HAYATI
Journal of Biosciences

Vol. 27 No. 1, January 2020 1-7
DOI:10.4308/hjb.27.1.1
ISSN: 1978-3019
EISSN: 2086-4094

Localization of Alkaloid and Other Secondary Metabolites in *Cinchona ledgeriana* Moens: Anatomical and Histochemical Studies on Fresh Tissues and Cultured Cells

Dian Rahma Pratiwi[1‡], Yohana Caecilia Sulistyaningsih[2], Diah Ratnadewi[2*]

[1]*Plant Biology Study Program, School of Post-Graduate Program. Bogor Agricultural University, Darmaga Campus, Bogor, Indonesia*
[2]*Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Darmaga Campus, Bogor, Indonesia*

ARTICLE INFO

Article history:
Received August 20, 2018
Received in revised form November 10, 2019
Accepted November 15, 2019

KEYWORDS:
Cinchona ledgeriana,
secretory cells,
histochemical test,
secondary metabolites

ABSTRACT

Cinchona ledgeriana **produces several secondary metabolites. The main quinoline alkaloid, quinine that is widely used as an antimalarial drug, is most commonly extracted from the bark of *Cinchona*, and its leaves contain several other metabolites. Many studies have revealed that cell culture of *Cinchona* also produces quinine. Nevertheless, the sites of secondary metabolites accumulation are still elusive. This study is aimed at describing specific anatomical structures where alkaloids and some other secondary metabolites are accumulated as well as their localization in leaves and barks of *C. ledgeriana*, compared to those found in cultured cells. Fresh leaves and barks, and cells of *C. ledgeriana* were used for anatomical observation and histochemical tests. It was found that these plant parts have specialized structures, idioblast cells with elliptical- and spherical-shapes, scattered in leaf hypodermis, stem cortex, and secondary phloem. Unspecialized structures such as epidermis and palisade mesophyll tissues were also found accumulating some metabolites. Histochemical tests showed that bark and leaves contained alkaloids, terpenoids, phenolic, and lipophilic compounds. Cultured cells presented positive results for alkaloids and terpenoids.**

1. Introduction

Cinchona ledgeriana (Rubiaceae) is widely known as yellow bark or ledger bark, an important source of quinoline alkaloids. The major quinoline alkaloids are composed of quinine, quinidine, cinchonine, and cinchonidine. Quinine is the main quinoline alkaloid found in the bark of *C. ledgeriana*. It can reach from 5-7% to 14%. Quinine was used as antimalarial drug for centuries prior to the advent of synthetic an antimalarial medicine (Staba 1988). Quinine is extensively used as an antipyretic drug and a tonic. In the soft drink industry, quinine is used as a bitter substance. It is also useful in tanning industry and as cosmetic ingredients (Anderson *et al.* 1986; Noriega *et al.* 2015). Other secondary metabolites such as terpenoids, phenolics, lipophilic compounds, and flavonoids are usually produced by *Cinchona* plants for adaptation and self-defense, which are also useful for human beings.

The bark is the most common tree part which is extracted for alkaloid, far before people revealed that its leaves, root, and seed also contain alkaloids (Anderson *et al.* 1986). Meanwhile, many studies have proven that cells culture from the *Cinchona* leaf can produce quinine (Verpoorte *et al.* 1985; Anderson *et al.* 1986; Robins *et al.* 1986; Ratnadewi *et al.* 2013). Organelle or specialized structures are expected to be places that accumulate certain secondary metabolites. Some specialized structures have been found containing secondary metabolites, such as idioblast, laticifer, salt gland, nectary gland, glandular trichome, and mucilage gland (Fahn 1979). In *Catharanthus roseus* cell culture, only one or two cells in a cluster have been detected exhibiting blue fluorescence from inside the vacuoles, indicating the presence of alkaloids (Neumann *et al.* 1983). Another study on cell aggregates of *C. roseus* has shown specialized structures, idioblast and laticifer, which accumulated alkaloids and terpenoids (Iskandar and Iriawati 2016). Even though many studies regarding the enhancement of alkaloids yield in *Cinchona* cells

