Malaysian Applied Biology (2024) 53(6): 105-114
https://doi.org/10.55230/mabjournal.v53i6.14

Research

Anatomical and Histochemical Analysis of *Hoya pentaphlebia* MERR. Flower: Insights into Structure and Chemical Composition

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ABSTRACT

Hoya R.Br. is an epiphytic plant known as an exotic ornamental plant with distinctive, unique, and fragrant flowers. Investigating its floral structure is crucial for understanding how these structures may contribute to the production and storage of secondary metabolites emitted by *Hoya*. This study aimed to identify the type and position of floral glands in *Hoya pentaphlebia*. The investigations began by identifying the type and position of the floral glands, utilizing light microscopy, electron microscopy, and histochemical staining techniques. Secondary nectaries (*sn*) were discovered in the corona lobe, while conical-shaped glandular trichomes (unicellular) (*ct*) were at the adaxial epidermis of the corolla. The secretory activity of proteins, lipids, polysaccharides, and starch grains was found in *sn*, whereas *ct* detected only lipids and proteins. Subsequent studies to identify the secondary metabolite profiles characterizing aroma emitted from *H. pentaphlebia* flowers using gas chromatography-mass spectrometry (GC-MS) showed 26 compounds were identified, with the methyl salicylate (MeSA) compounds being the most abundant. In conclusion, this study successfully identified the floral glands and secondary metabolites present as aromas in the species studied. *Sn* and *ct* were discovered to be present for the first time in *H. pentaphlebia*, providing new information into the *Hoya's* floral structures. The presence of floral glands indicates the existence of secondary aromatic metabolites that play a role in the interaction between plants and numerous environmental elements.

Key words: Anatomy, GC-MS, *Hoya*, methyl salicylate, nectary, trichome

Article History
Accepted: 2 November 2024
First version online: 25 December 2024

Cite This Article:
Basir, S., Talip, N., Bunawan, H. & Rahman, R.A. 2024. Anatomical and histochemical analysis of *Hoya pentaphlebia* MERR. flower: Insights into structure and chemical composition Malaysian Applied Biology, 53(6): 105-114. https://doi.org/10.55230/mabjournal.v53i6.14

INTRODUCTION

Hoya R.Br. is an ornamental plant that is gaining worldwide popularity due to its distinctive, alluring flowers and ability to emit various aromas (Rodda & Simonsson 2022). *Hoya* is the most extensive genus in the Apocynaceae family, consisting of 350–450 species of succulent vines and shrubs that are native to Asia and Australasia (Rodda *et al*., 2022; Daawia *et al*., 2023). The distinctive shape and aromas of the *Hoya* flower have garnered widespread recognition as an exotic ornamental plant in Europe and the United States since 1970 (Mochizuki *et al*., 2017; Jayagoudar *et al*., 2024). Some studies indicate that *Hoya* has medicinal properties and has been used in traditional medicine for various purposes such as treating burns, swelling, orchitis, phthisis (TBC), pyroderma, sedatives, stomach aches, swelling, encephalitis, orchitis, and pyroderma (Don *et al*., 2021; Rumaling *et al*., 2024).

In general, plants synthesize and release most of the aroma as a medium of interaction and response to the environment (Hirata *et al*., 2016). Aromas are released from various plant parts, such as flowers, leaves, fruits, stems, and roots (Yue *et al*., 2015). Secretory cells in plants are specialized structures responsible for synthesizing and releasing aromatic chemicals, these cells have different

functions depending on their location within the plant (Basir *et al*., 2022a). The synthesis of these metabolites is frequently associated with specialized structures such as osmophores, nectaries, and glandular trichomes. These structures are commonly found in flower parts and serve as food rewards to attract pollinators, ensuring successful pollination (Wiemer *et al*., 2009). The structure and characteristics of cells involved in the synthesis of secondary metabolites in floral scents have not been thoroughly studied in most plant species.

