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# Anatomy and Histochemistry of Structures Producing Aroma in Leaves of *Sygyzium aromaticum* (L.) Merr. and *Clausena excavata* Burm. f.

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## ABSTRACT

Anatomical and histochemical studies on leaves of *Syzygium aromaticum* and *Clausena excavata* have been carried out. This study was conducted in order to investigate the relationship between aroma production and a plant's secretory structures. Leaves from the two tropical aromatic plants were sampled from the Institute of Bioscience (IBS) Conservatory Park and transversely sectioned through lamina, midrib and petiole with a sliding microtome for anatomical investigation. Through light microscopy, oil cells and secretory cavities were distributed near the adaxial and abaxial epidermal layers with large in size, up to 60 µm length. Other leaf anatomical characters such as shape of petiole and midrib, pattern of vascular bundle, palisade and spongy mesophyll, the presence or absence of brachysclereids and crystals are also observed. This study also aimed to investigate the leaf's secretory structures responsible for plants' aroma production and to detect the presence of terpenes and essential oil in secretory structures histochemically.

*Keywords*: Aroma, secretory structures, terpenes, essential oils, oil cells, oil cavities

## INTRODUCTION

Asia is well known throughout the world as the land of aromatic plants, a fame it enjoys due to its favourable climatic conditions, which are suitable for the

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growth and development of aromatic plants (Chomchalow, 2002). Aromatic plants contain volatile aromatic compounds, most of which make up the essential oils of plants. Since ancient times, aromatic plants have been the source of spices and herbs, traditional medicines as well as raw materials for essential oil extraction (Chomchalow, 2002; Joy et al., 2002). Brenes and Roura (2010) stated that a plant's secretory structures are found in every part of the plant including its leaves, flowers, fruit, bark and roots. Examples of secretory structures are oil glands, secretory cavities and glandular trichomes (Fahn, 1988; Svoboda, 2000; Cutler et al., 2008). In this study, two species of tropical aromatic plants, *Syzygium aromaticum* and *Clausena excavat*a, were studied.

*Clausena excavata* (Rutaceae) is classified as a small tree or shrub. It ranges in height as a shrub that is only 1-2 m tall to a small tree that can grow up to 10 m tall. The leaves appear as leaflets that are lanceolate to crescent-shaped, measuring 3-7 cm in length, while the fruit has a grape-like taste (Arbab et al., 2011). Recent studies of *C. excavata* have been restricted to investigating the chemical constituents of this medicinal plant and its contribution to antimicrobial, anti-fungal and anti-insecticide preparations (Guntupalli et al., 2012; Albaayit et al., 2015).

A previous study by Kamatou et al. (2012) recorded the presence of the chemical constituent, Eugenol, in the essential oil of *S. aromaticum* (Arras & Usai, 2001; Ayoola et al., 2008; Santos et al., 2009) and emphasised that Eugenol is the main volatile compound of extracted oil from clove buds that is used in traditional medicine, bactericides, fungicides and other preparations but no study was done on the secretory structures that produce the aromatic volatiles from the genus *Clausena*, especially *C. excavata*. Many studies on the leaf anatomy of Myrtaceae and Rutaceae have been conducted. Metcalfe and Chalk (1979) described the anatomical structure of the leaf as having basic cells such as the epidermis, mesophyll, vascular bundle, parenchyma, sclerenchyma that include specialised secretory cells. Later, histochemical studies enhanced the search for detailed information about plants' internal structures. Several studies had stated that members of Myrtaceae possess oil cavities or oil glands in their leaves (Fahn, 1988; Arruda & Victorio, 2015; Dickson, 2000). Khatijah and Ruzi (2006) revealed the presence of oil glands in the transverse section of the lamina, midrib and petiole of *S. aromaticum*. Al-Edany and Malik (2012) reported that secretory oil cavities are in the lamina near to both the adaxial and abaxial surfaces.

Essential oils are distributed at different parts of the plant including the leaves, flowers, fruit, bark and roots (Chamchalow, 2002; Chamorro et al., 2012). The leafy part of the plant possesses external secretory structures such as glandular trichomes, resin ducts or canals, whereas the internal secretory structures include lacticifers and oil cells (Cutler et al., 2008). Other studies describe secretory cells in the leaf

as idioblasts containing a variety of oil and mucilage as specialised cells contributing to the scent of the plant (Dickison, 2000). Glandular trichomes were found to secrete a number of secondary metabolites of terpenes, flavonoids, alkaloids and essential oils (Svoboda and Svoboda, 2000; War et al., 2012).

