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Original Article

Comparative leaf and stem anatomy of ten Piper species from Indonesia

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Abstract

The genus *Piper* with over 700 species distributed in tropical and subtropical areas of the world has a considerable number of species with economic importance as spices and herbal medicine. In this study the anatomical characters of leaf and stem on 10 species of *Piper* from Bogor Botanical Garden, Indonesia were observed in relation with their function as oil-accumulating organs. The objective of this study was to explore the anatomical variability among species. Observations on anatomical characters were done on leaf epidermal and cross section as well as stem cross section prepared using paraffin embedding method. Results indicated that there were structural variations on the epidermal features, mesophyll, secretory cells and secretory cannals of leaves, as well as variation on the ratio of medullary and peripheral vascular bundles on stem. Results of this study provide additional taxonomic evidence to confirm differences between *Piper* species, and serve as supporting data for identification based on internal structures. Data on the comparative anatomy of leaf and stem also contributes to the selection of the right materials for the extraction of essential oils from various *Piper* species based on the presence of secretory cells.

Keywords: *Piper*, Plant internal structure, Medicinal plant, Taxonomic evidence

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Introduction

The genus *Piper* is one of the most diverse genera in Angiosperms, with more than 700 species distributed in tropical and subtropical regions (Parmar et al., 1997). *Piper* species have various growth habits, from erect or scandent herbs, shrubs, climbers, or rarely as trees (Jaramilo and Callejas, 2004). A number of *Piper* species are of economic importance and widely known for their use as spices such as *P. nigrum* and *P. guineense*, as herbal medicine such as *P. betle* and *P. methysticum*, or as condiment like *P. auritum* and *P. lolot* (Dyer et al., 2004; Jaramilo and Callejas, 2004). The wide range use of *Piper* is due to their secondary metabolites. One of the major secondary metabolites found in *Piper* is essential oils, which can be easily recognized from their specific aromas of the leaves (Oyen and Dung, 1999). Plant secondary metabolites are accumulated in external and internal secretory tissues. When focusing on internal secretory tissues, secondary metabolites can be accumulated in secretory cells, secretory glands, secretory cannals, or secretory cavities (Fahn, 1990). Internal secretory

tissues in leaves are normally located in mesophyll or multiple epidermal tissues, while in stem or root they are located in cortical parenchyma or surrounding vascular tissues (Esau, 1977). Meanwhile, external secretory tissues could be in a form of trichomes.

The importace of morpho-anatomical studies for medicinal plants in relation to their phytochemistry has been noted in different taxa, such as *Oxystelma esculentum* (Poornima et al., 2009) and *Cnicus benedictus* (Djamila et al., 2013). Anatomical characterization is especially useful in determining the originality and authethication of similar or closely related medicial plants. This approach has been applied for many plant species such as two species of herbal drug materials from different genus, in which *Cassytha filiformis* is often used as substitues for *Cuscuta reflexa* (Sharma et al., 2010). The use of anatomical features in botanical identification leading to authentication as an intergral part of product standarization has been repoted on *Piper betle* leaf (Periyanayagam et al., 2012). Information on types of cells or tissues serve as oil-accumulating structures in *Piper* is important for optimum extraction of essential oils. Therefore, the distribution of secretory cells on leaf and stem of variuos *Piper* species need to be explored accordingly.

Studies on the anatomy of *Piper* has been reported for various purposes. Anatomical comparison between *Piper sarmentosum* and *Piper betle* was reported as a means to distinguish between the two species that have similar leaves morphology and their uses as medicinal plants and culinary ingredients (Raman et al., 2012). The anatomy of *Piper lepturum* var. *lepturum* and *P. lepturum* var. *angustifolium* was studied to clarify the differences between the two and determine their taxonomical status whether they represent two varieties of one species or as two different species (Machado et al., 2015). Meanwhile, the anatomy of *P. amalago* was examined in order to distinguish it from other *Piper* species in the quality control process as a medicinal plant (dos Santos et al., 2015).