Flower aromas are composed of a wide range of secondary metabolites, and it is estimated that there are more than 2000 metabolites that have been identified through field studies covering 991 species from 90 plant families (Dunkel *et al*., 2009). Various secondary metabolites from different groups, such as terpenoid compounds, aldehydes, fatty acid derivatives, amino acid derivatives, and phenylpropanoid or benzenoid compounds in floral organs, have attracted the attention of researchers to better understand the structure and function of secondary metabolites synthesized in these flowering plants (Dudareva *et al*., 2004).

Recent technical advancements have demonstrated that techniques like spectrometry (GC-MS) have proven to be faster, simpler, more sensitive, and solvent-free methods for analyzing aroma metabolite profiles emitted from plants (Deng *et al*., 2004). The development of technology in chemical analysis, making the aroma of flowers, has been exploited and used by humans in various industries such as perfumery, cosmetics, food flavoring, medicine, and agriculture (Dudareva & Pichersky, 2006; Mileva *et al*., 2021).

A recent floral anatomical and histochemical investigation of *H. cagayanensis*, *H. lacunosa,* and *H. coriacea* revealed the presence of secondary nectaries and simple trichomes (Basir *et al*., 2022a). The volatile composition of *Hoya* revealed that β-ocimene and MeSA compounds dominate the aroma in *H. cagayanensis*; the 1-octane-3-ol compound was found highest in *H. lacunosa*; (Z)-acid butyric, 3-hexenyl ester compound was found highest in *H. coriacea* (Basir *et al*., 2022b). The aroma release in plants is due to the biosynthesis of secondary metabolites found in plant tissues and cells (Ramya *et al*., 2013). Floral secondary metabolites are believed to act as one of the attractants of pollination agents to assist in the pollination process (Wiemer *et al*., 2009). Unfortunately, there have been no reports on the floral structure and chemical studies on *Hoya pentaphlebia*. Therefore, this study aims to identify the flora glands and their chemical composition in *H. pentaphlebia* flowers using a light microscope (LM), a scanning electron microscope (SEM), histochemical tests, and gas chromatography-mass spectrometry (GC-MS) to add new information and a better understanding of floral glands and their relation to the aroma profile emitted by flowers in the genus *Hoya*.

MATERIALS AND METHODS

Plant materials

First-day bloomed flowers of *Hoya pentaphlebia* used in this study were collected in November 2020 at Kedah Malaysia. For light microscopy, scanning electron microscopy, and histochemical tests, the specimens were fixed in Carnoy's fixative (a 1:3 mixture of acetic acid solution & 70% ethanol) (Puchtler *et al*., 1968). As for phytochemistry studies, the fresh blooming flowers were weighed at 5 grams and placed in 20 mL vials. Dry ice was used to retain the samples at the right temperature and humidity, preventing the flowers from wilting and being damaged. The samples were immediately kept at -80°C upon arrival at the laboratory. Three replicates were used in this study.

Floral structure analysis

Light Microscopy (LM)

Light microscopy (LM) was used to examine the anatomical structures in the flower parts. Cross-sections were obtained by using a sliding microtome (Leica SM 2000 R) and stained for 20 min in Safranin (Sigma-Aldrich) and Alcian Blue (Sigma-Aldrich). The slides were then dehydrated in a graded ethanol series (50, 70%, 90% & 100%) and were mounted using Euparal (Noraini *et al*., 2019). The anatomical structures were analyzed using an Olympus BX43 light microscope coupled to an Olympus DP72 camera (Olympus Optical Co.) and a Canon EOS 700D (Canon). The anatomical floral glands were imaged and analyzed through image processes in Analysis Docu and EOS Utility 2 software. Images of floral glands were studied at magnifications of 40× and 60×. The replicates were subjected to the same technique.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to examine the micromorphological structures in the flower parts. First, the samples were cut into 0.5 cm × 0.5 cm on a wax plate (Cavex) (Basir *et al*., 2022a) to protect the samples. Next, the samples were rinsed three times in phosphate buffer solution (PBS) (Sigma-Aldrich) (0.1 M, pH 7.4) for 10 min to maintain the pH of the biological samples. Following that, dehydration was carried out in a graded ethanol series (35%, 50%, 70%, 80%, 90% & 99%) for 10 min for each concentration and three times for 99% ethanol (Wiemer *et al*., 2009; Kowalkowska *et al*., 2015). The samples were then dried by critical point dried (CPD) (Leica® EM CPD300) (Leica) to eliminate the ethanol before being coated with gold for 10 min. Finally, the micromorphological structures were studied using a scanning electron microscope (FESEM, Carl Zeiss group) at magnifications ranging from 50×–1500×.