The essential oils were found accumulated at the sub-cuticular cavity of glandular trichomes. The oils diffuse outwards through the cuticle, covering the outer surface of the hair gland after the rupture of the cuticle (Svoboda and Svoboda, 2000). According to Dickison (2000), essential oils that are produced in glandular trichomes carry discrete aroma and taste to the plant part possessing them. Secretory cells are known as cells specialised to secretion of one or more, often organic substances. Cutler et al. (2008) stated that secretory cells can be single cells, groups of cells or even tissue. Morphologically, they are usually larger than the surrounding elements and sometimes resemble enlarged, densely staining parenchyma cells. Secretory cavities are spherical intercellular spaces that are lined with secretory cells and filled with secretory products including essential oils (Dickison, 2000; Svoboda & Svoboda, 2000). However, other published studies have reported the use of histochemistry to detect and locate the active components within the plant cells such as terpenoids, lipids, carbohydrates and proteins (Gersbach et al., 2001; Dubey & Trivedi, 2012; Bosabalidis, 2014; Hassan & El-Awadi, 2013). Greathead (2003) reported the oil itself is a complex mixture of secondary metabolites comprising low-boiling point and molecular weight of phenylpropenes and terpenes. Several studies on aromatic plants affirmed the presence of essential oil as the contributing factor in such essence of these plants (Joy et al., 2001; Figueirdo et al., 2008; Chamorro et al., 2012).

Studies on leaf anatomy with special reference to secretory structure in genera *Syzygium* and *Clausena* are very few. Although the leaves of *S. aromaticum* and *C. excavata* are well known for their applications of essential oil, few studies have identified the anatomical structures that produce and secrete such aroma from these plants. This study examined the relationship of the cells responsible for producing and secreting aroma as well as the histochemical identification of the secreted materials within a plant's leaf and, furthermore, the histochemical analysis determined the chemical compounds of terpenes and essential oils within the secretory structures, vascular bundle and parenchyma cells, and terpenes in the essential oil of the plants have proven to be responsible for the aroma of the two species studied.

## Histochemical Study

Wick (2012) and Hassan and El-Awadi (2013) described several applications of histochemistry in plant research, which includes the detection and localisation of cellular components of active constituents. The constituents include protein, lipids, carbohydrate as well as a range of ionic elements occurring in cell solutions. Over

the past decades, a variety of chemical reagents have been used in the study of plant histochemistry. Johansen (1940) successfully came up with some such as concentrated sulfuric acid ($H_2SO_4$), ferric chloride ($FeCl_3$) and ruthenium red. Later, Jensen (1962) introduced reagents such as Nile Blue and Schiff's reagent following Sudan III, Sudan IV, Sudan Black B (Lison, 1960), Nadi reagent (David & Carde, 1964), Vanilin-HCl (Guerin et al., 1971) and many more. In the colour-staining technique of histochemistry, specific chemical reagents were used to test different compounds within a cell through the change in colouration (Table 1). Bakker et al. (1992) studied the distribution and systematic value of two secretory structures (idioblast) in reference to oil cells and mucilage cells for the genus *Cinnamomum* Schaeffer (Lauraceae). Later, Geng et al. (2012) performed a histochemistry test on oil cells. They localised the main chemical classes of metabolites present in the oil such as aldehydes, lipids and terpenoids. Besides the histochemistry of oil and mucilage cells, a number of other secretory structures captured the interest of researchers such as glandular trichomes.

Gersbach et al. (2001) studied the peltate and capitate glandular trichomes distributed over the adaxial and abaxial leaf surface. In addition, Christodoulakis et al. (2013) studied the localisation of secreting sites as well as the identification of the secreted material in the leaf of a Mediterranean aromatic plant. The study of histochemistry was not restricted to leaf secretory structures only, but was also applicable for secretory structures present in the flower, stem and root. Sajwan et al. (2014) revealed the presence of oil glands and clusters of calcium oxalate crystals in the parenchyma cells of the hypanthium of *Syzygium aromaticum*. They later verified a positive test on the glands containing oil globules with red colouration when stained with Sudan III.

## MATERIALS AND METHOD

This study aimed to investigate the secretory structures involved in aromatic leaves. Fresh leaf samples were fixed in (FAA), then sectioned transversely (TS) through the middle part of the midrib, lamina and petiole using a sliding microtome, stained with safranin and alcian blue, dehydrated in a series of alcohol solutions and mounted in Euparal. For the histochemical study, sections were submitted to NADI reagent for detection of terpenoid compounds and Sudan Black B for detection of essential oil cells. All laboratorial procedures were conducted at the laboratory of the Biology Department, Faculty of Science (UPM) and the Laboratory of Anatomy and Microtechnique, Faculty of Science and Technology (UKM). Aromatic plants of *S. aromaticum* and *C. excavata* were sampled from the Conservatory Park, Institute of Bioscience, Universiti Putra Malaysia.

