example, most of the *Avicennia* species studied exhibited the presence of tanniferous idioblasts cells, which is absent in other taxa of the Acanthaceae.

Variation in leaf anatomical characteristics is much greater. The anatomical variations are mainly between species belonging to *Avicennia*and the other taxa studied (Table 1). These characteristics are as follows:1) presence of brachysclereids 2) types of trichomes; 3) shape of vascular bundles in the midribs and petioles; 4) presence of hypodermal layers; 5) present of cystoliths; 6) types of margin outline; 7) presence of dents on abaxial surface at margin;8) areolar system in lamina venation; 9) presence of swollen tracheids; 10) shape of adaxial and abaxial anticlinal epidermal walls; and 11) types of stomata. For convenience purposes, the following discussion emphasizes more on the differences between *Avicennia* and other Acanthaceae taxa.

In this study, all *Avicennia* species studied have shown the presence of sclerenchyma cells, either completely or incompletely ensheathing the vascular tissue in the midribs and petioles. *Strobilanthes crispa* also showed similar findings, whereas *Asystasia gangetica* subsp. *micrantha* only showed the presence of sclerenchyma cells in the midribs. The similarity between sclerenchyma and collenchyma cells is to provide support for the plant's structure. However, sclerenchyma differs from collenchyma in term of elasticity (Coyle 2005). Based on Xiao et al. (2009), during waterlogged periods on *Avicennia marina*, no sclerenchyma was present at that time. The absence of sclerenchyma results in the reduction of support and protection in the leaves.

Three types of vascular bundle arrangement were found in the petioles. Type one only occurs in *Avicenna* species (opened system, the main vascular bundle is a stack or interrupted of U-shaped bundles, ending recurved inwards, two additional vascular bundles are situated at the above left and right side of main vascular bundle). Meanwhile, type two is classified for *Justicia* species and *Strobilanthes crispa* (opened system, main vascular bundle is continuous stack of U-shaped bundles, two additional vascular bundles are situated at the above left and right side of main vascular bundle near each wing) and type three was found in *Asystasia gangetica* subsp. *micrantha* (opened system, main vascular bundle is interrupted stack of U-shaped bundle, two additional vascular

bundles are situated at the above left and right side of main vascular bundle near each wing).

As for the midrib cross section, only *Strobilanthes crispa* and *Asystasia gangetica* subsp. *micranthahave* shown similarity in vascular tissues arrangement (opened system, U-shaped continuously vascular tissue, consists of one stack of vascular bundle). *Avicennia* species exhibited similar vascular bundles arrangement (opened system, O-shaped interrupted vascular tissue, consists of three to five separated vascular bundles) and slightly different compared with vascular bundles arrangement of *Justicia* species (opened system, V-shaped interrupted vascular tissue, consists of several (5-9) separated vascular bundles).The systematic significant of vascular bundles arrangement is relevant as it is proven in many studies, such as in the family Rhizophoraceae (Nurnida 2012) and in *Parashorea* (Dipterocarpaceae) (Noraini and Cutler 2009). Findings in these previous studies have shown that the vascular bundles arrangement can be used for identification at the genus and family level. In this study, the presence of O-shaped sclerenchyma cells in the midrib can be used to differentiate *Avicennia*f rom the other taxa studied.

The types of trichomes found in this study can be very useful to identify *Avicennia* species. Trichomes exist in a variety of shapes and forms that function as an aid to the plants' defending mechanism. The existence of trichomes is consistent in particular plants that enable researchers to use this characteristic in plants taxa delimitation (Weryszko-Chmielewska and Chernetskyy 2006). Metcalfe and Chalk (1979) stated that both glandular and non-glandular trichomes are present in species belonging to Acanthaceae. In this study, capitate glandular trichomes (terminal flattened) were present and were densely scattered on the abaxial side of leaf lamina in three Avicennia species studied (*A. alba, A. officinalis* and *A. lanata*) (Figure 3 D). This type of trichome was found to be absent in other species studied. Peltate glandular trichomes and simple multicellular trichomes are present in all taxa studied except in *Justicia betonica*. A study by Verdam et al. (2012) on *J. acuminatissima*, reported that trichomes are present in both sides of epidermal layers. However, in this study, only *J. gendarussa* showed the existence of simple multicellular trichomes on adaxial epidermis surface, while *J. betonica* does not possess any trichomes. In *Strobilanthes crispa*, trichomes (Figure 3 C) were present on both adaxial and abaxial epidermis but only on adaxial side in *Asystasia gangetica* subsp. *micrantha*. Verdam et al. (2012) suggested that the trichomes type can be used for identification of *Justicia* species even in powder form.