Histochemical analyses

Histochemical studies were performed on sections of corolla petals and the corona lobes region containing *sn* and *ct*. The presence of substances was detected using the following reagents: Sudan Black B (SBB) (Sigma-Aldrich) for total lipids (Demarco, 2017a). The periodic acid-Schiff reaction (PAS reaction) (Sigma-Aldrich) was used to identify the presence of polysaccharides (Spence, 2001). Lugol's reagent (LGL) (Sigma-Aldrich) for the existence of starch (Basir *et al*., 2022a) and Coomassie Blue (CB) (Sigma-Aldrich) was used to detect the presence of proteins (Demarco, 2017a). Three replicates were performed for each reagent. Photographs were taken with an Olympus DP72 camera and a Canon EOS 700D connected to an Olympus BX43 light microscope. Analysis Docu and EOS Utility 2 software were used to examine the structures at magnifications of 40× and 60×.

Phytochemical analyses

Solid Phase Micro Microextraction (SPME)

SPME fiber (DVB/C-WR/PDMS/10), grey color, needle 23 with 80m SPME (Agilent technologies) fiber coating was used for extraction. Before the extraction procedure, the SPME fiber was activated for 30 min by preheating it at 250°C in the CG-MS injector hole. The vials holding a 5-gram sample were then heated for 30 min at 35°C. Then, the SPME fiber was injected into the sample vials via a septum, with the PDMS-coated fiber part exposed to the sample space. Gas chromatography (Agilent 7890A) with mass spectrometry detector (Agilent 5975C), MSD (DB-5MS UI) (30 m × 0.25 mm × 0.25-µm) with 5% phenyl methylpolyxyxane fiber coating. The temperature of the column was raised from 50°C to 250°C (3°C/min), then to 250°C (5°C/min). The experiment was done three times for each species.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Quantitative analysis was carried out by MSD ChemStation software and identified using a library search of the National Institute of Standards and Technology's (NIST) database version 2.0. The analysis excluded compounds that were not generated from the plant. Compounds with less than 800 similarities and inversions were excluded from the final table. However, the percentage of compounds remaining was retained.

RESULTS

Floral anatomy and micromorphology

In cross-section, studies on the anatomical features show the several layers of nectariferous tissues in the epidermis and parenchyma layers of the corona lobe, a sign of the existence of secondary nectary (*sn*) (Figure 1b). In addition, the nectar pool (reward area) (R) was located adjacent to the corona lobe and corolla petals of the flower (Figure 1a). Corolla adaxial epidermis presents unicellular, conical-shaped glandular trichomes (*ct*) with non-uniform width and height (average width 40 µm, average height 98 µm) (Figure 1c, d & g).