### Preparation of Specimens

Preparing specimens before the sectioning process is very important for obtaining successful results. In this experiment, the fresh specimens were prepared into two main categories. One was for the leaf

anatomy study, which used fixed samples, and another for the histochemical study that directly used fresh samples with no fixation. The fixatives used in this experiment were FAA (Formalin-Acetic acid-Alcohol). The FAA mixture follows methods by Metcalfe and Chalk (1979) with 90 ml of 50% alcohol, 5 ml of 99.9% acetic acid and 5 ml of 40% formalin. Upon outdoor sampling for leaf anatomy, each leaf sample was placed in a re-sealable plastic bag filled with FAA for structural preservation. On the other hand, another batch of leaf samples was separately soaked in distilled-watered plastic bags and submitted for histochemical study.

**Leaf Anatomy Investigation**

Transverse sections of the lamina, midrib and petiole illustrated the anatomy and secretory structures of the two species. The method of study followed the standard method derived from Johansen (1940), Jensen (1962) and Metcalfe and Chalk (1979) with slight modifications from Khatijah and Ruzi (2006), Cutler et al. (2008) and UKM Anatomy Laboratory technicians and staff. Leaf sections of *S. aromaticum* and *C. excavat*a were hand-cut into 1 $cm^2$ pieces using a razor blade. The chosen part of the leaf was then manually embedded in hard polystyrene before transversely-sectioned using a sliding Microtome Reichert (model Leica Jung histoslide 200) of 15-25 µm thickness according to the plant sample (Soukup and Tylova, 2014). The sections were cleared by soaking in 20% sodium hypochlorite (NaHCl) until they turned a white colour. The sections were rinsed two to three times with distilled water before being submitted to staining procedure with Safranin and Alcian Blue. Dehydration sections were treated in a series of alcohol of increasing concentration. The sections were mounted on a microscopic slide in Euparal as permanent medium. Finally, the slides were left to dry in an oven at temperature of 60°C for two weeks. The slides were observed and viewed under light microscope (model: Olympus CH20).

**Histochemical Investigation**

Similarly, the leaf lamina, midrib and petiole of two species were transversely sectioned using the sliding microtome as described above and then directly submitted to the following reagents: NADI reagent for the detection of terpene compounds and Sudan Black B for the detection of essential oil and total lipids (Machado et al., 2006).

**Staining Process of Histochemistry of Leaf Sample**

Two types of staining process were carried out in this study. The first stain used was the NADI reagent: 5 drops of NADI reagent (5 g of α-naphtol in 125mL of 50% ethanol + 5 g of N,N-dimethyl in 125mL of phosphate buffer of pH 7) were dropped onto tissue sections in a Petri dish for 15 min. The tissue sections were rinsed twice with distilled water, then transferred onto a glass slide using a camel brush and covered with a cover slip. Observations were made under a light microscope (model: Olympus CH20). The second stain was Sudan Black B: 5

drops of Sudan Black B (500 g SBB in 20 mL acetone + 15 mL AA + 35 mL d/w) were dropped onto tissue sections in a Petri dish. The tissue sections were rinsed twice with distilled water and were then transferred onto a glass slide using a camel brush and covered with a cover slip. Observations were made under a light microscope (model: Olympus CH20). The images of the sample slides were captured using a Leitz Diaplan light microscope at magnification 4X, 10X, 20X and 40X.

Table 1
*List of common chemical reagents used in plant histochemistry*

| Chemical Reagents | Target Compounds | Observed Colour |
|---|---|---|
| Sudan III & Sudan Black B | Total lipids/Essential oil | Orange red/Black |
| Mix of Sudan III & IV | Suberin | Red |
| Conc. $H_2SO_4$ | Sesquiterpene | Orange |
| Ferric chloride | Polyphenols/Phenolics | Emerald-green |
| Nile Blue | Neutral/Acidic lipid | Red/Blue |
| Vanilin-HCl | Flavanoids | Yellow |
| Ruthenium Red | Acid Polysaccharides/Mucilage/Pectin | Red/Pink |
| Schiff's reagent | Water insoluble polysaccharides/Aldehydes | Magenta-red |

## RESULTS AND DISCUSSION

The morphology of leaf anatomy described includes the shape of the petiole, pattern of vascular bundle, cuticle, abaxial and adaxial epidermis, palisade and spongy mesophyll, ground tissue, secretory (idioblast) cells, crystals and trichomes. The leaf's anatomical characteristic is described based on Melcalfe and Chalk (1979), Bakker et al. (1992), Baruah and Nath (2006), Cutler et al. (2008), Arruda and Victoria (2011), Muntoreanu et al. (2011), Al-Edany and Malik (2012) and Geng et al. (2012).

