

MICROMORPHOLOGICAL ANALYSES OF STOMATA AND EPIDERMAL CELL AMONG DIFFERENT SPECIES IN THE GENUS *Premna* L. (LAMIACEAE)

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Abstract. The genus *Premna* in Lamiaceae family was categorized into two groups namely *P. serratifolia*-group and *P. trichostoma*-group by de Kok in 2013. The division was based on three distinct morphological characters which are twigs, calyx and fruit. This group category lacks of anatomical and micromorphological evidence and solely based on those morphological characters. Therefore, in this study, anatomical and micromorphological characters were analysed on six *Premna* species (*P. interrupta*, *P. serratifolia*, *P. trichostoma*, *P. tomentosa*, *P. parasitica* and *P. odorata*) from few localities in Peninsular Malaysia. They were investigated to determine the size of stomata and some epidermal characters among the species members to provide some evidence towards the division. The methods involved in this research were epidermal peel preparation and field emission microscopy (FESEM) technique. The stomata were observed under the microscope and also photographed at different magnifications. Based on the observation, primary sculpturing pattern of leaf surface showed four types of patterns and labelled as type 1, 2, 3 and 4. All six species of *Premna* have the same undulating, four sides and number of anticlinal walls which showed shared characteristics of the genus. However, the stomatal size, sculpturing and distribution show some taxonomic significance in identification of the species. Stomatal size shows significant difference between species which ranged from 3 µm to 20 µm. Three types of stomata presented at the abaxial surface, anisocytic, paracytic and diacytic while four different types of stomatal sculpturing were observed which are type 1, 2, 3, 4 and stomatal distribution resulted in variances in stomatal density. Therefore, the division of *Premna* as suggested by de Kok was supported based on stomatal type and secondary stomata sculpturing evidences.

Keywords: *Premna* sp., stomata, density, size, micromorphological

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Introduction

The genus *Premna* was first described by Linnaeus in 1771 based on two species, *Premna serratifolia* and *Premna integrifolia*. These two species now were treated as one species. The generic name is derived from the Greek ‘premnōn’ which means the tree stump refer to the short and twisted tree trunks of *P. serratifolia*. This genus occurs from Africa to China, throughout Indochina and Malesia, Australia and the Pacific [1]. The number of species throughout the areas estimated from 50 [2] to 200 [3]. This family consists of climbers, shrubs and small trees. The morphological characters of this family are simple leaves, small whitish inflorescence, in terminal clusters: calyx with 3-5 teeth: corolla bilaterally symmetrical, 2-lipped with 4-5 lobes and 4 stamens of a floret. *Premna* is also known as *Buas-buas*, *Berbuas* and *Berbuat* in Malay. Around 50 species were found in the warmer region of the Old World (Africa, Asia, including Australia) while 13 species of Malaya were found in the lowlands [4].

The species in *Premna* was categorized into two groups, *P. serratifolia*-group and *P. trichostoma*-group [5]. The first group *P. serratifolia*-group, with these common species: (*P. serratifolia*, *P. odorata* and *P. tomentosa*), while species in the second group tend to be rare and generally geographically more restricted (*P. trichostoma* group) as in Table 1 [6]. The group determination was based on three distinct morphological characters of twigs, calyx and fruit. Two fruit types can be identified, which the first one is a typical globose drupe-like fruit consisting of four somewhat fleshy mericarps with one seed each. The second type is a clavoid, single seeded drupe-like fruit consisting of one fleshy mericarp, endocarp encompassing 1-4 seeds within the drupe-like fruits. Table 1 shows two groups of *Premna* species in Flora Malesia area and their distribution based on de Kok categorization [6].

Table 1: *Premna* species in Malesia region and their distribution based on de Kok categorization [6]

Taxon	Distribution
<i>P. serratifolia</i>-group	
<i>P. odorata</i> Blanco	India and China to Australia
<i>P. pubescens</i> Blume	Java, Sumatra, the Lesser Sunda Islands and the Philippines
<i>P. serratifolia</i> L.	East Africa to Tahiti
<i>P. sterculiifolia</i> King & Gamble	Peninsular Malaysia
<i>P. tomentosa</i> Willd	India and China to Australia, except Borneo
<i>P. trichostoma</i> group	
<i>P. clavata</i> de Kok	Sabah
<i>P. decurrens</i> H.J.Lam	Sumatra
<i>P. herbacea</i> Roxb.	Southeast Asia to Australia
<i>P. interrupta</i> Wall. Ex Schauer	India and China to Malaysia
<i>P. oblongata</i> Miq.	Sunda Islands and Sulawesi
<i>P. pallescens</i> Ridl.	Borneo
<i>P. parasitica</i> Blume	Java and Bali
<i>P. regularis</i> H.J.Lam	Philippines and New Guinea
<i>P. trichostoma</i> Miq.	Myanmar and Vietnam to New Guinea