Comparative studies of leaf and stem anatomy on three species representing three different genus within Piperaceae revealed the structural diversities, and thus suggesting their role in taxonomy of this family (Souza et al., 2004). In this study the anatomical characters of stem and leaf of 10 species of *Piper* from Bogor Botanical Garden was examined for exploring their anatomical variability. Information on anatomical variability could be used as supporting data in plant taxonomy, namely as taxonomic evidence for identification and anatomical characterization of *Piper* species based on their internal structures. The anatomical characterization is useful in the authentication of herbal medicinal ingredients, especially if the material available is only in the form of dried leaves and stems without the presence fresh plant materials. Accurate identification of dry materials based anatomical characters is very important for quality assurance of medicinal plants and to avoid the problem of adulteration of herbal ingredients.

Material and Methods

Materials used in this study were 10 species of *Piper* from Bogor Botanical Garden, Indonesia. These species were *P. baccatum, P. betle, P. porphyrophyllum, P. firmum, P. acutilimbum, P. lowong, P. flavomarginatum, P. crocatum, P. majusculum,* and *P. caninum*. Fresh leaves on the third order from the growing shoot and stems were collected from mature plants maintained at the green house of Bogor Botanical Garden. Cross sections of leaf and stems were prepared using paraffin method (Ruzin, 1999). Freshly collected samples were cut into small pieces and fixed in FAA (formaldehyde – acetic acide – alcohol) overnight, followed by dehydration process using a series of alcohol solutions (70%, 80%, 90% and 100%) and dealcoholized by passing through graded series of xylene : alcohol solutions up to 100% xylene, 5 minutes for each step. The tissues were then embedded into paraffin and left setlled down for 24 hours. Tissues embedded in blocks of paraffin wax were sectioned at the thickness of 6 to 12 µm using rotary microtome. The sections were stained by safranin solution and counter stained with fast green solution, and then mounted in canada balsam. The epidermal peels of both upper and lower surfaces of leaves were made manually. The median area of the leaves were placed on a clean glass slide, and then the epidermis of the desired surface was peeled-off carefully with sharp razor blade. The epidermal peels were stained in 1% aqueous solution of safranin for 5 minutes, rinsed carefully in water to remove excess stain and then mounted in 10% glycerol. Data on anatomical characters were obtained based on the examination of five slides replicate under Nikon SE light microscope at the magnification of 10x40, and captured using OptiLab microscope camera.

Table 1: List of anatomical characters examined on 10 *Piper* species

No	Character code	Anatomical character
		Leaf
1.	EPC	thickness of epidermis cell wall and cuticula
2.	UPT	thickness of upper epidermis
3.	PAT	thickness of palisade tissue
4.	SPT	thickness of spongy tissue
5.	MET	thickness of mesophyll
6.	LPT	thickness of lower epidermis
7.	UPL	length of upper epidermis cells
8.	UPW	width of upper epidermis cells
9.	LPL	length of lower epidermis cells
10.	LPW	width of lower epidermis cells
11.	STL	length of stomatal cavity
12.	STW	width of stomatal cavity
13.	STI	stomatal index *
14.	PER	ratio of palisade to epidermal cells *
15	SCN	number of secretory cells *
16.	SCD	diameter of secretory cells
17.	STN	number of stomata *
18.	PAD	density of palisade *
		Stem
19.	EPT	thickness of epidermis outer cell wall and cuticula
20.	COT	thickness of cortex area
21.	COD	diameter of cortex parenchyma cells
22.	SED	distance between schlerenchyma and epidermis
23.	SCT	thickness of schlerenchyma layer
24.	SCD	diameter of schlerenchyma cells
25.	SWT	thickness of schlerenchyma cell wall
26.	SET	diameter of stele
27.	PVL	length of peripheral vascular bundle
28.	PVW	width of peripheral vascular bundle
29.	MVL	length of medullar vascular bundle
30.	MVW	width of medullar vascular bundle
31.	PVN	number of peripheral vascular bundle *
32.	MVN	number of medullar vascular bundle *
33.	PID	diameter of pith

Note: measurement was made in µm, except for those marked with asterisk (*)

A total of 33 anatomical characters on 10 *Piper* species were observed in this study. These 33 characters consisted of 15 stem anatomical characters and 18 leaf anatomical characters (Table 1). The data is analyzed descriptively by comparing the anatomical characters between species.