### Anatomical Features of *Syzygium aromaticum*

**Petiole.** The petiole outlines in transverse sections are U-shaped extending outwards in adaxial surface, whereas the abaxial surface is generally U-shaped (Figure 1A). There was an abundance of secretory cavities (up to 20) present in the ground tissue near the epidermis (Figure 1A and 1E). The general vascular system of the petiole was open type and crescent-shaped with intraxylary phloem and brachysclereid (Figure 1B). The parenchyma cells were observed to be of more than 10 layers. Idioblast tannin was present in parenchyma cells, appearing black in colour. Also present were druse and solitary crystals (Figure 1C).

**Midrib.** Based on observation, the adaxial surface was straight with an arc-shaped abaxial surface (Figure 3A). Secretory cavities were present in the ground tissue near the abaxial and adaxial surface (Figure 3A). *Syzygium aromaticum* has an open type and V-shaped vascular tissue with

intraxylary phloem. Sclerenchyma cells were present around the vascular bundle (Figure 3A). Druse and solitary cuboidal crystals were present in the parenchyma cells (Figures 3B and 3C).

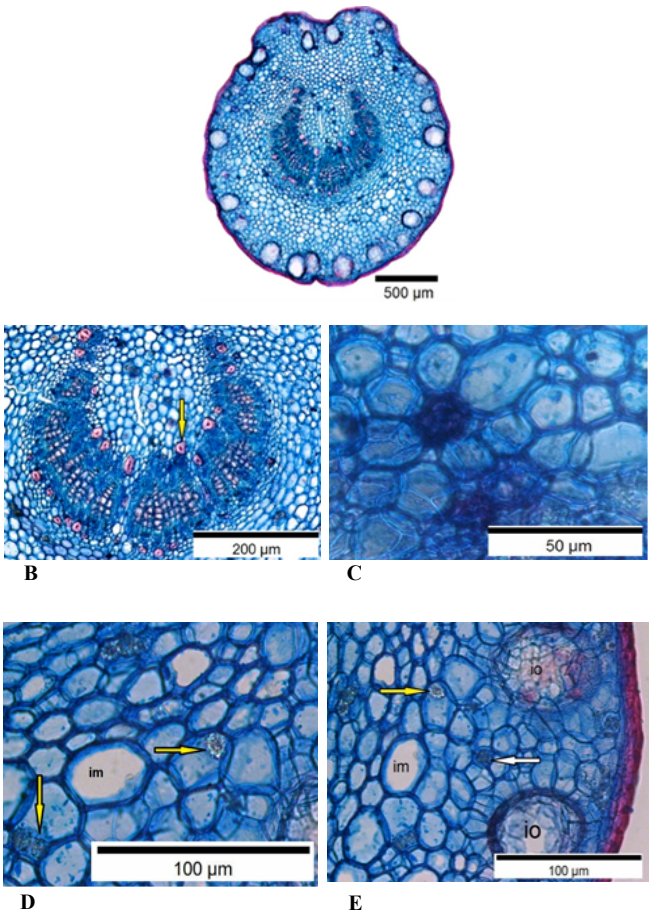


*Figure 1.* Transverse section of petiole *Syzygium aromaticum* (A) Sub-circular external shape of petiole, (B) Open-type vascular bundle with presence of intraxylary phloem; brachysclereid cell presence (yellow arrow); (C) Idioblast tannin cell; (D&E) Idioblast mucilage cell (im); Idioblast oil cells (io); Druse crystal (yellow arrow) and solitary crystal (white arrow) in parenchyma cell

**Lamina.** Based on observation, the adaxial epidermis was thicker than the abaxial epidermis. Secretory cavities were present between the palisade and spongy mesophylls (Figure 4A and 4D). The lamina also had an open-type vascular tissue (V-shaped) with intraxylary phloem (Figure 4A). In the parenchyma cells, solitary and druse crystals were present (Figure 4B and 4C).