The pattern of anticlinal walls for both adaxial and abaxial epidermal cells can also be used to differentiate species belonging to *Avicennia* from other Acanthaceae taxa studied. All *Avicennia* species exhibited straight to curved anticlinal walls, *Justicia* species exhibited wavy to sinuous anticlinal walls, and *Strobilanthes crispa* and *Asystasia*

gangetica subsp. *micrantha* showed sinuous anticlinal walls. According to Garland (1984), systematic studies usually neglected the aspect of cuticular variation and focused only on other features. His study on old world plants showed that the leaves commonly exhibit low diversity and thus, attracted the least attention. However, modern flowering plants usually exhibit variation in their anticlinal walls and thus have some systematic values.

The hypodermal cells were present in all *Avicennia* species studied with a greater number of hypodermal layers (up to six layers). Layer of hypodermal cells was observed underneath adaxial epidermis in the leaf lamina and margin, and underneath both adaxial and abaxial epidermis in the midribs. Other species which also were shown to possess of one layer of hypodermal cells were *J. gendarussa* and *Asystasia gangetica*. *Avicennia alba* exhibited the thickest layer compromising of 5-7 layers, while *A. officinalis* and *A. lanata* showed 2-4 and 3-5 layers (Figure 3 A), respectively. A study by Sheue (2003) on *Rhizophora* also gave similar results in term of numbers of layers of hypodermal cells (4-5 layers).

Brachysclereids was observed to be present at the marginal part in all *Avicennia* species studied and absent in others (Figure 3 A, B). Sclereids were present in many forms (macrosclereids, astrosclereids, brachysclereids) and were well-distributed in the leaves and also in the flower parts (Rao, 1957). The forms of sclereids depend on its structures and shapes. Sclereids' main function has yet to be determined, although it was reported to provide mechanical protection by giving additional strength and rigidity to the plants (Rao, 1957). Based on Fahn (1982), brachysclereids were developed in plants as responses towards physiological disturbances. Rao (1957) stated that brachysclereids were commonly found in the bark of the plant, which adds incompressibility to the plant. As such, these findings may be useful in taxa delimitation.

Venation in the leaf lamina also gives a significant difference between *Avicennia* and other taxa studied. One of the characteristics related to venation in the leaf lamina is the presence of swelling and non-swollen tracheids at the ending veinlets. Findings have shown that all of *Avicennia* species studied have swollen tracheids, while being absent in others (Figure 3 E, F). These *Avicennia* species also possessed short terminal tracheids compared to others. According to Metcalfe and Chalk (1979), certain anatomical characteristics in the veins can be used as an indicator in identifying a particular species and genus. Leaf lamina venation has been used in many plant families, such as in Theaceae, Euphorbiaceae and Lauraceae, for their taxonomic characteristics (Hickey, 1973). The short terminal tracheids of *Avicennia* species in this study are similar with the findings by Das (2001),which stated that short terminal tracheids are one of the key characteristics in *Avicennia* species.

There are three types of stomata observed in this study, namely as cyclocytic, diacytic and anisocytic stomata. Cyclocytic stomata present

in species belonging to *Avicennia*, (Figure 3 G) and the other two types of stomata present in the rest of Acanthaceae taxas tudied. *Justicia gendarussa*was shown to have heterostomatic character by having two types of stomata which are diacytic (Figure 3 H) and anisocytic stomata, while the others are homostomatic with only one type of stomata. *Asystasia gangetica* subsp. *micrantha*, *Justicia gendarussa*and *J. betonica* showed to have stomata on both adaxial and abaxial epidermis. This character is known as amphistomatic character; nevertheless, the stomata on adaxial epidermis normally are very sparsely scattered. The other four species are having stomata only on the abaxial epidermis and this character is known as hypostomatic. Out of 25 main types of stomata recognized by Metcalfe and Chalk (1979), most taxa shows to have diacytic and paracytic stomata (Kannabiran 1977; Verdam et al. 2012). Thus findings in this study do not support the statement by Metcalfe and Chalk (1979) or other previous study on *Avicennia*.