‡ Current address: Indonesian Oil Palm Research Institute. Jl Brigjen Katamso No. 51, Medan-North Sumatra, Indonesia
* Corresponding Author
E-mail Address: dratnadewi@apps.ipb.ac.id

have been conducted, and the metabolites extraction from *Cinchona* bark has been practiced for centuries, the information of specific anatomical structures and the site of secondary metabolites accumulation is still lacking. Therefore, this study aims to explore specialized structures and their localization in *Cinchona ledgeriana* leaf and bark as well as *in vitro* cultured cells that accumulate particular secondary metabolites.

2. Materials and Methods

2.1. Samples Preparation

Plant materials of *Cinchona ledgeriana* clone QRC 315 were collected from Gambung-West Java. Fresh leaves samples were taken on the second and third positions from the top of the tree. Bark was collected from 75-100 cm above the root neck, with 10-20 cm length. The trees are 3 years old and have 2.0-2.5 m height. The collected samples were used for histochemical tests and anatomical studies.

2.2. Histochemical Tests on Fresh Tissues and Cultured Cells

Histochemical tests were carried out on fresh leaves and bark samples, without fixation or embedding. Leaf and bark were sectioned by using a razor blade to thin slices. Cuts were subjected to the following tests for alkaloids, terpenoids, flavonoids, lipophilic, and phenolic compounds. Wagner reagent was used for alkaloids detection; positive reactions were shown by brownish-red staining. As a negative control, the sample was soaked in 5% tartaric acid dissolved in 95% ethanol for 48 hours at room temperature, then treated with Wagner reagent (Furr and Mahlberg 1981). Terpenoids detection was carried out by 5% copper acetate. Brownish-yellow staining indicated positive reactions (Martin *et al.* 2002). Ferric trichloride was dropped and added with several flakes of sodium carbonate for the detection of phenolic compounds; positive results showed black or blackish green (Johansen 1940). Sudan IV was used for the detection of lipophilic compounds; positive reactions of the oil cells were marked by red or yellow to orange color (Boix *et al.* 2011). Flavonoids were detected by aluminum chloride, and then the observation was done under UV light. Positive reactions of flavonoids was demonstrated by the appearance of blue, yellow, or green colors (Guerin *et al.* 1971). For each procedure employed on the samples, slides without any treatment (blanks) were also mounted for negative controls. All documentations of the results were performed with microscope photography equipment, Optilab Advance.

Histochemical observation on the cells from *in vitro* cultures used the same method as with the fresh tissues. The cells were collected from 7 weeks old cultures having treated with certain elicitors. A small amount of cells were placed on an object-glass then smoothly scattered after staining. Then it was ready for observation.

2.3. Leaf Sections and Secretory Structure Observation

Leaf peridermal sections were made in semi-permanent preparations with whole-mount method to observe the secretory structures according to Sass (1951). Leaf was fixed in 70% alcohol, rinsed with distilled water for 3 minutes and soaked in a solution of 50% HNO_3 for 40 minutes, then rinsed again with distilled water for 3 minutes. Abaxial and adaxial parts of leaf were slashed using a razor blade, then soaked in 5.25% sodium hypochlorite for 3-5 minutes, rinsed with distilled water for 3 minutes. Then the samples were stained with 1% safranin. The sections were mounted in 30% glycerin.

Transversal and longitudinal sections of leaves and barks were made using fresh samples to observe their anatomy. The samples were slashed using a razor blade to obtain thin slices. Subsequently, sections were stained by 1% safranin then mounted in 30% glycerin.

The observations made on secretory structures found in the samples were their position and size, using Olympus C21 microscope, in 5 replications.