During the leaf growth process, some epidermal cells of the leaf differentiate into a complex of cells that regulate the movement of gaseous into and out of the leaf. Stomata are important structures used in exchanging matter between plants and the environment. The movement of gases in and out of a leaf is controlled by stomata and made carbon dioxide available for the process of photosynthesis. Through transpiration, stomata also control the loss of water from the leaf. Stomatal conductance, or the ability of materials to pass through the stomata of a leaf is dependent on both the size of the stomata and the total density of stomata on the leaf [7]. This variable should relate to the rate at which water passed through the stomata of each plant during the transpiration. As stomata is an important structure in plant, this study was carried out by analysing the stomatal and the epidermal cell of *Premna* species to prove that the character can be used in identification and delineation of *Premna* members up to the species level.

The wood of the larger species of some *Premna* are sometimes used in house building or in the making of furniture and tools as the wood is durable and resembles teak [8]. Historically, species of the family Lamiaceae have enjoyed a rich tradition of use for flavouring, food preservation, and medicinal purposes, due to their curative and preventive properties [9]. It is known that each species of Lamiaceae has a special, complex mixture of bio-active compounds in which each component contributes to its overall bio-activities. This value lays in the production of a wide range of secondary metabolites with potent antibacterial, antioxidant, anti-inflammatory, antimicrobial, antiviral, and anticancer activities. Some of the inflorescence part in Lamiaceae also used as antidepressant and was tested in mice [10]. The *Premna* genus can be used traditionally in treating various ailments like rheumatism, asthma, dropsy, cough, fever, boils and scrofulous diseases [11].

Few recent studies on phylogenetic analyses of Lamiaceae placed *Premna* in subfamily Premnoideae which contains three genera: *Premna* (50-200 spp. in tropical to subtropical Asia, Africa, Australia and the Pacific Islands), *Gmelina* (31 spp. in tropical and subtropical Asia to Australia and western Pacific Islands), and *Cornutia* (12 spp. in tropical America) [12]. These three genera were traditionally placed in subfamily Viticoideae and then transferred to Lamiaceae together with the subfamily and then excluded from Viticoidea based on molecular evidence. The analyses were carried out using 79 protein-coding plastid genes from 175 accessions representing 170 taxa, 79 genera, and all 12 subfamilies consequently proposed an updated phylogenetically informed tribal classification for Lamiaceae that is supplemented with a detailed summary of taxonomic history, generic and species diversity, morphology, synapomorphies, and distribution of each subfamily and tribe [13].

Premna now ranks among the most taxonomically difficult and complicated genera of Lamiaceae. Most of the recent studies focused on *Premna*'s medicinal value and lack of micromorphological study. The classification of the species was basically based on its fruit characteristics and less on its leaves characteristics. The most complete review on the genus and family was presented in Flora Malesiana by Bramley [14] but not much reported on micromorphological and anatomical study. Variation between species can be observed through several features such as stomatal shape and type, epidermal anticlinal wall, epidermal pattern either on abaxial or adaxial epidermis [15]. Leaf architecture morphological characters of vegetative parts can also be used in identification to species, subspecies and variety level of genus and propose some changes to the placement of taxa hence can be devoted as an improvement for the *Vitex* study by Syanawiyah et al. [16]. In some studies of other genus in Lamiaceae family such as *Clerodendrum*, selected characters such as the

presence and shape of stipule, vein spacing, leaf venation, 5th vein category, leaf texture, agrophic, leaf shape, presence or absence of hair at the epidermal layers, areolation, petiole outline and presence of hair and leaf texture, can be very useful in species delimitation. A study carried out by Mazatul Azrin et al., [17] concluded that leaf apex, base, margin and most of serration and sinuses features can be used as diagnostic characters to several species identification such as *C. johorensis*, *C. chinensis* and *C. paniculatum*.

In study of *Vitex* species by Nor Nafizah et al., [18] other genus in family Lamiaceae, its epidermal characters show great similarities. They possess anomocytic stomata where surrounding epidermal cells and subsidiary cells are not differentiated, open areoles and looped marginal venation. Significant variations in anatomical characters between the studied species is type of anticlinal wall of the epidermal cells. The identification of species based on leaves was carried out due to its availability all year round compared to the flowers or fruits which are dependent on the flowering and fruiting season. Therefore, this study was conducted to use alternative characters as tools to differentiate and identify species in *Premna*.