Results

Results of the observation and measurement on 33 anatomical characters used in this study was presented in Table 2.

Piper leaves were dorsiventral in structure which was indicated by clear differentiation of palisade and spongy parenchyma composing the mesophyll. In general the anatomy of *Piper* leaves was consisted of epidermis, mesophyll, and vascular bundles. The epidermis showed two different shapes of epidermal cells, the cuboid and rectangular. These epidermal cells has a modification in structure with the presence of papillae (Figure 1, a and b). The outer side of epidermis was covered with a thick layer of cuticle. Tetracyclic stomatas were located at the lower surface of the leaves. Two kinds of trichomes were present, the multicellular glandular and non-glandular trichomes (Figure 1, c and d).

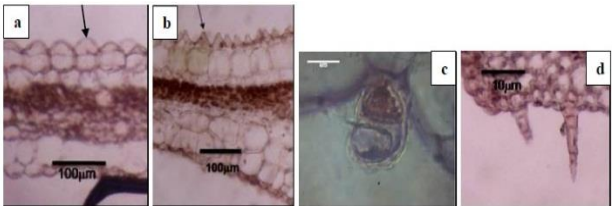

Figure 1. Variation on the shape of epidermal cells and trichomes:
(a) rectangular epidermal cells with rounded papillae, (b) cuboid epidermal cells with pointing papillae, (c) glandular trichome, (d) non-glandular trichome.

Densely arranged collenchyma cells was found in the sub-epidermal area of the midrib which could be clearly distinguished from the surrounding parenchyma cells based on their smaller size and thicker cell wall. Secretory cells were sparsely distributed in the sub-epidermal area, in the mesophyll, and the parenchymatic tissue around the midrib. The occurrence of secretory canals could be found in mesophyll or sub-epidermal area. These secreatory canals could be recognized by their appearance as round cavity surrounded by dense epithelial cells (Figure 2).

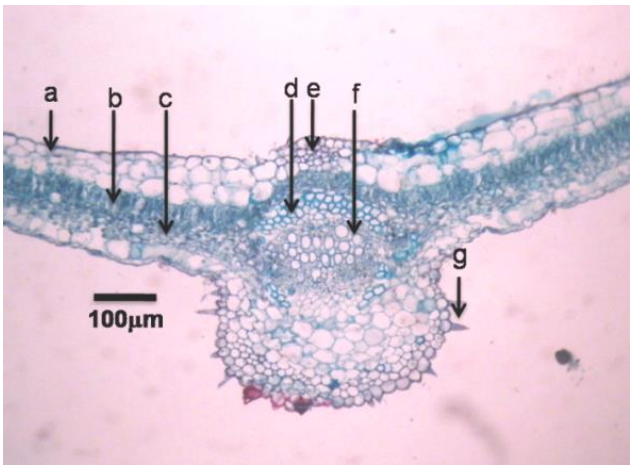

Figure 2. Cross section of *Piper betle* leaves: a. epidermis, b. palisade parenchyma, c. spongy parenchyma, d. sclerenchyma, e. collenchyma, f. vascular bundle, g. trichome.

The big secretory canal located above the midrib is very distinctive character on *Piper* leaves was shown in Figure 3.

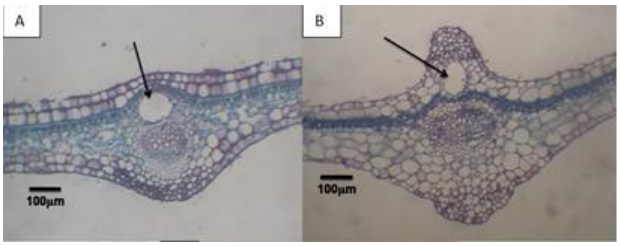

Figure 3. Secretory canal on the leaf of *P. crocatum* (A) and *P. lowong* (B)

Observation on leaf anatomical characters of 10 *Piper* species revealed differences on the shape of epidermal cells, number of epidermal layer, the occurrence of secretory cells, thickness of palisade parenchyma, and thickness of spongy parenchyma. Most of the species had epidermal cells of cuboid to rectangular shapes. Structural modification on the epidermal cells was observed such as conical papillae, as found in the epidermis of *P. porpirophyllum*.