Findings in this study it has been proven that leaf anatomical characteristics can be used for identification at the genus and species levels. This was proven as a dichotomy key was constructed based on the diagnostic and variations in leaf anatomical characteristics found in all taxa species studied.

Key to the taxa studied based on anatomical characters

1. O-shaped vascular bundles in the midrib; brachysclereids present in the petiole and lamina; tracheids swollen; both anticlinal walls on the abaxial and adaxial surfaces straight to curved; cyclocytic stomata; cystoliths absent; marginal outline Type 1….....2
1. U or V-shaped vascular bundles in the midrib; brachysclereids absent in the petiole; tracheids not swollen; both anticlinal walls on the abaxial and adaxial surfaces wavy to sinuous or sinuous; diacytic stomata; cystoliths present; marginal outline either Type 2, 3 or 4 4

2. Midrib adaxial surface convex; abaxial surface U shaped...……...*Avicennialanata*
2. Midrib adaxial surface flat; abaxial surface arch-shaped....……3

3. Four hypodermal layers present underneath adaxial epidermis…… ……...*A. alba*
3. Three hypodermal layers present underneath adaxial epidermis…… ……*A. officinalis*

4. V-shaped vascular bundles in the midrib; marginal outline Type 1...…….....…… 5
4. U-shaped vascular bundles in the midrib; marginal outline either Type 2 or 4……6

5. Trichomes absent; areolar venation closed system; no free ending vienlets……………………………………………..*Justiciabetonica*
5. Trichomes present; areolar venation majority opened, minority closed system; majority closed endings and minority are free ending vienlets…………………………………..……*J. gendarussa*

6. Areolar venation closed system with no free ending veinlets; complete marginal venation; hypostomatic stomata; margin outline obtuse or rounded (Type 4) ..……….....................................*Strobilanthes crispa*
6. Areolar venation majority closed system, ending veinlets majority closed system; incomplete marginal venation; amphistomatic stomata; marginal outline truncate or rounded (Type 2).…..……..……..……..… …..…..…..…..…..…..…..…..……*Asystasia gangetica* subsp. *micrantha*

In conclusion, findings of this study have shown that leaf anatomical characters vary between species of *Avicennia* and the other Acanthaceae taxa studied. Therefore based on these findings, the leaf anatomical characteristics clearly do not support the placement of *Avicennia* into Acanthaceae. However, since this study has only emphasized leaf anatomy, it is quite hard to conclusively reject the placement of *Avicennia* in the Acanthaceae. A comprehensive study involving various organs of each species must be done. Studies on molecular systematics should also be carried out involving various species and genera from this region to truly support and correlate with the anatomical studies.

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Table 1. List of species studied.

Specimen	Voucher specimen	Collectors	Localities
Avicennia alba	CNA 51	Nurul-Aini, Ruzi, Ummu-Hani & Arrabe	Larut Matang Mangrove Forest, Taiping, Perak
Avicennia alba	CNA 90	Muhamad Razali Salam	Station Setiu Wetlands, Setiu, Terengganu
Avicennia officinalis	CNA 48	Nurul-Aini, Ruzi, Ummu-Hani & Arrabe	Larut Matang Mangrove Forest, Taiping, Perak
Avicennia officinalis	CNA 47	Nurul-Aini, Ruzi, Ummu-Hani & Arrabe	Larut Matang Mangrove Forest, Taiping, Perak
Avicennia lanata	CNA 91	Muhamad Razali Salam	Station Setiu Wetland, Setiu, Terengganu
Justicia gendarussa	CNA 5	Nurul-Aini & Ruzi,	UKM Herbs Garden. Bangi, Selangor
Justicia gendarussa	CNA 94	Nurul-Aini, Ruzi & Ummu-Hani	Lata Belatan Recreational Forest, Besut, Terengganu
Justicia betonica	CNA 6	Nurul-Aini & Ruzi,	UKM Herbs Garden. Bangi, Selangor
Justicia betonica	R2	Radhiah	UKM Herbs Garden. Bangi, Selangor
Strobilanthes crispus	CNA 36	Nurul-Aini, Suni, Nik-Norafida & N.A.	Ethnobotany Garden, Rimba Forest, Gunung Berlumut, Kluang, Johor
Strobilanthes crispus	R1	Radhiah	UKM Herbs Garden. Bangi, Selangor
Asystasia gangetica subspecies micrantha	CNA 2	Nurul-Aini & Ruzi,	UKM Herbs Garden. Bangi, Selangor
Asystasia gangetica subspecies micrantha	CNA 44	Nurul-Aini, Ruzi, Ummu-Hani & Arrabe	Larut Matang Mangrove Forest, Taiping, Perak