3. Results

3.1. Histochemical Evidence

The qualitative results of the histochemical tests employed on leaves and bark tissues of *C. ledgeriana* are summarised in Table 1. In bark and leaf, positive results were demonstrated for alkaloid, terpenoid, phenolic, and lipophilic compounds, while flavonoids showed a negative result. In leaves, the accumulation of metabolites was detected in epidermis, hypodermis, and palisade; alkaloids and terpenoids were found in hypodermal and palisade tissues, while lipophilic substance was in epidermis;

Table 1. Histochemical results on *Cinchona ledgeriana*'s tissues and cells

Metabolic group	Leaf				Bark			Cultured Cell
	Epi	Hyp	Pa	Spo	Co	Ph	Interspc	
Alkaloids	-	+	+	-	+	+	+	+
Terpenoids	-	+	+	-	-	+	+	+
Lipophilic	+	-	-	-	+	-	+	-
Flavonoids	-	-	-	-	-	-	-	-
Phenols	-	-	+	+	-	+	+	-

Secondary metabolites (+) present, (-) absent. epi: epidermis, hyp:hypodermis, pa: palisade mesophyll, spo: spongy mesophyll, co: cortex, ph: secondary phloem, interspc: intercellular space

phenols was in palisade. Cortex, secondary phloem, and intercellular spaces were the sites in the bark containing the secondary metabolites. The color intensity may correlate with the amount of substance accumulation. Palisade was the site where the concentration of alkaloids, terpenoids and phenols occurred intensively (Figure 1a, e, i). A bigger accumulation of lipophilic compounds was detected only in the epidermis (Figure 1m) compared to the other parts.