In general the stem is consisted of epidermis, cortex, and the cental part which occupied the largest portion of the stem, consisted of parenchyma tissue with scattered vascular bundles, defined as the stele. These three parts showed rather continuous gradual transition, and thus make them less clearly distinguished from one part to another. Epidermal cells were cuboid to rectangular in shape with convex outer cell wall, where two kinds of non-glandular trichomes were found. The parenchyma tissue composing cortex consisted of polygonal, thin-walled cells. Some species has secretory cells within the cortex which could be recognized from their rounded shape and their reflective appearance from surrounding parenchyma cells. The occurrence of secretory cells was found only on three species, *P. betle, P. baccatum,* and *P. lowong*. Meanwhile the existence of secretory canals was observed in five species of *Piper*, they were *P. firmum*, *P. caninum*, *P. lowong*, *P. flavomarginatum* and *Piper betle*. Comparison on cross section of *Piper* stem showing variations on number, size, and location of secretory canal was presented in Figure 4. Stele occupied major part of the stem, consisted of vascular bundles and parenchyma tissue. The vascular bundle of all *Piper* species was closed-collateral type, with phloem located at the outer part of the xylem. Two groups of vascular bundles were recognized based on their location, i.e., the peripheral vascular bundles on the outer side of sclerenchyma cylinder, and the medullary vascular bundles on the inner side. The number of these two groups of vascular bundles varied among species.

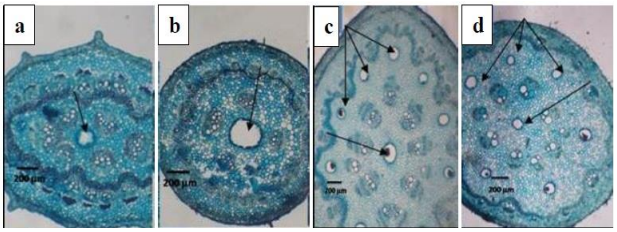

Figure 4. Comparison of stems cross section on four *Piper* species, showing different number, size, and location of secretory canal (arrow): (a) *P. firmum*, (b) *P. caninum*, (c). *P. flavomarginatum*, (d) *P. betle*

Table 2: Quantitative variation on 33 anatomical characters of leaf and stem on 10 *Piper* species