## Anatomical Features of *Clausena excavata*

**Petiole.** The petiole outlines in transverse section had an irregular shape of the adaxial

surface and circular abaxial surface (Figure 2A). The vascular system was organised in a open-free vascular bundle (Figure 2A). The trichomes were unicellular and multiseptate, present around the petiole especially on the abaxial epidermis (Figures 2A and 2F). Secretory cavities were present in the ground tissue near the epidermis, and were observed to be obviously larger in size than the adjacent cells in the mesophyll (Figures 2A and 2E). Idioblast tannin stained dark red-black was present in the vascular bundle (Figure 2C). Also, brachysclereid cells occurred above the vascular bundle as well as in between the cells towards the abaxial of the petiole (Figure 2B). Solitary crystals with a diamond and cuboid shape were present in the parenchyma cell (Figures 2B and 2 E).

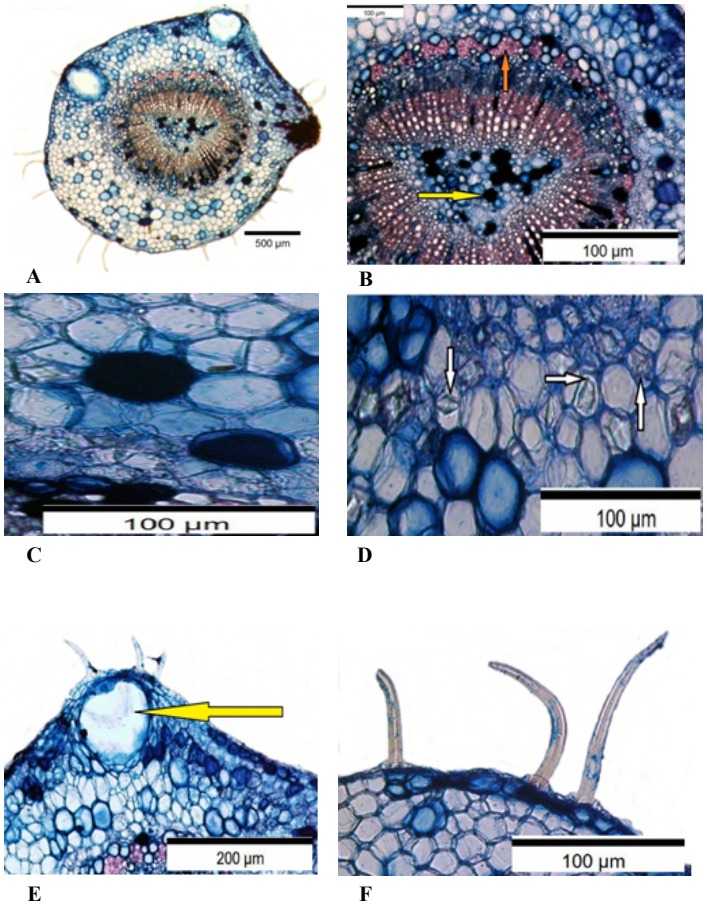


*Figure 2.* Transverse section of petiole *Clausena excavata* (A) Circular external shape of petiole and pattern of vascular bundle; (B) Sclerenchyma cells (brown arrow) above the vascular bundle; tannin cells (yellow arrow) in the vascular bundle; (C) Idioblast tannin in ground tissue with black colour; (D) Solitary crystals in parenchyma cells; (E) Idioblast oil cell (yellow arrow) near epidermis; (F) Unicellular (unbranched) and multiseptate trichomes

**Midrib.** Based on observation, both adaxial and abaxial surfaces had a convex shape (Figure 3E). The midrib had close-type vascular tissue surrounded with pericyclic fibres (Figure 5G). In the parenchyma cells, solitary rhombic crystals were found present (Figure 5F). Unicellular and non-glandular trichomes were present on the abaxial epidermis (Figure 5G). Secretory cavities were found in the ground tissue below the vascular bundle and near the epidermis (Figure 5H).