Table 2. Summary of leaf anatomical characteristics of taxa studied

Characters	**taxa**				
	A. alba, A. officinalis, A. lanata	*Justicia gendarussa*	*J. betonia*	*Stobilanthes crispa*	*Asystasia gangetica* subsp. *micrantha*
Vascular bundles arrangement in the petiole	Type 1	Type 2	Type 2	Type 2	Type 3
Vascular bundles arrangement in the midrib	Type 1	Type 2	Type 2	Type 3	Type 3
Areolar venation	Open system	Majority open; Minority close System	Close	Close system	Majority close; minority open
Ending veinlets	Open system	Majority open; Minority close System	Close system	Close system	Majority close; minority open
Swollen tracheid	Present	Absent	Absent	Absent	Absent
Hypodermal cells	5-6 layers	1 layer	Absent	Absent	1 layer
Intracellular spaces	Absent	Present	Present	Present	Absent
Brachysclereids	Present	Absent	Absent	Absent	Absent
Types of stomata	Cyclocytic	Diacytic	Diacytic	Diacytic	Diacytic
Cystoliths	Absent	Present	Present	Present	Present
Marginal outline	Type 1	Type 2	Type 2	Type 3	Type 4
Dentation at leaf margin	Present	Absent	Absent	Absent	Absent

Characters	**taxa**				
Patterns of anticlinal walls	Straight to curve	Wavy to sinuous	Wavy to sinuous	Sinuous	Sinuous
Types of trichomes	Capitates glandular (terminal flattened)	Simple multicellular	Absent	Simple unicellular and multicellular, capitate glandular (terminal multicellular)	Simple and unicellular, capitate glandular (terminal multicellular)
Presence of stomata	Hypostomatic	Amphistomatic	Amphistomatic	Hypostomatic	Hypostomatic

