HISTOCHEMICAL ANALYSIS OF THE LEAVES OF FOUR ERICALES MEDICINAL PLANTS

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ABSTRACT

Active chemical compounds from medicinal plants are secondary metabolites that can be histochemically tested. It enables the identification and localization of diverse secondary metabolites within the tissue, which is not possible with screening phytochemicals. In this study, the histochemistry of the leaf blades of Ericales plants i.e., cantigi (*Vaccinium varingiaefolium* (Blume) Miq.), persimmon (*Diospyros kaki* Thunb.), sapodilla (*Manilkara zapota* (L.) van Royen), and star apple (*Chrysophyllum cainito* L.) were evaluated. These species have been reported as folk medicines that have secondary metabolites. Each leaf tissue was observed under a microscope, and phytochemical compounds' presence and location were detected using specific reagents. The results showed that those four leaves contained phenolic compounds, flavonoids, and terpenes, while quinones were only found in cantigi leaves. The secondary metabolites were present in the idioblast of parenchymal and epidermal tissues. The persimmon and star apple leaves had secretory cavities in trichomes with terpenes and phenolic compounds.

Key Words: Ericales, histochemistry, secondary metabolites.

ANALISIS HISTOKIMIA EMPAT DAUN TUMBUHAN OBAT DARI ORDO ERICALES

ABSTRAK

Senyawa kimia aktif tanaman obat sebagian besar merupakan metabolit sekunder yang dapat diuji secara histokimia. Metode ini memungkinkan identifikasi dan lokalisasi golongan senyawa metabolit sekunder di dalam jaringan, yang tidak mungkin dilakukan dengan skrining fitokimia. Pada penelitian ini dilakukan analisis histokimia pada daun tumbuhan bangsa Ericales yaitu cantigi (*Vaccinium varingiaefolium* (Blume) Miq.), kesemek (*Diospyros kaki* Thunb.), sawo (*Manilkara zapota* (L.) Van Royen), dan sawo duren (*Chrysophyllum cainito* L.). Spesies tumbuhan ini telah dilaporkan sebagai obat tradisional yang memiliki metabolit sekunder. Jaringan daun dapat diamati di bawah mikroskop dan keberadaan serta lokasi senyawa metabolit sekunder dideteksi dengan menggunakan pereaksi spesifik. Hasil penelitian menunjukkan bahwa keempat daun tersebut mengandung fenol, flavonoid, dan terpen, sedangkan kuinon hanya terdapat pada daun cantigi. Metabolit sekunder ditemukan terutama pada idioblas sel parenkim dan epidermis. Daun kesemek dan daun sawo duren memiliki sel sekresi pada trikoma dengan kandungan terpen dan fenol.

Kata kunci: Ericales, histokimia, metabolit sekunder

INTRODUCTION

Active chemical compounds are generally included in secondary metabolites such as alkaloids, flavonoids, and terpenes (Dewick 2009). Most of these compounds are stored in secretory structures, while only a limited amount of secondary metabolites are stored in vacuoles and the cytosol of parenchymal cells (Dickison 2000). One way to determine the distribution of secondary metabolites in plants is through histochemical tests, which allow quick and inexpensive preliminary tests in searching for active chemical compounds (Coelho *et al.* 2012, Royo *et al.* 2015).

Histochemistry has a tremendous long-term impact on cell and tissue biology, embryology, and pathology and continues at the forefront of research in these disciplines. Histochemical techniques are now more widely used than ever and, in conjunction with new developments in microscopical imaging and analysis, will continue to have an important position in the life sciences and medicine (Coleman *et al.* 2000). The histochemical analysis is related to the localization and identification of compounds, metabolic activity, and aspects of cell biology from cells and tissues. This technique is used to visualize the type of compounds present in tissues (Lavis 2011). The histochemical test's advantage is locating where the metabolites are produced or accumulated in plant tissues (Mulyani and Laksana 2011).

Among the Angiosperms plant group, Ericales is one of the plant's orders used in traditional medicine for various diseases. The main chemical compounds are triterpenoids and flavonoids (Rocha *et al.* 2015). According to APG IV system (2016), Ericales comprise about 22 families, Balsaminaceae, Marcgraviaceae, Tetrameristaceae, Fouquieriaceae, Polemoniaceae, Lecythidaceae, Sladeniaceae, Pentaphylacaceae, Sapotaceae, Ebenaceae, Primulaceae, Theaceae, Symplocaceae, Diapensiaceae, Styracaceae, Sarraceniaceae, Roridulaceae, Actinidiaceae, Clethraceae, Cyrillaceae, Ericaceae, and Mitrastemonaceae. Several common medicinal plants of Ericales found in Indonesia are cantigi (*Vaccinium varingiaefolium* (Blume) Miq.), persimmons (*Diospyros kaki* Thunb.), sapodilla (*Manilkara zapota* (L.) van Royen.), and star apple (*Chrysophyllum cainito* L.). The leaves of *V. varingiaefolium* (Ericaceae) are traditionally used for the treatment of wrinkles, external wounds, burns, and inflammation (Yulyana *et al.* 2016). *D. kaki* (Ebenaceae) is used as an antioxidant, anti-inflammatory, hypolipidemic, antidiabetic, and antibacterial (Xie *et al.* 2015). The leaves of *M. zapota* (Sapotaceae) are useful for the treatment of inflammation, skin diseases, diarrhea, and improving blood circulation (Ganguly and Rahman 2013). Leaf infusion of *C. cainito* (Sapotaceae) is used to treat diabetes and articular rheumatism (Shailajan and Gurjar 2014).

To our knowledge, the pharmacognostic analysis including phytochemical and histochemical studies on Ericales is still limited. Therefore, this study aimed to analyze the accumulated metabolite compounds in the secretory structure and the phytochemical content in four medicinal plants of Ericales using histochemical tests.