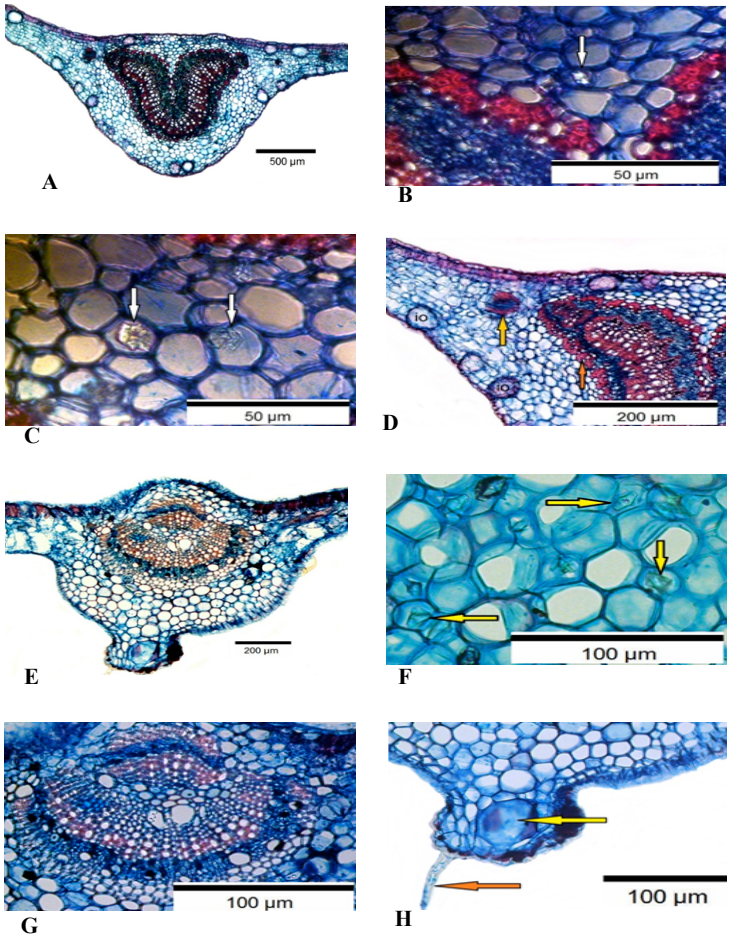


*Figure 3.* Transverse section in midrib of *Syzgyium aromaticum* (A) External shape of midrib and pattern of vascular bundle; open vascular tissue (V-shaped) with intraxylary phloem; (B&C) Solitary (cuboid shape) and druse crystals (white arrow) present in parenchyma cells; (D) Idioblast (oil) cells (io) in ground tissue near the epidermis; secondary vascular bundle (yellow arrow), sclerenchyma cells on vascular bundle (brown arrow), Transverse section in midrib of *C. excavata*; (E) External shape of midrib; (G) Pattern of vascular bundle; (F) Single rhombus crystal in mesophyll/parenchyma cell; (H) Idioblast (oil) cell (yellow arrow) in ground tissue near adaxial epidermis, unicelluar, multiseptate trichomes on adaxial epidermis.

**Lamina.** Based on observation, the adaxial epidermis was thicker than the abaxial epidermis with the presence of unicellular trichomes on both sides (Figures 4D, 4E and 4G). Large secretory cavities existed below the epidermis in between the palisade and spongy mesophyll (Figures 4D and 4F) and solitary crystals were present in the parenchyma cells (Figure 4H).