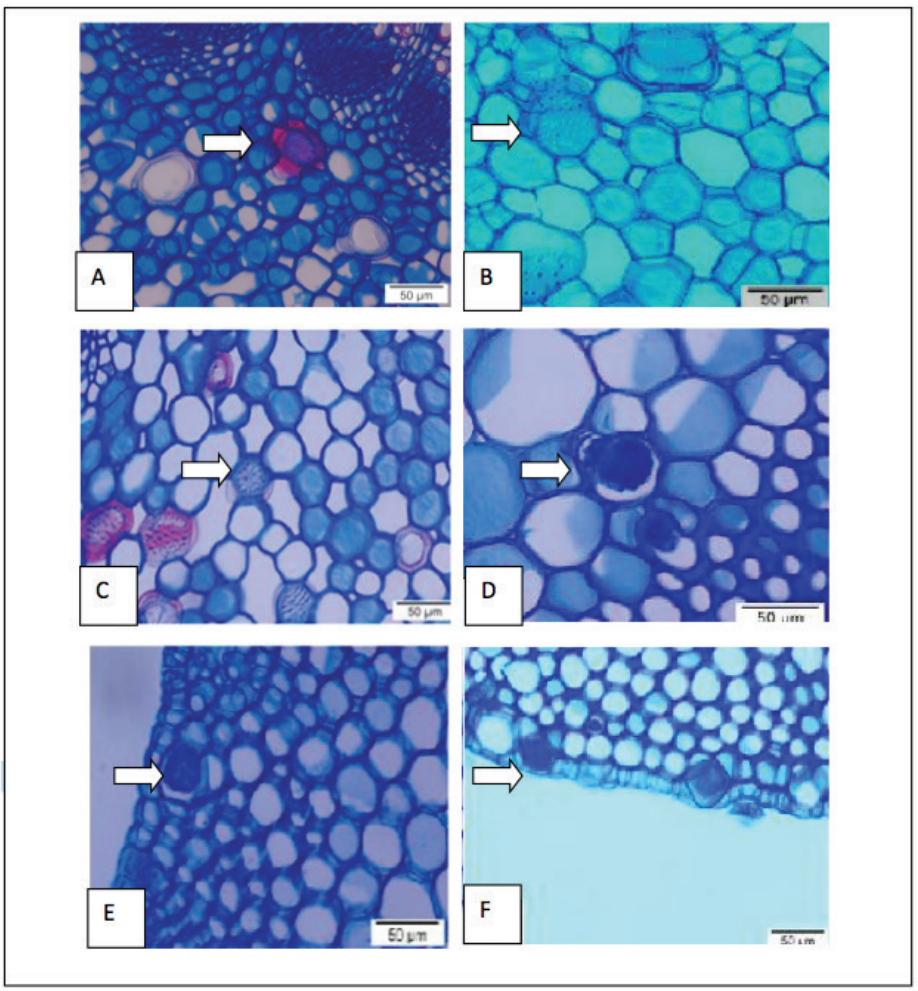

Figure 1. Cross-section of petioles. Arrow indicates the presence of mucilaginous idioblast cells A) Petiole of *Avicennia alba*, B) Petiole of *A. officinalis*, C) Petiole of *A. lanata*, D) Petiole of *Justicia gendarussa*, E) Petiole of *J. betonica*, F) Petiole of *Strobilanthes crispa*.

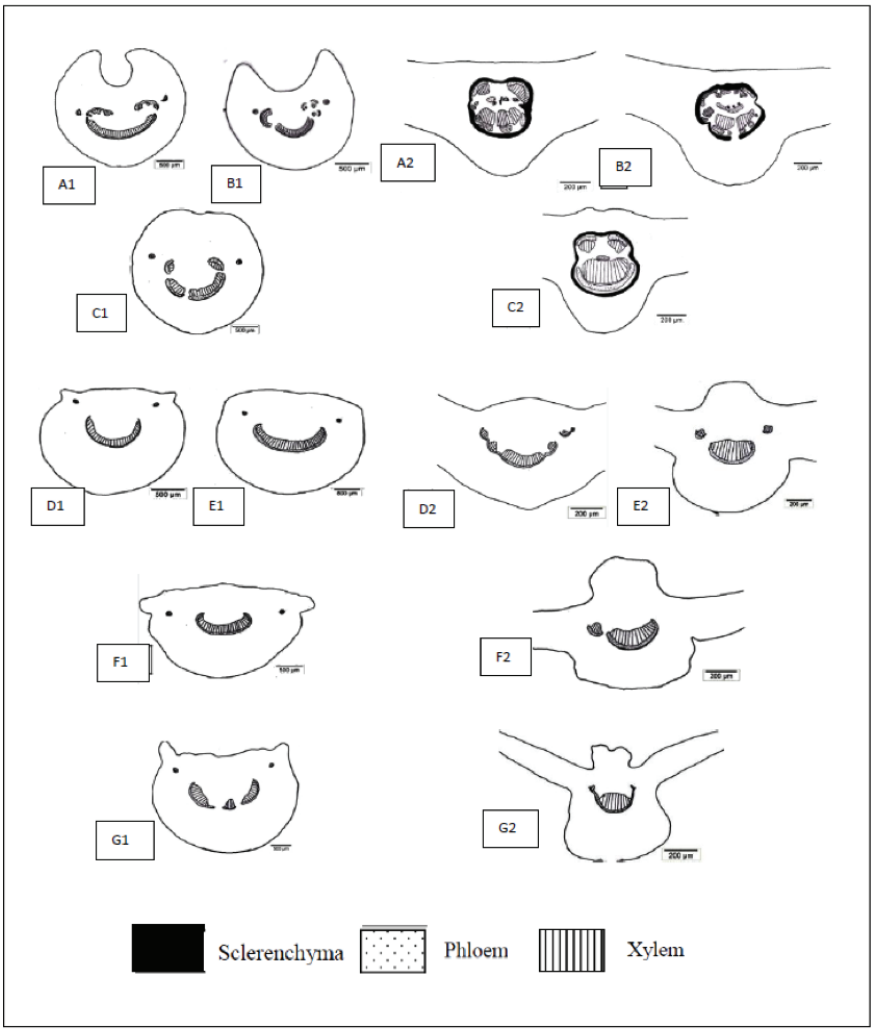

Figure 2. Illustrations of 1) petiole cross-sections 2) midrib cross-sections: A) *Avicennia alba*, B) *A. officinalis*, C) *A. lanata*, D) *J. gendarussa*, E) *J. betonica*, F) *S. crispa*, G)*Asystasia gangetica* subsp. *micrantha*. Scale bar: A-G) 200µm.