Materials and Methods

Samples used in this research were collected from several forest reserves in Perak and Negeri Sembilan as shown as in Table 2. Meanwhile Table 3 shows the list of species collected from the sampling areas.

Table 2: Location of sampling areas

No.	Location	State
1.	Sungai Dara Forest Reserve	Perak
2.	Gunung Liang Forest Reserve	Perak
3.	Pasoh Forest Reserve	Negeri Sembilan

Table 3: List of species collected

Species	Voucher no.	Locality
<i>Premna serratifolia</i> L.	ANA013	Sungai Dara Forest Reserve
	ANA008	Gunung Liang Forest Reserve
<i>Premna interrupta</i> Wall. Ex Schauer	ANA004	Sungai Dara Forest Reserve
	ANA005	Sungai Dara Forest Reserve
	ANA010	Gunung Liang Forest Reserve
<i>Premna trichostoma</i> Miq	ANA003	Sungai Dara Forest Reserve
	ANA007	Gunung Liang Forest Reserve
<i>Premna tomentosa</i> Willd.	ANA001	Pasoh Forest Reserve
	ANA002	Pasoh Forest Reserve
<i>Premna parasitica</i> Blume	ANA012	Sungai Dara Forest Reserve
<i>Premna odorata</i> Blanco	ANA011	Sungai Dara Forest Reserve

A total of six *Premna* species were collected. The three replicates for each species were also collected. Herbarium specimens were investigated mainly in Forest Research Institute Malaysia FRIM (KEP) and Universiti Pendidikan Sultan Idris Herbarium (FP).

Specimens were also observed virtually from Singapore Botanic Garden (SING), Royal Botanic Gardens, KEW (K), University of South Florida (USF) and New York Botanical Garden (NY). Fresh samples for this study as in Table 3 were collected through a series of fieldwork in Peninsular Malaysia. Voucher herbarium prepared was deposited at Universiti Pendidikan Sultan Idris Herbarium (FP).

Herbarium Samples

Figure 1 shows the herbarium of *Premna* sp.

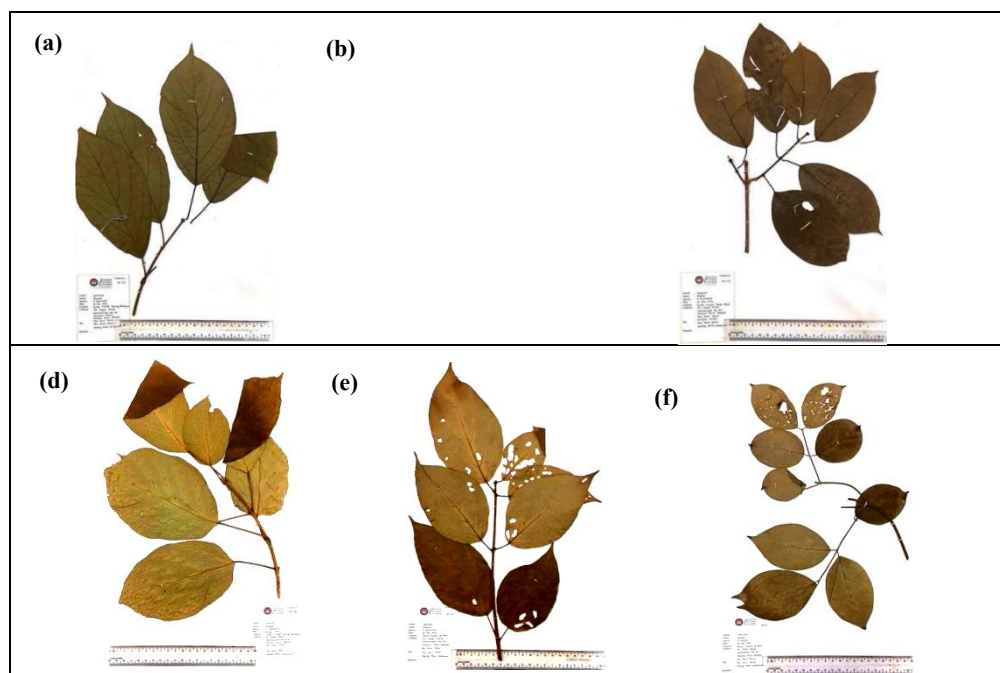


Figure 1: Herbarium samples of (a) *Premna serratifolia* (b) *Premna interrupta* (c) *Premna trichostoma* (d) *Premna tomentosa* (e) *Premna parasitica* and (f) *Premna odorata*