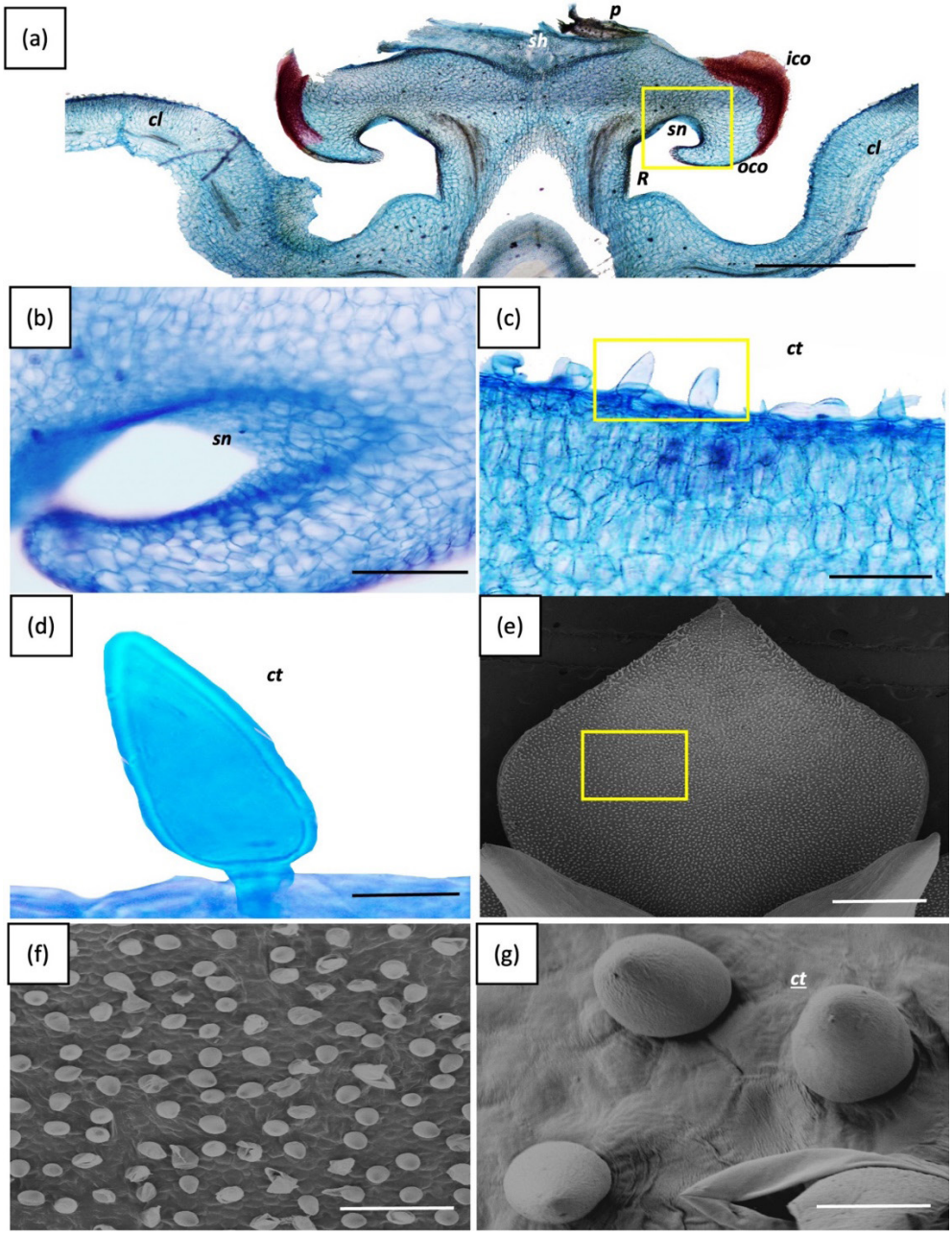

Fig. 1. Floral anatomical and micromorphological structure of *H. pentaphlebia.* (**a)** –(**d**) Anatomy features under light microscope (LM); (**b**) The overview cross-section of the flower. (**b**) Secondary nectaries (*sn*) were observed in the corona lobe (*co*); (**c**) Cross-sections of corolla petals (*cl*) showed the presence of unicellular conical-shaped glandular trichomes (*ct)* (yellow square); (**d**) enlargement of *ot* in (c). (**e)** –(**g**) SEM features of the corolla petals (*cl*);. (**f)** & (**g**) enlargement of (**e**) (yellow square), showed the presence of numerous *ct*. *cl* =corolla petals; *co* =corona; *ico* =inner corona; *oco* =outer corona; *sn* =secondary nectaries; *p* =pollinia; *R* =nectar pool; Scale: (**a**) = 1000 µm; (**b&c**) = 200 µm; (**f**) = 100 µm; (**d**) = 20 µm; (**g**) = 10 µm; (**e**) = 1 mm.