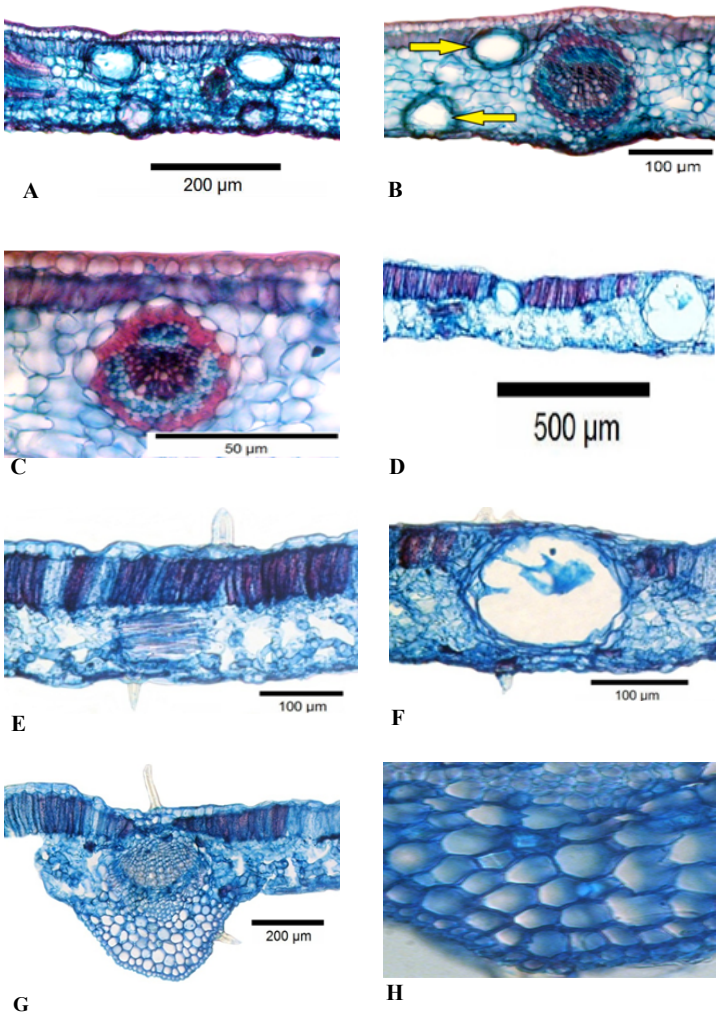


*Figure 4*. (A) Transverse section of lamina in *Syzygium aromaticum*; (B) Idioblast (oil) cells (yellow arrow) in palisade and spongy mesophyll; (C) Main vascular bundle with sclerenchyma tissue present; (D) Transverse section of leaf lamina *Clausena excavata*; (E) Unicellular (single) multiseptate trichome (brown arrow); (F) Idioblast oil cells in mesophyll; (G) Main vascular bundle; (H) Solitary crystal in mesophyll parenchyma (yellow arrow)

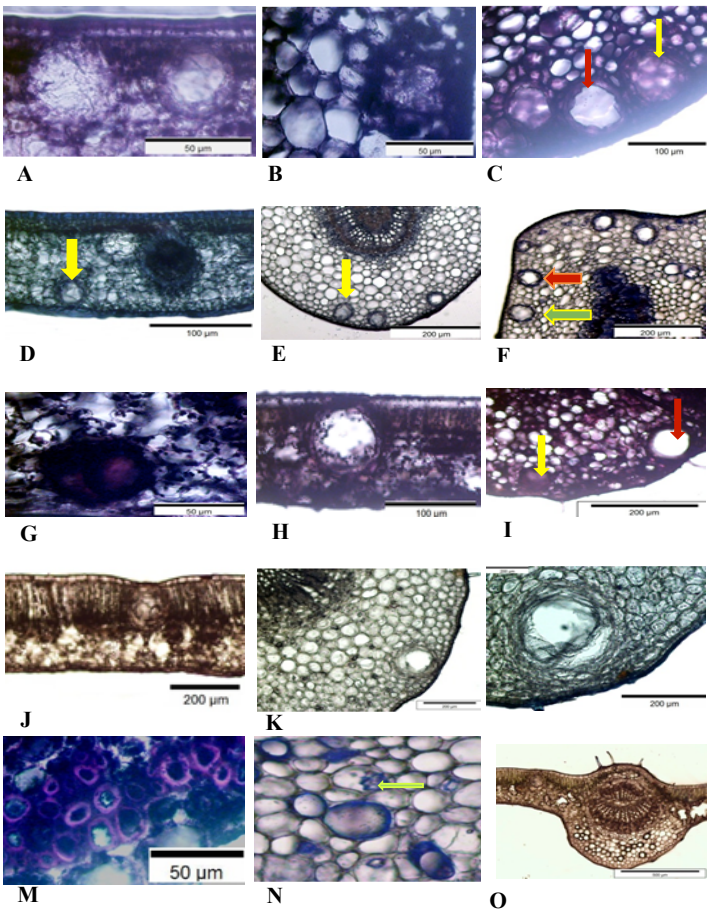


*Figure 5*. Photomicrographs (LM) of specimens stained with NADI reagent and Sudan Black (A-F) *Syzygium aromaticum*; (A&D) Lamina; (B&E) Midrib; (C, F) Petiole; notice full accumulation of terpenes (yellow arrow) and emptied oil cavity (brown arrow); (G-L) *Clausena excavata*; (G) Midrib; notice intense purple colouration; (O) Midrib; notice absence of oil cavity, (H, J) Lamina; black colouration; (I, K, L) Petiole; notice full accumulation of terpenes (yellow arrow) and emptied oil cell (brown arrow); (M) Sclerenchyma cells of *C. excavata* stained pinkish-violet in petiole; (N) Druse crystals of *S. aromaticum* stained blue black (green arrow) in petiole

## Anatomical Analysis

The presence of secretory structures is one of the characteristics for Myrtaceae and Rutaceae (Metcalfe & Chalk, 1979). It was found that *S. aromaticum* possessed secretory cavities that secrete oil in the leaf. Morphologically, the cavities are glandular, spherical or elliptical in shape, larger than neighbouring cells and surrounded with parenchyma cells. This finding was supported by previous studies on the secretory cavities present in *S. aromaticum* (Fahn, 1988; Khatijah & Ruzi, 2006; Al-Edany & Malik, 2012; Arruda & Victorio,

2015). Although earlier studies had similar findings, they mentioned little about the distribution of the secretory cavities and the specific leaf part in which the structures were present. In the present study, the secretory cavities were found located abundantly in all parts of the leaf involving the lamina, midrib and petiole.