Epidermal Peel Procedure

In epidermal peel procedure, fresh leaves were washed with running tap water for approximately 20 minutes and fixed in FAA for at least 48 hours. The middle part of the specimens were washed with running tap water for about 20 minutes. The middle part of each leaf lamina was soaked in 100% Sodium Hypochlorite (Clorox) for a few minutes up to an hour. The leaf was then, scraped using a razor blade or brush to remove the epidermis and mesophyll tissues, leaving only the remaining epidermal surface to be examined. The prepared epidermal layer was stained with Safranin O and underwent dehydration process through series of alcohols as shown in Table 4.

The specimens then were transferred to a glass slide by using paint brush and mounted with Canada Balsam or Euparal and left on the slide warmer to dry. Dried anatomical slides were observed under light microscope to observe stomatal and epidermal characters. Micrographs were captured using Nikon Eclipse 2000-U microscope at several magnifications.

Table 4: Dehydration process for epidermal peel preparation

Percentage of alcohol solution	Duration (minutes)
50%	5
70%	5
95%	5
100%	4
Alcohol 100% : xylene	2
Xylene	2

Field Emission microscopy (FESEM) technique was carried out to examine plant surfaces at high resolution. In FESEM study, high resolution field emission electron microscope, Hitachi SU 8200 were utilized to capture the microstructure image of the samples. Sample of leaf was sectioned in the size of 2 cm² from the herbarium sample prepared. Double sided adhesive was used to stick the specimens on aluminium stubs and then coated with gold palladium to a thickness of 40-50 nm. The specimens were examined at different several magnifications and the best images were selected. The micrographs were taken at an accelerating voltage of 5.0 kV. The type of stomata were described and classified.

Results and Discussion

Lamiaceae was known as the stoma with diacytic with a few anomocytic intermixed and these observations are confirmed by Ramayya and Rao on the cotyledons of three species of *Ocimum* [19]. Diacytic, transitional between diacytic and anomocytic stomata types were observed in thirty-three species of this family. The first type mentioned as the most frequent in all taxa, with perigenous and mesogenous ontogenetic patterns were reported [20]. On the other hand, the stomata are predominantly diacytic, anomocytic, anisocytic and a mixture of anomocytics and anisocytics [21]. In this research, the stomata were found absent on adaxial. Whilst, anisocytic, paracytic and diacytic stomata present at the abaxial surface as in Table 5. From the leaf surface observation, the *Premna* species can be categorized in three groups based on stomatal type. *P. serratifolia* and *P. trichostoma* in group 1 (anisocytic), *P. interrupta* in group 2 (paracytic) and group 3 (diacytic) for *P. tomentosa*, *P. parasitica* and *P. odorata*.

This result showed that most of the *Premna* species followed the categorization by the previous research [19-21]. The stomatal number of these six species vary from 10 to 17 stomata per leaf. The actual number of stomata per sq mm may vary from the same plant grown in different environment or under different climatic conditions.

Figure 2 shows that abaxial epidermal cell and stoma of *Premna serratifolia*, *Premna interrupta*, *Premna trichostoma*, *Premna tomentosa*, *Premna parasitica* and *Premna odorata*. Based on the anatomical observation on leaf surface, the adaxial anticlinal wall of epidermal surface are straight to wavy for *P. serratifolia* as in Figure 2(a) while all *Premna* has wavy anticlinal wall for abaxial as in Figures 2(b)-(f). Table 5 shows the anatomical observation on leaf surface in term of stomatal types.

Table 5: Anatomical observation on leaf surface

No.	Species	Stomata Abaxial
1.	<i>P.serratifolia</i>	Anisocytic
2.	<i>P.interrupta</i>	Paracytic
3.	<i>P.trichostoma</i>	Anisocytic
4.	<i>P.tomentosa</i>	Diacytic
5.	<i>P.parasitica</i>	Diacytic
6.	<i>P.odorata</i>	Diacytic

Stomata sculpturing could be used as an additional character to define each particular species. Primary sculpturing pattern of leaf surface in Figure 3 shows only four types labeled as type 1, type 2, type 3 and type 4 based on Figure 3.