Histochemical

The histochemical test showed the presence of various chemical substances; polysaccharides, starch, protein, and lipid in *sn* and *ct* (Figure 2). The polysaccharides present in the *sn* were stained pink after the application of PAS reagents (Figure 2a). The abundance of starch grain was present with dark blue in the *sn* with Lugol's solution (Figure 2b). The Coomassie blue (CB) showed the presence of proteins in *sn* and *ct* (Figure 2c & e). The lipid droplets (blue-black) in *sn* and *ct* with Sudan Black B (SBB) (Figure 2d & f). Table 1 shows the accumulation of polysaccharides, starch grains, protein, and lipids in *sn* and *ct* of *H*. *pentaphlebia*.

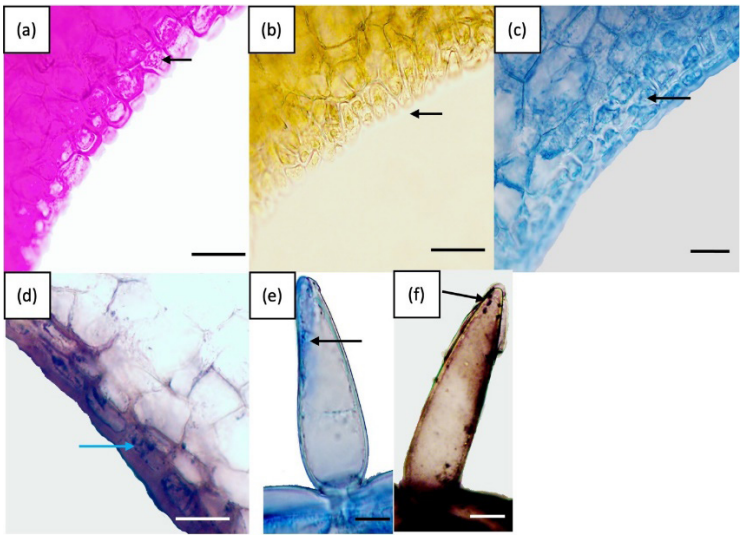

Fig. 2. Histochemical test of the secondary nectaries and glandular trichomes of *H. pentaphlebia*. (**a**)–(**d**) Secondary nectaries (*sn*); (**e**) & (**f**) conical-shaped glandular trichomes (*ct*). (**a**) Polysaccharides in *sn* stained pink after PAS reagent treatment; (**b**) starch grains were detected in *sn* (IKI) reagent; (**c**) & (**e**) s*n* and *ct* stained blue with protein (arrow, CB); (**d**) & (**f**) lipid droplets were detected in the s*n* and *ct* after (arrow, SBB). PAS = periodic acid-Schiff reaction; IKI = Lugol's reagent; CB = Coomassie Blue; SBB = Sudan Black B. Scale: (**a**),(**c**),(**d**) = 50 µm; (**b**) = 100 µm; (**e**) & (**f**) = 20 µm

Table 1. Histochemical test of secondary nectaries (*sn*) and conical-shaped glandular trichomes (*ct*) of *H. pentaphlebia* flowers

Tests	Target compounds	Colour observed	*sn*	*ct*
PAS reagent	Polysaccharides	Pink	+	-
Lugol's solution	Starch grains	Dark blue to black	+	-
Coomassie Blue	Protein	Blue	+	+
Sudan Black B	Lipids	Dark blue to black	+	+

Phytochemistry analyses

The volatile profile of a floral bouquet of *H. pentaphlebia* was determined using HS-SPME and GC-MS techniques. A total of 26 metabolite compounds were discovered during the experiment. However, only methyl salicylate (benzenoids) compound (Figure 3) was found to have a relative proportion more significant than 10% and dominate the floral aroma of *H. pentaphlebia* with a relative percentage of 59.46 percent (Table 2).