	P. bac.	*P. betle*	*P. por.*	*P.fir.*	*P. acu*	*P. low*	*P. fla*	*P. cro*	*P. maj*	*P. can*
EPC	16,2	14,2	9,3	4,9	2,0	4,5	17,4	6,9	3,4	4,8
UPT	38,4	17,1	21,6	35,5	43,9	10,8	35,1	25,7	14,6	19,4
PAT	35,6	33,8	35,0	36,0	48,6	32,7	33,0	31,0	19,8	31,7
SPT	44,9	41,1	53,9	39,8	78,3	42,2	155,8	41,8	19,3	53,3
MET	77,3	72,4	87,3	76,0	118,4	37,3	84,9	70,0	41,0	58,6
LPT	22,6	10,9	17,3	13,4	17,5	19,2	30,6	20,9	10,1	22,4
UPL	58,5	35,9	41,6	65,9	65,8	38,5	74,1	42,8	28,6	43,1
UPW	38,4	17,1	17,3	34,9	43,3	17,2	47,7	25,5	12,8	18,8
LPL	48,9	30,3	35,5	24,1	44,4	33,6	53,1	25,7	18,8	40,6
LPW	22,6	10,9	21,6	23,8	27,0	19,6	32,6	22,6	19,0	30,9
STL	10,8	12,0	7,1	19,3	16,8	11,0	8,1	14,4	6,2	11,0
STW	3,5	3,2	2,5	5,1	6,0	3,3	2,3	3,4	1,5	3,5
STI	13,3	14,2	11,6	7,7	5,2	6,0	14,6	10,4	11,5	12,5
PER	3,0	3,0	4,0	5,0	7,0	3,0	6,0	5,0	4,0	5,0
SCN	6,0	6,0	6,0	5,0	23,0	28,0	14,0	2,0	1,0	3,0
SCD	30,1	30,6	26,0	28,9	19,7	28,7	22,4	26,5	23,8	20,1
STN	5,4	5,8	4,2	2,8	2,3	1,8	5,6	3,5	5,0	3,3
PAD	5,2	8,6	7,4	5,0	6,2	6,2	8,0	8,4	9,6	7,4
EPT	10,5	15,0	5,0	7,0	5,2	4,9	20,1	8,6	6,8	10,8
COT	456,2	674,3	585,6	89,7	171,6	166,8	269,2	188,0	129,8	186,2
COD	30,4	25,8	32,8	21,9	33,5	31,9	17,3	23,7	26,7	23,8
SED	209,8	204,4	240,6	298,0	264,0	231,0	355,8	214,4	184,0	220,0
SCT	80,1	76,9	66,5	74,2	79,0	61,0	74,6	97,3	76,4	56,6
SCD	20,3	19,0	15,3	13,6	15,1	13,7	16,8	10,2	13,4	27,4
SWT	3,7	4,7	7,0	4,8	4,5	3,8	2,9	2,6	2,7	7,6
SET	947,1	1450,0	1234,0	1437,0	749,1	1495,0	1871,0	1962,0	848,5	778,7
PVL	140,6	76,7	230,4	112,9	152,8	119,0	141,0	215,6	116,2	100,2
PVW	136,2	69,3	185,8	125,0	164,6	78,1	177,8	153,1	84,4	74,5
MVL	274,2	346,8	241,6	265,2	264,6	145,6	249,8	281,8	197,6	240,8
MVW	282,8	299,4	176,8	266,8	285,4	138,4	313,2	336,4	226,4	198,0
PVN	20,0	16,0	30,0	22,0	24,0	12,0	29,0	20,0	15,0	20,0
MVN	5,0	12,0	13,0	6,0	11,0	5,0	11,0	9,0	7,0	5,0
PAD	396,8	737,6	673,4	815,5	152,1	474,4	508,1	583,9	413,7	370,1

Note: measurement was made in µm, except for those marked with asterisk (*)

Discussion

There were notable variations on the number of epidermal layers in leaf cross section among *Piper* species. *P. baccatum*, *P. caninum*, *P. acutilimbum*, *P. crocatum*, and *P. majusculum* had two layers of epidermis on both upper and lower leaf surfaces, while *P. firmum* had three layers on both surfaces. *P. betle*, *P. porpirophyllum* and *P. lowong* had three layers on upper surface and two layers on lower leaf surface, whereas *P. flavomarginatum* had three epidermal layers on its upper leaf surface and two layers on

lower surface. The occurrence of multiseriate epidermis was common for *Piper* as a result of periclinal cell division of protoderm (Fahn, 1990). The occurrence of multiple epidermis have been observed in species of *Piper*, as reported by Raman et al. (2012).

Glandular trichomes on leaves provide a characteristic feature for identification and classification of plants on genus and species level. In the case of *Piper*, the occurrence of trichomes on their leaves was related to their properties as oil-producing organ, since glandular trichomes were involved in the secretion of essential oils (Cutler, 1978; Fahn, 1990). The existence of trichomes has been reported as a character for differentiating varieties or species of Piper (Machado et al., 2015).

An apparent variation on the thickness of palisade layer was an interesting fact. The thickest palisade layer was found on *P. baccatum* (48.12µm), and the thinnest one was found on *P. betle* (25.12 µm). It was suggested that environmental factor such as light intensity might affect the thickness of palisade (Cutler, 1978; Fahn, 1990). In this case, plants grow in the environment with high light intensity had thicker palisade than those grow in shaded places. Variations in the characteristics of the palisade are common to *Piper*, as stated in which the number of layers of palisade and spongy parenchyma may vary (dos Santos et al., 2015).