Table 6 shows the sculpturing pattern, intercellular flanges and leaf surface observation of six species of *Premna*. Meanwhile, Table 7 shows the stomatal observation in term of its size, type and number of stomata on the leaf surface. Majority of the studied species have type 1 and type 2 primary sculpturing (Table 6). Type 1 stomata sculpturing shows two distinct flanges on the parallel side of the guard cell with little or no distance between it. Several flanges run periclinally toward both polar of the stomata and on both central side of the guard cells. Type 2 protrusion, flanges run sinuously and touching each end of stomata polar side. Type 3 protrusion, flanges run in an almost perfect cycle with the stomata were located at the centre. Single flanges connect the circle with other flanges on one of the stomata pole. Type 4 flanges run in an almost perfect cycle with the stomata place on the centre. Primary and secondary stomatal sculpturing were observed in this study and four different types of stomatal primary sculpturing were identified as in Figure 2 and stomatal distribution resulted in variances in stomatal density. Several species shared type 1 and type 2 primary sculpturing. However, only *P. odorata* has type 3 and *P. interrupta* has type 4 stomatal primary sculpturing respectively in which could no be found in other species.

P. serratifolia, *P. tomentosa* and *P. odorata* shared type 2 secondary sculpturing therefore support the de Kok [5] division of the species in *P. serratifolia*-group. In the other hand, type 1 was observed in *P. trichostoma*, *P. parasitica* and *P. interrupta* also supported the placement of the species into *P. trichostoma*-group. All six species of *Premna* have the same numbers of anticlinal walls and shared genus characters. However, the stomatal sizes, sculpturing and distributions shows some taxonomic significance in identification of the species. Stomatal sizes showed significant difference between the species which ranged from 3 μm to 20 μm as in Table 7.