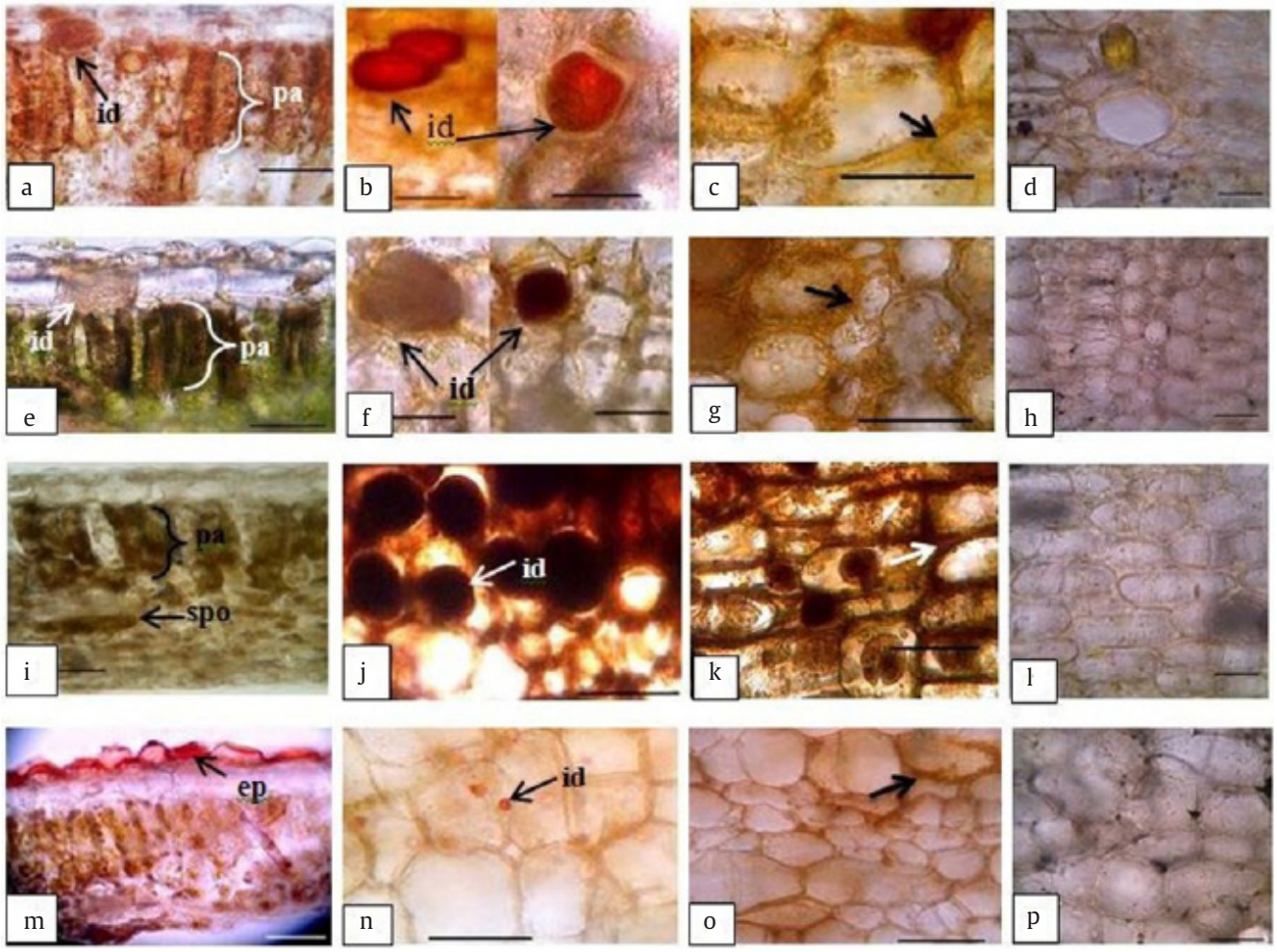

Figure 1. Secretory structure and tissues localizing secondary metabolites in fresh leaves and bark of *Cinchona ledgeriana*. Bar≈50 µm. (a) Alkaloids: idioblast cells in leaf hypodermis and palisade tissue, (b) idioblast cells in cortex and secondary phloem, (c) intercellular space of the bark, (d) negative control for alkaloids, (e) terpenoids: idioblast cells in leaf hypodermis and palisade tissue, (f) idioblast cells in secondary phloem, (g) intercellular spaces of the bark, (h) control, (i) phenols: leaf palisade and sponge, (j) idioblast cells in secondary phloem, (k) intercellular space of the bark, (l) control, (m) lipophilic substances: leaf epidermal tissue, (n) idioblast cells in cortex, (o) intercellular space of the bark, (p) control, ep: epidermis, pa: palisade, spo: sponge, id: idioblast cells. (d, h, l, p) The results of negative control for alkaloids, terpenoids, phenols, and lipophilic compounds

3.2. Anatomical Structure and Secondary Metabolites Storage Sites

The leaves and bark of *C. ledgeriana* revealed special anatomical structure accumulating secondary metabolites. Those structures are divided into specialized and unspecialized cells. The specialized structure found was idioblast. The idioblast cells have elliptical-shape and spherical-shape. The size of idioblast with elliptical-shape averages 35.10±4.64 µm length and 31.77±5.26 µm width, while idioblast with spherical-shape has a diameter of 14.70±3.18 µm on average. Unspecialized structures containing secondary metabolites were epidermis, hypodermis, palisade, and spongy mesophyll tissues (Figure 1).

Idioblasts accumulating alkaloids located in the leaf hypodermal tissues (Figure 1a), while in the bark, idioblasts were found in the cortex and secondary phloem (Figure 1b). Leaf palisade as well as intercellular spaces of the bark also indicated the presence of alkaloids (Figures 1a and c). Terpenoids were found in idioblast of leaf hypodermis and secondary phloem of the bark (Figures 1e and f), also in palisade and bark intercellular spaces (Figures 1e and g).

Idioblast cells accumulating phenols were only found in the secondary phloem of the bark (Figure 1j), while in leaf, phenol was detected in palisade and spongy tissues (Figure 1i). High color intensity of phenols was found in the bark intercellular spaces (Figure 1k). Lipophilic substances were detected in idioblasts located among the leaf epidermal tissue and in the cortex of the bark (Figure 1m and n). The bark apoplast also demonstrated lipophilic compounds (Figure 1o).