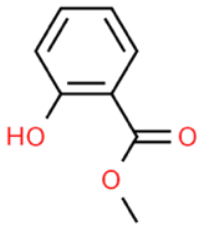

Fig.3. Methyl salicylate structure compound

DISCUSSION

In this study, the flowers of *Hoya pentaphlebia* showed the presence of floral secondary nectaries (*sn*) in the corona lobes. In addition, numerous nectareous tissue layers were detected in the corona lobes' epidermal and parenchyma layers as described by Basir *et al*., (2022a) for other *Hoya* species. This species' secondary nectaries features are similar to those reported for certain genera in the Asclepiadoideae. Additionally, secondary nectaries in this subfamily are composed of the epidermis in the staminal corona lobe or multiple layers of epidermal and parenchymal nectariferous in the circular region of the corona lobe (Monteiro & Demarco, 2017).

Table 2. List of secondary metabolites compounds found in flowers of *H. pentaphlebia***.** The relative amount (in %) of scent compounds is listed according to retention time (RT)

Formula	CAS. No	RT (min)	Compound	Relative percentage (%)
$C_4H_9NO_2$	003913-67-5	1.34	N-Methylalanine	0.46
$C_4H_8O_2$	000141-78-6	1.63	Ethyl Acetate	0.28
$C_2H_8O_2Si$	001066-42-8	2.16	Dimethylsilanediol	0.21
C_5H_8O	000107-86-8	3.33	Senecialdehyde	0.39
$C_6H_{12}O$	000066-25-1	3.58	Hexanal	1.89
$C_6H_{10}O$	006728-26-3	4.77	(E)-2-Hexenal	0.57
$C_8H_9NO_2$	1000222-86-6	6.05	methyl N-hydroxybenzenecarboximidate	0.20
$C_{10}H_{16}$	000080-56-8	6.70	α-Pinene	0.39
C_7H_6O	000100-52-7	7.62	Benzaldehyde	2.58
$C_8H_{24}O_4Si_4$	000556-67-2	7.99	Octamethyltetrasiloxane	1.43
$C_8H_{16}O$	003391-86-4	8.17	3-Octenol	5.44
$C_8H_{16}O$	000106-68-3	8.24	3-Octanone	2.70
$C_{10}H_{16}$	005989-27-5	9.33	D-Limonene	0.42
$C_{10}H_{18}O$	000470-82-6	9.42	Eucalyptol	0.26
$C_{10}H_{16}$	003779-61-1	9.53	trans-β-Ocimene	0.19
$C_{10}H_{16}$	003338-55-4	9.84	cis-β-ocimene	7.57
$C_{10}H_{16}$	000099-85-4	10.11	γ-Terpinene	0.14
$C_{13}H_{22}O_4$	1000373-80-3	10.49	Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl) propan-2-yl carbonate	0.30
$C_{10}H_{14}O$	000539-52-6	11.20	Perillene	0.30
$C_{10}H_{18}O$	000078-70-6	11.30	Linalool	1.98
$C_{10}H_{30}O_5Si_5$	000541-02-6	12.04	Decamethylcyclopentasiloxane	6.66
$C_{10}H_{16}$	007216-56-0	12.30	allo-Ocimene	0.22
$C_{10}H_{18}O_2$	014049-11-7	13.11	Linalool oxide	0.58
$C_8H_8O_3$	000119-36-8	13.91	Methyl Salicylate	59.46
$C_{10}H_{16}O$	000106-26-3	14.78	β-Citral	0.57
$C_{10}H_{16}O$	000141-27-5	15.49	α-Citral	0.55