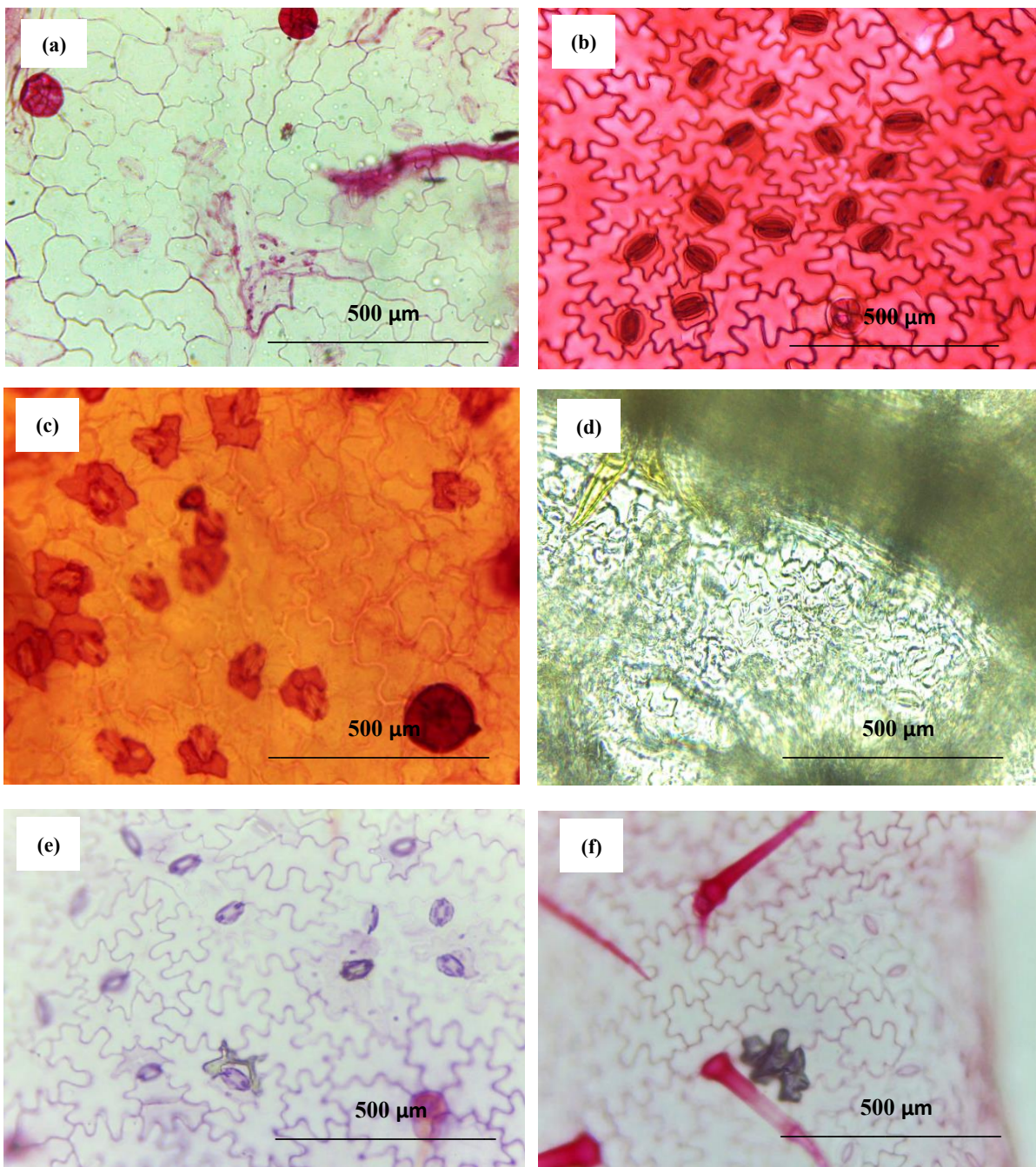


Figure 2: Abaxial epidermal cell and stoma (a) *Premna serratifolia* (b) *Premna interrupta* (c) *Premna trichostoma* (d) *Premna tomentosa* (e) *Premna parasitica* and (f) *Premna odorata*

Table 6: Sculpturing pattern, intercellular flanges and leaf surface observation

No.	Species	Leaf Surface	Primary Sculpturing	Secondary sculpturing	
				Intercellular flanges Straight (St) or Undulating (Un)	Anticlinal wall (sided)
			Type		
1.	<i>P. serratifolia</i>	Abaxial	2	Un	4
2.	<i>P. interrupta</i>	Abaxial	4	Un	4
3.	<i>P. trichostoma</i>	Abaxial	2	Un	4
4.	<i>P. tomentosa</i>	Abaxial	1	Un	4
5.	<i>P. parasitica</i>	Abaxial	1	Un	4
6.	<i>P. odorata</i>	Abaxial	3	Un	4

Table 7: Stomatal observation

No.	Species	Leaf Surface	Secondary sculpturing		
			Stomata		
			Size (µm)	Type	Number of stomata
1.	<i>P.serratifolia</i>	Abaxial	3-9	2	11
2.	<i>P.interrupta</i>	Abaxial	4-10	4	17
3.	<i>P. trichostoma</i>	Abaxial	7-11	1	15
4.	<i>P. tomentosa</i>	Abaxial	8-20	2	11
5.	<i>P. parasitica</i>	Abaxial	6-8	1	13
6.	<i>P. odorata</i>	Abaxial	6-8	2	6

Figure 3 shows the stomatal flanges sculpturing types. Meanwhile, Figure 4 shows the abaxial surface micrograph results of *Premna serratifolia*, *Premna interrupta*, *Premna trichostoma*, *Premna tomentosa*, *Premna parasitica* and *Premna odorata*.

Table 8 shows the general features of *Premna* sp. From the herbarium study, all *Premna* sp. share quite a lot of similarities between each species. *Premna* sp. share the same type of leaves (simple), leaves arrangement (opposite), leaf venation (pinnate), 3rd venation course (sinuous), agrophic (simple), marginal ultimate (looped), intercostal area (irregular), F.E.V.S (absent), leaf rank (4^f), blade petiole separation (good), petiole feature (base swollen), position of petiole attachment (marginal) petiole hair (present) and stem shape (round). Leaf apex of the species also shows taxonomic significance to some extent as almost all sample has its own type of leave apex caudate, acuminate, cuspidate and acute to acuminate. However, three species share the same type of leaves apex (cuspidate) namely *P. serratifolia*, *P. trichostoma* and *P. odorata*. Leaf base further divided the genus into two groups with majority of the species have acute leaf base (*P. interrupta* and *P. odorata*) and

rounded leaf base (*P. tomentosa* and *P. parasitica*). Leaf abaxial hair further help distinguished the genus as only some of the studied sample did not have abaxial hair namely *P. serratifolia* and *P. tomentosa*. In term of adaxial hair only one of the studied *Premna* species have them and prove to be of taxonomic significance toward species level (*P. parasitica*).