Histochemical tests on the cultured cells of *C. ledgeriana* revealed the existence of alkaloids and terpenoids. Phenols, lipophilic, and flavonoids examinations gave negative results. Alkaloids and terpenoids were accumulated in undifferentiated cells (Figure 2).

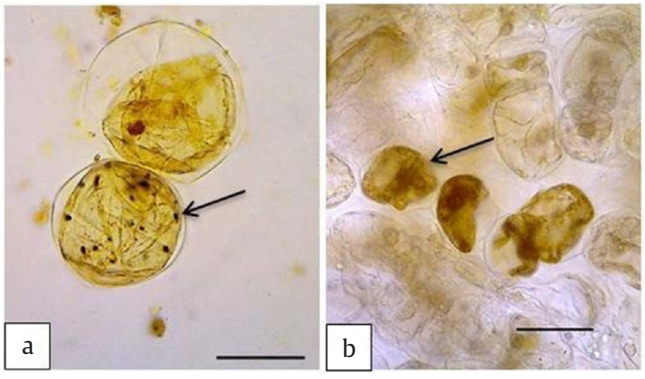

Figure 2. (a) Undifferentiated cells of *Cinchona ledgeriana* presenting positive results in the assay for alkaloids and (b) terpenoids. Bar 50 µm. Arrows show the accumulation of secondary metabolites in cells

4. Discussion

4.1. Structures Containing Secondary Metabolites

In *Cinchona*, it has previously been reported that idioblast cell contained calcium oxalate (Abdelgawad 2016). According to Metwaly (2015), quinoline alkaloids reside in the bark of *Cinchona* within the internal gland or canals, such as schizogenous, lysigenous, and schizo-lysigenous cavities. Those structures contain alkaloids, oils, volatile compounds, and resins. This research reconfirms that alkaloids, terpenoids, phenols, and lipophilic compounds were found in bark intercellular spaces (periderm) with various levels of color intensity which may indicate their amount of accumulation.

Idioblast cells in leaf hypodermis had never been reported in Rubiaceae, especially in *Cinchona* spp. Concerning hypodermis, however, *Psychotria carthagenesis*, which belongs to Rubiaceae, has a subepidermal layer in leaves adaxial (Moraes *et al.* 2011). The existence of this subepidermal/hypodermal layer has also been reported in *Cycas rumphii* and *Ceratozamia mexicana* from the family Cycadaceae which contained tannin (Vovides 1991). The subepidermal/hypodermal layer is formed by genetic factors and is not influenced by the environment (Coelho *et al.* 2013).

In barks, idioblasts in cortex contained alkaloids and lipophilic compounds, while those in secondary phloem localized alkaloids, terpenoids, and phenols. Alkaloids might be more abundant in the bark than the other metabolites since it was deposited both in the cortex and secondary phloem.

Flavonoids were not detected in this *C. ledgeriana*. Research conducted by Guerin *et al.* (1971) showed only flavon, flavonol, and flavanone that could be detected by $AlCl_3$ reagent, so it suggested that the flavonoids in *C. ledgeriana* were not from those types. Four flavonoids, catechin, kaempferol, apigenin, and quercetin in *Cinchona pubescens* were revealed through thin layer chromatography (TLC) analysis (Noriega *et al.* 2015) for TLC might be more sensitive for detecting compounds present in a small amount (Coelho *et al.* 2012). *Cinchona* leaf and bark tissues might have a very small amount of flavonoids that could not be detected through histochemical tests. Sundowo *et al.* (2017), however, reported that leaf of *C. ledgeriana* extracted by 70% ethanol contained flavonoids, tannins, saponins, other than alkaloids, and terpenoids.