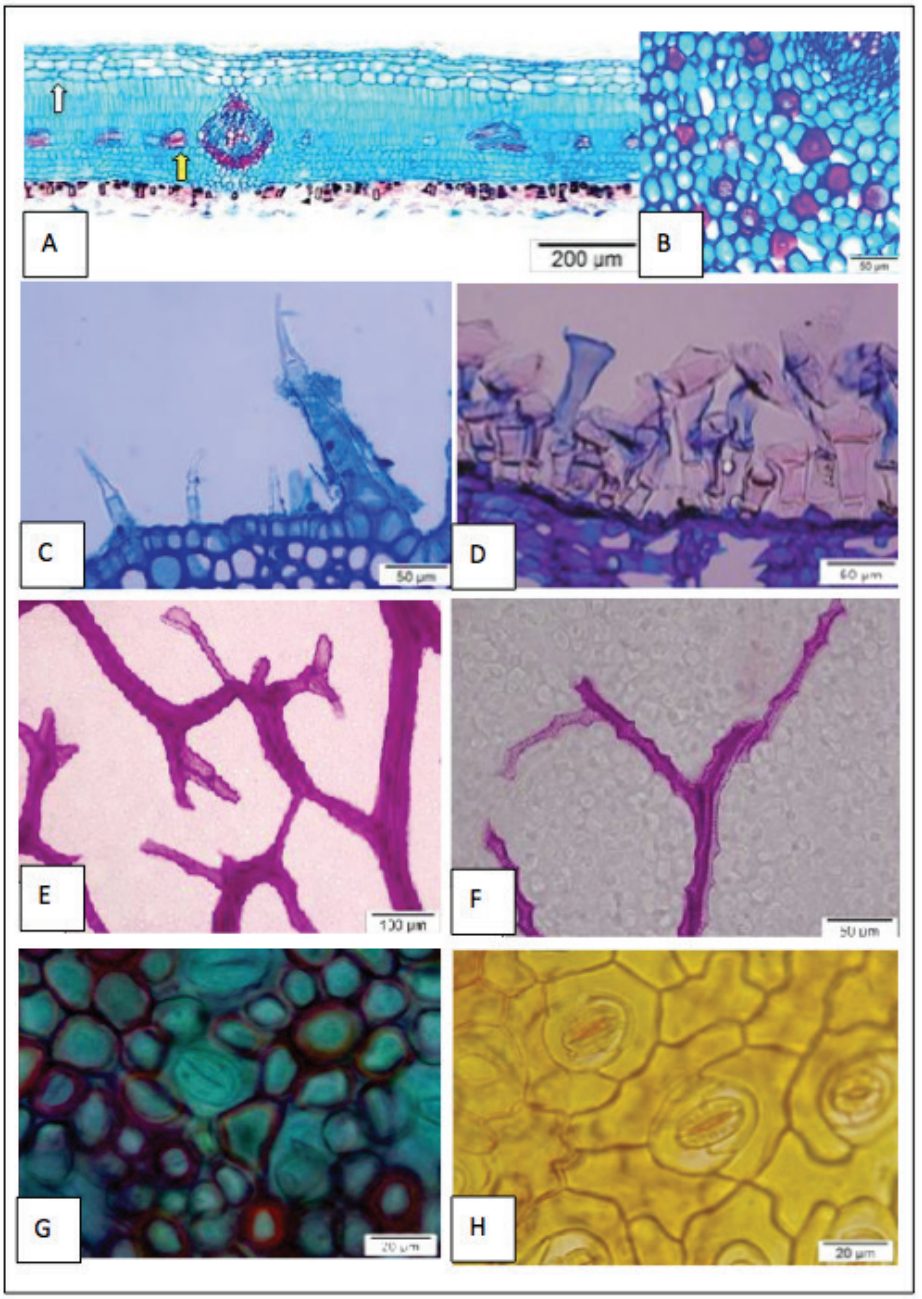

Figure3. A) *Avicennia alba*, Cross sections of leaf lamina, white arrow indicates hypodermal layers, yellow arrows indicate brachysclereids. B) *A. alba*, brachysclereids in the midrib, C) *S. crispa*, Simple multicellular trichomes, D) *A. lanata*, capitate glandular (terminal flattened) trichomes, E) *A. officinalis*, veinlets: open endings, branched and swollen