Moreover, the histochemical tests showed the existence of polysaccharides, lipids, proteins, and starch grains as constant components of the nectar. Nectar stores sweet substances like sugars (sucrose, glucose & fructose), proteins, amino acids, and lipids (Chwil *et al*., 2019; Poinar & Poinar, 2020; Konarska & Masierowska, 2020) which play an essential role as a reward to pollinators such as insects, birds, and bats in the successful active pollination process. The presence of starch grain in floral glands indicates that starch is employed as an energy source in the synthesis of nectar and aroma released (Nepi, 2007; Pacini & Nepi, 2007). Recent studies have indicated the presence of nectar-secreting cells in angiosperm plants and have shown that these cells have undergone multiple evolutionary changes (Dudareva & Pichersky, 2006). The position of the secondary nectaries (*sn*) in the corona lobe is unique and described for the first time in the studied species, providing a new understanding of the structure of the *Hoya* flower.

Aside from that, this investigation also revealed the presence of conical-shaped glandular trichomes (unicellular) (*ct*) on the adaxial epidermis of the corolla petals. In the histochemical test, the *ct* in our study showed positive results for lipids and proteins. Trichomes are plants' physical resistance structures, such as hair growth from the epidermis, which can be unicellular, multicellular, elongated, prickly, or scaly (Hewson, 1988). Trichomes are exceedingly variable in shape, size, structure, and secretion materials and may have evolved due to several evolutionary processes (Huchelmann *et al*., 2017). There are two types of trichomes: glandular trichomes and non-glandular trichomes (Noraini *et al*., 2019). A trichome's type is determined by its form and ability to store or release primary or secondary metabolites that improve a plant's environmental function (Kolb & Muller, 2004; Demarco, 2017b). Secondary nectaries (*sn)* and conical-shaped glandular trichomes (unicellular) (*ct)* in this study were found near the nectar pool, which is a reward area that implies the presence of specific secondary metabolites involved in pollination, defense, and interactions with the surrounding environment (Kundan *et al*., 2019).

Aroma release in plants is often associated with the floral glands, secondary metabolites generated in specific cells, and genes involved in metabolite biosynthesis **(**Kundan *et al*., 2019**)**. Secondary

metabolites in plants were discovered to have multiple functions, such as protecting plants from pathogens, repelling pests, attracting pollination agents, and as a mechanism of plant interaction (Gershenzon & Dudareva, 2007; Abbas *et al*., 2017). For example, flowering plants have been discovered to generate and emit various distinct and specific aromas to attract particular pollinators (Grison-Pigé *et al*., 2002; Farré-Armengol *et al*., 2017).

Generally, terpenoids, phenylpropanoids, and benzenoids are the most common constituents of floral aroma (Xiang *et al*., 2007). In this study, methyl salicylate (MeSA) (benzenoids) was the major component of the volatile chemical compound of *H. pentaphlebia*. Benzenoid groups (benzaldehyde, benzyl alcohol, benzyl acetate, and MeSA) frequently contribute to floral scents (Knudsen & Tollsten, 1993; Springob & Kutchan, 2009). Furthermore, our previous study on the volatile compound in *Hoya* also found that MeSA is one of the higher compounds in *Hoya cagayanensis* (24.67%) (Basir *et al*., 2022a). In the other *Hoya* species studied MeSA is also one of the main volatile compounds in *Hoya carnosa* (Altenburger & Matile, 1988).