In cultured cells, positive results were presented for alkaloids and terpenoids in undifferentiated cells, while phenol, lipophilic, and flavonoids tests performed negatively. The difference in secondary metabolites content between cultured cells and fresh tissues was commonly encountered. Differences may even be detected among cell lines of the same source (Constabel *et al.* 1981). Availability of precursors,

physiological conditions and genetic of the cells, made up by somaclonal variation, contribute to diversity in the quantity of products synthesized and accumulated (Vasil 2012).

4.2. Sites of Secondary Metabolites Biosynthesis and Accumulation

There are two cell types involved in the metabolism of secondary compounds, i.e cells in which biosynthesis and excretion of metabolites occur, and cells that absorb and store the metabolites (Verpoorte *et al.* 1985). If a secondary metabolite is detected in an organ or tissue, it is not necessarily suggested that the compound is synthesized in that part. The possibility of long-distance transport is to be considered. The site of biosynthesis may be determined from the site of the enzymes involved in the biosynthesis pathway of the secondary compound, or the presence of competent tissues to convert the intermediates into the secondary product (Wink 1987). The enzymes can be localized in certain tissues, and the products are then transported to other tissues. As an example, Hyoscyamine biosynthesis in *Hyoscyamus niger* takes place in the root pericycle. Through immunohistochemical assay, the accumulation of 6β-hydroxylase (H6H) that catalyzes the reaction of hyoscyamine to scopolamine is found only in intact root and root culture; from this root, the product scopolamine is transported to the aerial part via xylem or phloem (Hashimoto *et al.* 1991).

On the other hand, biosynthesis and subsequent accumulation of metabolites in certain cells or tissues might occur because the enzymes that control the biosynthetic pathway and their activity are localized in several compartments within the same cell (Facchini and St Pierre 2005). At the cellular level, enzymes can be found in the cytosol, endoplasmic reticulum, vacuole, or chloroplast (plastid) (De Luca and St Pierre 2000; O'Connor and Maresh 2006).

Enzyme activity is often restricted to a certain region of the plant. Cinchona alkaloids (quinoline alkaloids) belongs to terpenoid indol alkaloids (TIAs). Strictosidine synthase (STR), the key enzyme in the biosynthesis of TIAs, is most abundant in roots than in leaves and stems of *Catharanthus roseus* (Pasquali *et al.* 1992). STR in *Cinchona ledgeriana* demonstrated its activity in the upper stem, young leaves, and roots. In those parts there was a higher accumulation of TIAs (Aerts *et al.* 1991). TIAs biosynthesis, including quinolines, is very likely to occur mainly in leaves, then the intermediates or the final products are transported and stored in the bark (Verpoorte *et al.* 1985). The localization of TIA biosynthetic enzymes shows a complex relationship between intracellular and intercellular intermediates throughout the pathway (O'Connor and Maresh 2006). The other secondary metabolites have not been elicidated in Cinchona yet. From this research, the discovery of alkaloids in the bark intercellular spaces, in addition to idioblasts in the cortex and secondary phloem, convinces that the peridermal bark tissues is the major storage site of Cinchona alkaloids.

Alkaloids and terpenoids were detected in undifferentiated cultured cells. The whole-cell becomes the storage of metabolites due to the loss of compartmentation, compared with the existing compartmentation of intact plant cells (Vasil 2012). The presence of cells capable of storing secondary metabolites in culture is a common occurrence. Some of the cells can be distinguished from other cells like idioblast, but some are not easily distinguishable. Through transmission electron microscopy assay, the idioblast cell feature is remarkable by the presence of many small vacuoles in it; but other studies reported the difficulties in distinguishing idioblast cell morphology in cultured cells since the cytoplasm and its organelles resembled with the neighboring cells (Eilert *et al.* 1986).

Alkaloids and terpenoids detected in cell suspension culture suggested that unspecialized cells are capable of producing both substances in the absence of specific structures. It also ensures that in *Cinchona*, the enzymes and precursors involved in the biosynthetic pathway of those two compounds at low level also reside in the same cell, and cell differentiation is not a necessity.