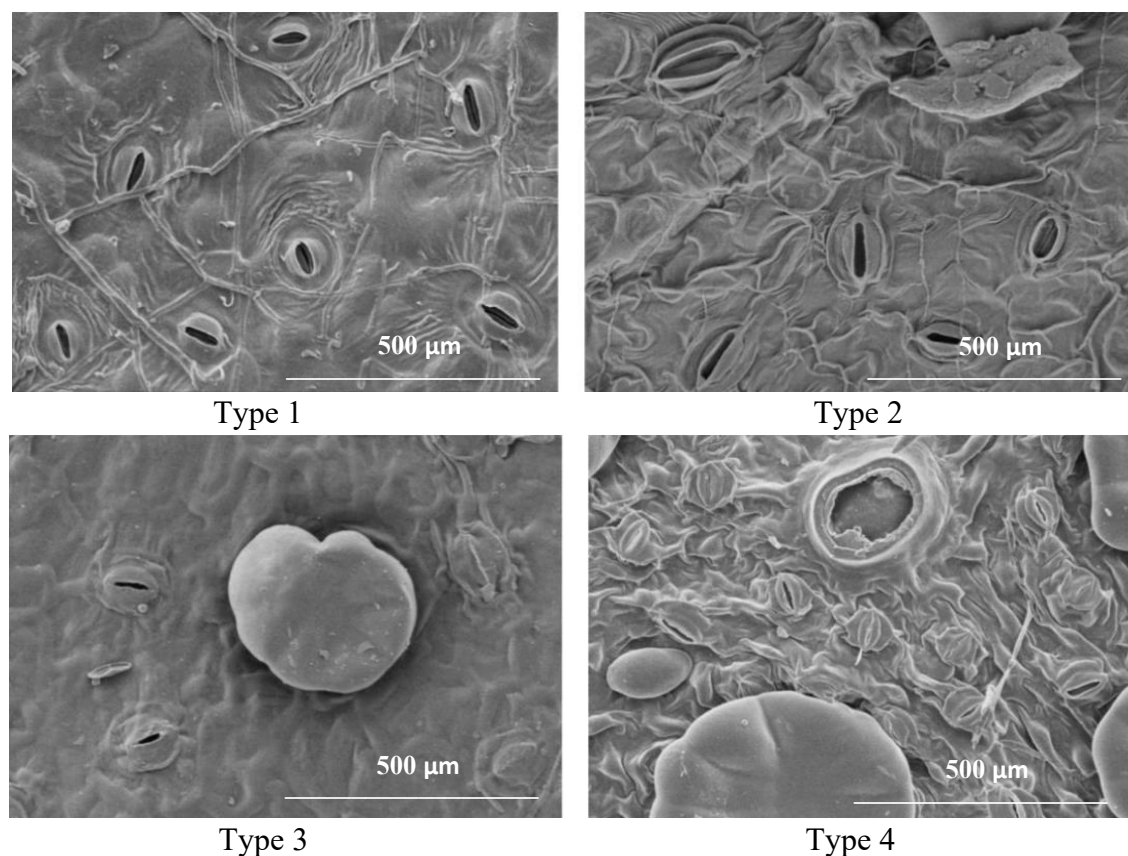


Figure 3: Stomatal flanges sculpturing types

Table 8: General features of *Premna* sp.

No.	Species	Type of tree	Leaf blade	Leaf apex
1.	<i>P. serratifolia</i>	Shrub	Ovate to obovate	Cuspidate
2.	<i>P. interrupta</i>	Climber	Elliptic to ovate	Acuminate
3.	<i>P. trichostoma</i>	Shrub or scrambling tree	Ovate to obovate	Cuspidate Acute to
4.	<i>P. tomentosa</i>	Shrub	Ovate to obovate	Acuminate
5.	<i>P. parasitica</i>	Large liana	Elliptic to rounded	Caudate
6.	<i>P. odorata</i>	Shrub	Elliptic to rounded	Cuspidate

The significance of the data obtained from the leaf anatomy and morphology will be discussed in relation to the current taxonomic opinions on Lamiaceae. It is hoped that this

comprehensive study of leaf anatomy will contribute towards the reclassification of the family at specific and generic level. The findings of the study able to provide more data on the previous study of this genus in Peninsular Malaysia for future researchers to be used as a reference in conducting more research on the genus. The combination of morphological, anatomical, micromorphological and distribution studies is hoped to produce more complete data for each studied species. However, in this research only six species of *Premna* over a total of 14 in Peninsular Malaysia were investigated. Therefore it is suggested that, in future a more comprehensive study involving all *Premna* species in Peninsular Malaysia will be conducted to gain better insight.