*Clausena excavata* was found to possess secretory cavities as its secretory structure in Rutaceae (Groppo et al., 2008). The glandular cavities were capitate with a unicellular stalk surrounded by secretory cells and located near the epidermis in the petiole (Figure 2). The transverse section of the midrib showed the presence of multiseptate trichomes on the adaxial epidermis (Figures 3E-H). According to Metcalfe and Chalk (1979), members of Rutaceae have both glandular and non-glandular trichomes. However, only non-glandular trichomes are found abundantly on the abaxial epidermis. This shows that certain characteristics may occur in only some members of the family and not necessarily in every genus and species.

**Histochemistry Analysis**

Through histochemical testing, most secretory cavities showed positive reaction to terpenes, indicating that it does accumulate within the structure. A previous study by Sajwan et al. (2014) proved the presence of oil globules located in cavities of the hyphantium region of *S. aromaticum* when tested with Sudan III. This work revealed that the oil cavities not only existed in the flower but also in the leaf of *S. aromaticum*. In addition, the oils in the cavity could also be tested using other staining reagents not necessarily Sudan III but NADI reagent and Sudan Black B. Therefore, the findings showed that the existence of volatile terpenes and essential oil in the cavity might be the contributing factor to the production of leaf aroma (Tables 2 and 3).

The absence of terpenes and essential oil in the midrib of *C. excavata* is related to the fact that the oil cells and cavity were not filled with these secreted substances. Based on observation, all the empty oil cells and cavities were colourless and unstained when treated with staining reagents. Only the area surrounding the oil cells or cavities were stained dark, whereas the centre area was brighter under transmitted light (Figures 5C, F, H, I, K, J). This shows that cells surrounding the cavity contained terpenes and lipids but not the inside of the structure. A similar observation was previously recorded by Bakker et al. (1992) when they stained oil cells using Alcian blue. The absence of essential oil in the midrib of C. excavata was due to the absence of secretory cavities in that region.

The application of NADI reagent onto each plant part not only revealed the secretory structures to be stained purple violet, but also included other cells. This can be seen in the TS of the *C. excavata* petiole where the sclerenchyma cells were also stained pinkish purple (Figure 5M). Also, the application of Sudan Black B gave a blue-black colouration to the druse crystals found in the parenchyma of *S. aromaticum* (Figure 5N). Black and violet colouration

could also be seen on the cuticle located above the adaxial layer of both of species observed. Such colouration shows that terpenes and lipids also accumulate within ground tissue and not necessarily in the secretory structures. Although druse crystals are classified as ergastic substances whose function is not yet known, it is possible that they contribute to the production of aroma in plants.

Table 2
*Summary of the secretory structures in the leaves of S. aromaticum and C. excavata*

| Aromatic Plants | Plant Parts | Secretory Structures | | |
|---|---|---|---|---|
| | | Secretory Cavity | Secretory Cell | Glandular Trichome |
| *S. aromaticum* | Lamina | + | - | - |
| | Midrib | + | - | - |
| | Petiole | + | - | - |
| *C. excavata* | Lamina | + | - | - |
| | Midrib | + | - | - |
| | Petiole | + | - | - |

*Notes:* +: present, - : absent

Table 3
*Histochemistry of secretory structures in the leaves of S. aromaticum and C. excavata.*

| Aromatic Plants | Plant Parts | Compounds Investigated | |
|---|---|---|---|
| | | Terpenes | Essential Oil |
| *S. aromaticum* | Lamina | ++ | ++ |
| | Midrib | +++ | ++ |
| | Petiole | +++ | + |
| *C. excavata* | Lamina | + | ++ |
| | Midrib | +++ | - |
| | Petiole | +++ | + |

*Notes:* - : negative, + : positive, + (low), ++ (medium), +++ (high) indicating the vividness of stained colour

## CONCLUSION

In this study, the morphology and anatomy of the secretory structure of two plants were determined using light microscopy. Secretory cavities were found to be the source of aroma production in *S. aromaticum* and *C. excavata*. The substances accumulated in the secretory structure were detected using histochemical testing. Terpenes, responsible for the aroma of *S. aromaticum* and *C. excavata*, were found in the secretory cells and secretory cavities in their leaves. Essential oils were found accumulated in the secretory cavity of S. *aromaticum* and *C. excavata*. Hence, the presence of either one of the volatile oils indicates they are the

source of aroma in these tropical aromatic plants. The findings of this work can be strengthened by conducting a chemical analysis of the essential oil obtained from both species.

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