5. Conclusion

Four secondary metabolites were found in the fresh tissues of *Cinchona ledgeriana*. In leaves, idioblasts contain alkaloids and terpenoids. Palisade mesophyll, a non-secretory tissue, accumulates alkaloids, terpenoids, and phenols. Lipophilic substances reside in the epidermal layer. The accumulation of alkaloids, terpenoids, phenols, and lipophilic compounds also occur in the bark periderm. The four secondary compounds might be synthesized in leaves; then, they are accumulated in

idioblast cells which are scattered in cortical tissues and secondary phloem. They are also present in the intercellular spaces of the bark.

Undifferentiated cells contain two kinds of secondary metabolites, i.e. alkaloids and terpenoids. Both are consistently produced in the fresh tissues and cultured cells. This finding suggests that the biosynthesis and accumulation of quinoline alkaloids and terpenoids can occur both in differentiated and undifferentiated cells.

Acknowledgements

The authors would like to acknowledge The Indonesia Tea and *Cinchona* Research Institute for supplying the plant materials of *Cinchona ledgeriana* clone QRC 315 for this research, and also to The Indonesia Endowment Fund for Education (LPDP) for the financial support to this research.

References

Abdelgawad S. 2016. Scheme for Description of Cinchona bark. Available at: https://www.slideshare.net/ShimaaAbdElGawad/scheme-for-description-of-cinchona-bark [Date accessed: 2 April 2017]

Aerts RJ *et al.* 1991. The distribution of strictosidine-synthase activity and alkaloids in *Cinchona* plants. *Planta* 183:536–541. DOI:10.1007/BF00194275

Anderson LA *et al.* 1986. *Aspect of alkaloid Production by Plant Cells Cultures*. Cambridge: Cambridge University Press.

Boix YF *et al.* 2011. Glandular trichomes of *Rosmarinus officinalis* L.: Anatomical and phytochemical analyses of leaf volatiles. *Plant Biosyst* 145:848-856. DOI:10.1080/11263504.2012.751067

Coelho VPDM *et al.* 2012. Anatomy, histochemistry and phytochemical profile of leaf and stem bark of *Bathysa cuspidata* (Rubiaceae). *Aust J Bot* 60:49-60. DOI:10.1071/BT11315

Coelho VPDM *et al.* 2013. Colleters in *Bathysa cuspidata* (Rubiaceae): Development, ultrastructure and chemical composition of the secretion. *Flora* 208:579-590. DOI:10.1016/j.flora.2012.08.00

Constabel F *et al.* 1981. Alkaloid production in *Catharanthus roseus* (L.) G. Don. VI. Variation in alkaloid spectrum of cell lines derived from one single leaf. *Plant Cell Rep* 1:3-5. DOI:10.1007/BF00267645

De Luca V, St Pierre B. 2000. The cell and developmental biology of alkaloid biosynthesis. *Trends Plant Sci* 5:168-173. DOI:10.1016/S1360-1385(00)01575-2

Eilert U *et al.* 1986. Ultrastructure of acridone idioblasts in roots and cell cultures of *Ruta graveolens* L. *Can J Bot* 64:1089-1096. DOI:10.1139/b86-149

Facchini PJ, St Pierre B. 2005. Synthesis and trafficking of alkaloid biosynthetic enzymes. *Curr Opin Plant Biol* 8:657-666. DOI:10.1016/j.pbi.2005.09.008

Fahn A. 1979. *Secretory Tissues in Plants*. London: Academic Press.

Furr M, Mahlberg PG. 1981. Histochemical analyses of laticifers and glandular trichomes in *Cannabis sativa*. *J Nat Prod* 44:153-159. DOI:10.1021/np50014a002

Guerin HP *et al.* 1971. Localisations histochimiques II: procédés simples de localisation de pigments flavoniques. Application à quelques Phanérogames. *Bull Soc Bot France* 118:29-36. DOI:10.1080/00378941.1971.10838874

Hashimoto T *et al.* 1991. Hyoscyamine 6 beta-hydroxylase, an enzyme involved in tropane alkaloid biosynthesis, is localized at the pericycle of the root. *J Biol Chem* 266:4648-4653.