MeSA is widely released by plants and has a variety of roles in interspecific interactions, depending on the plant parts (Koschier *et al*., 2007). Plants emit MeSA in response to pathogen infection, insect feeding, and abiotic stress (Chen *et al*., 2003). In addition, MeSA is transformed into salicylic acid, an antibacterial molecule that stimulates plant defense responses and enables plants to develop resistance responses to recurrent pathogen invasion (Kobayashi, 2015). Furthermore, it is also herbivore-induced plant volatile that, depending on the dosage, can attract, repel, or discourage target species (Koschier *et al*., 2007). In some studies, the MeSA compound was found to attract pollinators (Brouat *et al*., 2000; James, 2003; De Boer & Dicke 2004). For example, MeSA was discovered to be a key attraction of *Eulaema*, *Euglossa*, and *Euplisia* bees, pollinators in most *Catasetum* orchid species (Hills *et al*., 1972). In most volatile mixtures, MeSA is not the main volatile. This methyl ester, on the other hand, is a substantial component of the flower volatile, for examples in *Clarkia breweri* (Onagraceae), the benzyl acetate constitutes up to 40% of the total scent, whereas the benzyl benzoate and MeSA only about 5% of the scents (Dudareva *et al*., 1998).

Based on the field observation, we found various ant visits on *H. pentaphlebia*. Brouat *et al*., (2000) study on *Leonardoxa Africana* showed that a high level of MeSA is released, and ant species of *Petalomyrmex phylax* species are drawn to MeSA and use it as a pheromone or as antiseptic for their nests. Plant chemicals may also elicit responses from ants because they are similar or identical to semi-chemicals produced by the ants themselves (Brouat *et al*., 2000). Other GC-MS studies showed that MeSA (12.8%) was one of the major compounds of the volatile extract of *Filipendula ulmaria* and subsequent studies in the behavioral assay's studies showed that although there was some evidence of attraction, 1,8-cineole showed little activity. Still, MeSA was substantially repellent (Chermenskaya *et al*., 2001). Therefore, it is likely that the appeal of the plant samples is due to a complex of chemical compounds rather than single compounds (Chermenskaya *et al*., 2001). However, synthetic MeSA attracts natural enemies under laboratory and field conditions, and commercial products have been produced for this purpose (Woods *et al*., 2011; Allsopp *et al*., 2014).

MeSA is acknowledged as a flavoring ingredient and is frequently synthesized to add flavor to various types of food (Effmert *et al*., 2005). MeSA is also used in diverse industries, such as cosmetics, perfumes, and medicine (Parker *et al*., 2004). MeSA is commonly used as a treatment for anti-irritant, analgetic, and local anesthetic purposes for alleviating muscle pain and stiffness (Parker *et al*., 2004). Therefore, this study on *H. pentaphlebia* species holds significant potential for commercialization and exploitation due to its high MeSA content, suitability for generating pure MeSA extract, and widespread use across diverse industries. Further studies using biological synthetic approaches can be conducted by expressing pathway genes to produce specific metabolites synthetically to achieve high concentrations of specific metabolites.

CONCLUSION

The anatomical and histochemical analysis of the *H. pentaphlebia* flower shows crucial insights into its structure, chemical composition, and ecological significance, which are important for fields such as horticulture, pharmacology, and conservation biology. In conclusion, this investigation successfully discovered two key floral structures: secondary nectaries (*sn*) and conical-shaped glandular trichomes (*ct*). Additionally, it revealed that MeSA metabolites are present in the highest concentrations in the aroma of *H. pentaphlebia*. These findings enhance our understanding of floral structures and secondary metabolite production, as well as the aroma profile emitted by flowers in the genus *Hoya*.

ACKNOWLEDGEMENTS
We would like to express our gratitude to the Faculty of Science and Technology at Universiti Kebangsaan Malaysia (UKM) for providing the instruments and facilities used in this study. Additionally, we wish to acknowledge the Scanning Electron Microscopy Unit FST, UKM and *Makmal Pencirian Struktur Molekul* (MPSM), Centre for Research and Instrumentation Management (CRIM), Universiti Kebangsaan Malaysia (UKM).

ETHICAL STATEMENT
Not applicable.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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