The stem of 10 *Piper* species showed a special type of vascular bundle which differed from the normal open-collateral type on Dicotyledoneae. The vascular bundles found in stem of *Piper* were closed-collateral type, which were distributed in both cortex and stele. This structure is called as atactostele. This type and distribution of vascular bundles were characteristic of Piperaceae family (Simpson, 2006). The same result was reported for *Piper amalago*, and also for other species of Piper, that vascular bundles are arranged in two circles within the vascular cylinder, and the arrangement of two concentric circles bundles is separated by sclerenchyma (dos Santos et al., 2015).

The occurrence of vascular bundles in cortex and stele brought a consequence that they were differentiated into peripheral and medullary bundles. There were variations on the ratio of medullary to peripheral vascular bundles. The ratio of these two vascular bundles in *P. majusculum, P. crocatum, P. acutilimbum,* and *P. lowong* were 1:2. The ratio in *P. firmum* dan *P. flavomarginatum* were 1:3, while the ratio in *P. caninum,* and *P. baccatum* were 1:4.Variation on the ratio of medullary and peripheral vascular bundles which was calculated based on the number of medullary vascular bundles to those of peripheral could not be considered as distinguishing characters from taxonomical point of view. This variation was reported as being affected by the age of plants and stem size.

Another variation on stem anatomy of 10 *Piper* species was the occurrence of secretory cells and canals. The variation could be in the position or the number of the secretory glands in particular tissues. In some species, secretory cells were found in both cortex and stele. The same phenomenon was observed for secretory canal characterized by its circular shape surrounded by a layer of epithelial cells. In this case, it was noted that secretory canals in *Piper* were formed as a result of schizogenous process (Lakshmi and Naidu, 2010). Observation on the distribution and number of secretory canals revealed differences among *Piper* species, that this kind of canals did not form a continuous channel along the stem.

The existence of schlerenchyma forming a cylindrical structure served protective and supportive functions on the stem. This phenomenon could be a compensation for the under-developed intervascullary cambium on medullary vascular bundles which resulted to the lack secondary growth. This structure was common for plants with creeping and climbing habits, in which schlerenchyma fibers were found in the inner part of cortex and on the peripheral part of stele such as on *Aristolochia* dan *Cucurbita* (Esau, 1977; Evert, 2006).

Anatomical study on medicinal plants is important for the purpose of species characterization in relation to the localization of pharmacologically active compound, as suggested by Ferreira et al. (2011). Moreover, Hartini and Nugroho (2017) stated that the neolignan concentration in the leaves of *Piper crocatum* is higher than that of the stem or flower. This is because neolignan is formed through the shicimic acid pathway. Some precursor compounds in the shicimic acid pathway are synthesized or accumulated in the plastids. Moreover, leaves are organs with high plastids content. This the reason why leaves contained the highest neolignane compared to those of other organs. In the genus *Piper*, anatomical study is very essential as supporting data for species identification and it is closely related to phytochemical aspects to distinguish the original species from their substitute materials and to avoid adulteration of materials as mentioned by Mubeen et al. (2014). In this case the

occurrence of secretory cells and secretory canals is one of the anatomical charateristics in *Piper*. The important role of anatomical study for correct identification of medicinal plants was also revealed by dos Santos et al. (2018) for *Piper caldense* and by Babu et al. (2018) for *Ipomoea pes-tigridis*.

Conclusion

The anatomy of 10 *Piper* species showed variation on the quantitative characters.The variation on leaf anatomy were the shape of epidermal cells, number of epidermal layer, the occurrence of secretory cells, thickness of palisade parenchyma, and thickness of spongy parenchyma. Meanwhile, anatomical variations found on the stem were the ratio of medullary to peripheral vascular bundles and the occurrence of secretory cells and canals.

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Contribution of Authors

Nugroho LH: Conceived Idea, Designed Research Methodology, Manuscript final reading and approval.
Sutikno: Conceived Idea, Designed Research Methodology, Literature Search,
Susandarini R: Literature Review, Data Interpretation, Manuscript Writing.
Yuliati IR: Literature Search, Data Collection.
Priyono Y: Literature Search, Data Collection.
Munawaroh E: Literature Review, Data Interpretation.
Astuti IP: Literature Review, Manuscript Writing.

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