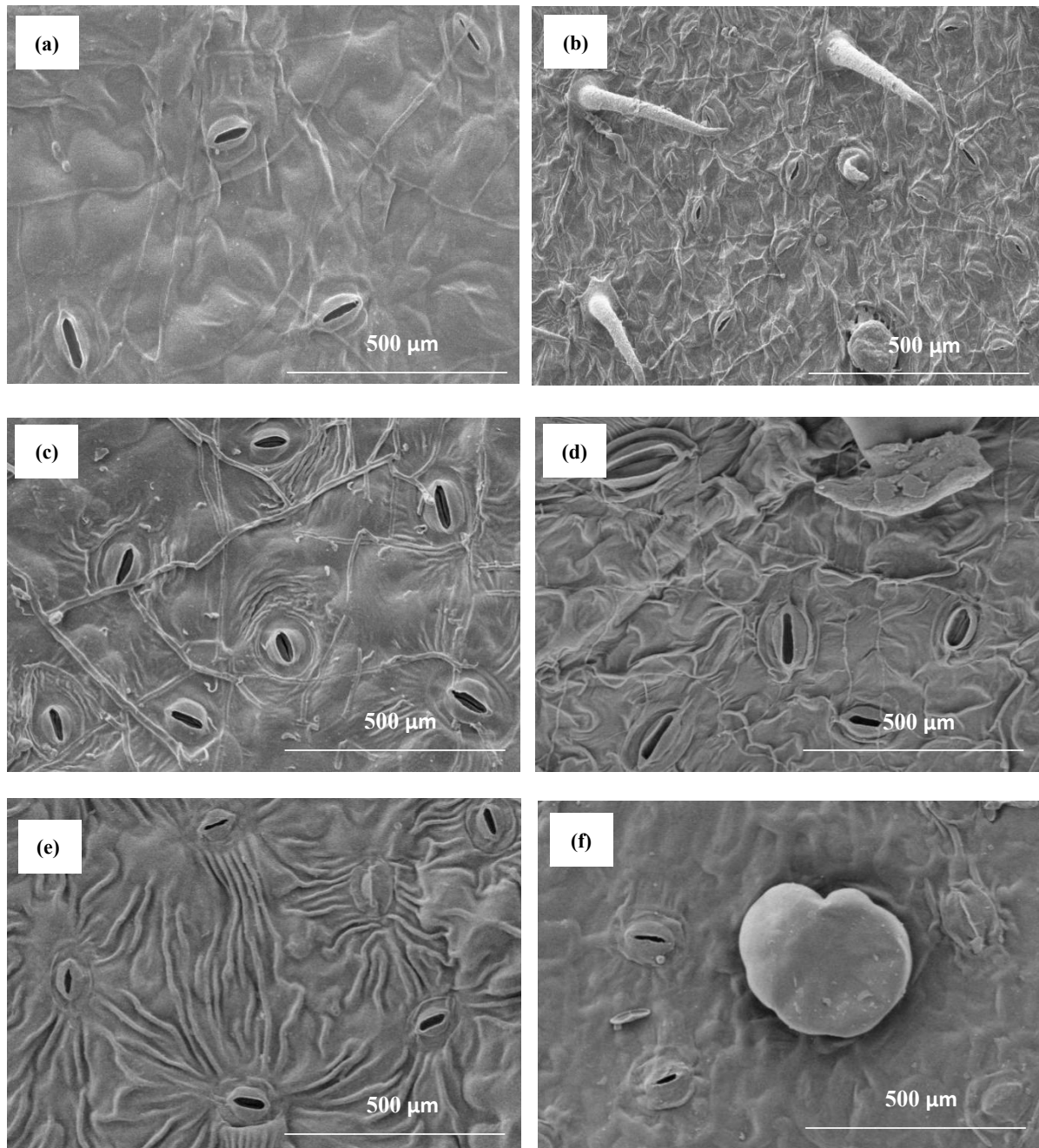


Figure 4: Abaxial surface micrograph results of (a) *Premna serratifolia* (b) *Premna interrupta* (c) *Premna trichostoma* (d) *Premna tomentosa* (e) *Premna parasitica* and (f) *Premna odorata*

Conclusions

In summary, all the evidence gathered from anatomical observation including sculpturing pattern, types of primary sculpturing and stomata observation (size and number) do not support the categorization of *Premna* by the previous researcher. The findings, however, support the group division and followed the categorization of genus *Premna* by the characters of stomatal types and secondary stomatal sculpturing from this research. The results of the study revealed that important features of leaf have taxonomic value to assist and support species identification and classification.

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Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of conflict of interest

The authors have no disclosures to declare.

Compliance with ethical standards

The work is compliant with ethical standards.

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