Iskandar NN, Iriawati. 2016. Vinblastine and vincristine production on Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don) callus culture treated with polethylene glycol. *Makara J Sci* 20:7-16. DOI:10.7454/mss.v20i1.5656

Johansen DA. 1940. *Plant Microtechnique*. New York: McGraw-Hill.

Martin D *et al.* 2002. Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. *Plant Physiol* 129:1003-1018. DOI:10.1104/pp.011001

Metwaly A. 2015. Bark, Wood and Galls. Bark 1-Cinchona. Available at: http://www.slideshare.net/AhmedMetwaly3/bark-1 [Date accessed: 2 April 2017]

Moraes TMS *et al.* 2011. Comparative leaf anatomy and micromorphology of *Psychotria* spesies (Rubiaceae) from the Atlantica rainforest. *Acta Bot Bras* 25:178-190. DOI:10.1590/S0102-33062011000100021

Neumann D *et al.* 1983. Indole alkaloid formation and storage in cell suspension cultures of *Catharanthus roseus*. *JMPR* 48:20-23. DOI:10.1590/S0102-33062011000100021

Noriega P *et al.* 2015. Cosmetic antioxidant potential of extracts from species of the *Cinchona pubescens* (Vahl). *IJPNI* 2:1-4. DOI:10.15171/ijpni.2015.14

O'Connor SE, Maresh JJ. 2006. Chemistry and biology of monoterpene indole alkaloid biosynthesis. *Nat Prod Rep* 23:532-547. DOI:10.1039/b512615k

Pasquali G *et al.* 1992. Coordinated regulation of two indole alkaloid biosynthetic genes from *Catharanthus roseus* by auxin and elicitors. *Plant Mol Biol* 18:1121-1131. DOI:10.1007/BF00047715

Ratnadewi D *et al.* 2013. Enhanced production level of quinine in cell suspension culture of *Cinchona ledgeriana* Moens by paclobutrazol. *Biotropia* 20:10-18. DOI:10.11598/btb.2013.20.1.291

Robins RJ *et al.* 1986. Cell suspension cultures of *Cinchona ledgeriana*; I. Growth and quinoline alkaloid production. *Planta Med* 53:220-226. DOI:10.1055/s-2007-969128

Sass JE. 1951. *Botanical Microtehnique*. Iowa: Iowa State Coll Press.

Staba EJ. 1988. Alkaloid production from *Cinchona* cell and organ systems. In: Hanover JW (Eds.). *Genetic Manipulation of Woody Plants*. New York: Plenum Press. pp. 314-328.

Sundowo A *et al.* 2017. Phytochemical screening, total phenolic, total flavonoids contents and antioxidant activity of *Cinchona ledgeriana* leaves ethanol extract. In: *Proceedings of The 3rd International Symposium on Applied Chemistry (ISAC)*. Jakarta: AIP Conference Proceedings. pp. 1904:020067. DOI:10.1063/1.5011924

Vasil IK. 2012. *Cell Culture and Somatic Cell Genetics of Plants*. London: Academic Press Inc.
Verpoorte R *et al.* 1985. Plant cell and tissue culture of *Cinchona* species. In: Neumann KH, Barz W, Reinhard E (Eds.). *Primary and Secondary Metabolism of Plant Cell Cultures*. Berlin: Springer. pp. 196-208.
Vovides AP. 1991. Cone idioblasts of eleven cycad genera: morphology, distribution, and significance. *Bot Gaz* 152:91-99. DOI:10.1086/337867
Wink M. 1987. *Cell Culture and Somatic Cell Genetics of Plants*. New York: Academic Press.