MATERIALS AND METHODS

Leaves of *V. varingiaefolium* (Blume) Miq., *D. kaki* Thunb., *M. zapota* (L.) Van Royen, and *C. cainito* L. were collected in Bandung. Specimen was deposited at the Herbarium Bandungense, School of Life Sciences and Technology Institut Teknologi Bandung.

The transverse section for histochemical experiments were sectioned by hand-sliced from fresh leaves (3rd node) with three replications. The sample was prepared using a polystyrene mount according to the method of the American Herbal Pharmacopeia (2011). The sample is carefully sliced by running the blade evenly along the flat top surface of the mount during the sectioning technique, which uses a very sharp razor blade. The fresh section is stained in a few drop of reagent solution for 2–5 minutes, carefully washed with water, and covered with a cover slip. The stained sample was observed under the microscope with magnification of 100–400x. The specimen measurements are taken by calibrating an eyepiece with an integrated eyepiece micrometer with a precisely measured stage micrometer.

Histochemical tests were performed using the following reagents: 5% ferric chloride (Johansen

1940) and 10% potassium dichromate (Gabe 1968) for phenolic compounds; aluminum chloride (Guerin *et al.* 1971), citroboric acid (Pedro *et al.* 1990), sodium hydroxide (Robinson 1983), and vanillin-HCl (Mace and Howell, 1974) for flavonoids; cupric acetate (Rupa 2015) for terpenes; potassium hydroxide (Farnsworth 1966) for quinones; Dragendorff and Wagner (Furr and Mahlberg 1981) for alkaloids. The fresh and unstained sections were used as a negative control. The positive control was performed according to the reference of the respective author. The presence of brown or dark brown color in the tissue indicated a positive test for phenolic compounds. The positive result for flavonoids was indicated by the appearance of yellow with aluminum chloride, citroboric acid, sodium hydroxide, and pink with vanillin-HCl. A positive test for terpenoids was shown by the appearance of yellow or brownish-yellow color in the tissue. The presence of red color in the tissue indicated a positive test for quinone compounds. Alkaloid presence was shown by the presence of reddish-brown or yellow deposits. Image recording was performed using a light microscope (Olympus CX21LED) coupled with a digital camera (SXY-I50L). The histochemical test slides were then analyzed in the Laboratory of Pharmacognosy at the Institut Teknologi Bandung (ITB) for a detailed description and secondary metabolite of the leaf tissues

RESULTS AND DISCUSSION

Upon histochemical analysis on the transverse section of leaves from four Ericales plants, we found the presence of flavonoid, terpenes, and phenol containing tissues/ cells, and the absence of alkaloids (Table 1). In case alkaloid is present, the cells would be stained reddish-brown with Wagner's reagent (Mercadante-Simoes *et al.* 2014, Barbosa *et al.* 2021) and stained brown with Dragendorff (Matias *et al.* 2016, Gomez *et al.* 2019), which did not show in this present study. Meanwhile, quinones were only detected in *V. varingiaefolium* leaves. Secondary metabolites can be stored in internal and external secretory structures (Dickison 2000). In this study, the internal secretory structure was found in the midrib (the central vein of a leaf) and intercostal (the part between the leaf bone and leaf edge) area, especially in idioblast cells. The idioblast cells that contain the metabolites are in the epidermal tissue, parenchyma, and vascular tissue of all four leaves. The external secretory structures such as trichomes, especially glandular trichomes, are only found on *D. kaki,* and *C. cainito* leaves.

Table 1. Results of histochemical tests for the secretory structures on the leaf blade of the four species studied

Secondary metabolites	Reagents	Secretory structures	Species			
			V. varingiaefolium	***D. kaki***	***M. zapota***	***C. cainito***
Phenolic compounds	Ferric chloride	Idioblast	+	+	+	+
	Potassium dichromate	Idioblast	+	+	+	+
Flavonoids	Aluminum chloride	Idioblast	+	+	+	+
	Citroboric acid	Idioblast	+	+	+	+
	Sodium hydroxide	Idioblast	+	+	+	+
	Vanillin-HCl	Idioblast	+	-	+	-
Terpenes	Cupric acetate	Trichomes	-	+	-	+
Quinones	Potassium hydroxide	Idioblast	+	-	-	-
Alkaloids	Dragendorff	-	-	-	-	-
	Wagner	-	-	-	-	-

Note: (+) = positive result; (-) = negative result

The transverse sections of *V. varingiaefolium* leaves without reagent solution is showed in Figure 1(A-D). The chemical compounds were visualized by adding various reagent solutions as in Figure 1(E-N). Phenolic compounds were observed in idioblast cells, especially in the midrib and intercostal areas (Figure 1E-F). Flavonoid compounds in idioblast cells are found in the parenchyma of the intercostal (Figure 1H-J) and midrib area (Figure 1K). Quinone compounds showed positive results in palisade and spongy parenchyma of the intercostal site (Figure 1G). The terpenoid compounds were detected in the lower epidermis (Figure 1L). No histochemical were found in the literature to demonstrate the chemical nature of *V. varingiaefolium*. The chemical compound of *V. varingiaefolium* leaves has been reported by Yulyana et al. (2016) with phytochemical screening. The positive result showed flavonoid, saponin, steroid/triterpenoid, and tannin. Anthraquinone was not present based on the phytochemical screening test. The difference in the results of this quinone compound was due to the growing location of *V. varingiaefolium* in Yulyana's research, which was obtained from East Java, Indonesia. In contrast, this study was obtained from West Java, Indonesia. Plants that grow in different places will synthesize other secondary metabolites depending on many factors, such as light, temperature, soil water, soil fertility, salinity, the presence, or absence of stress, etc (Yang *et al.* 2018).

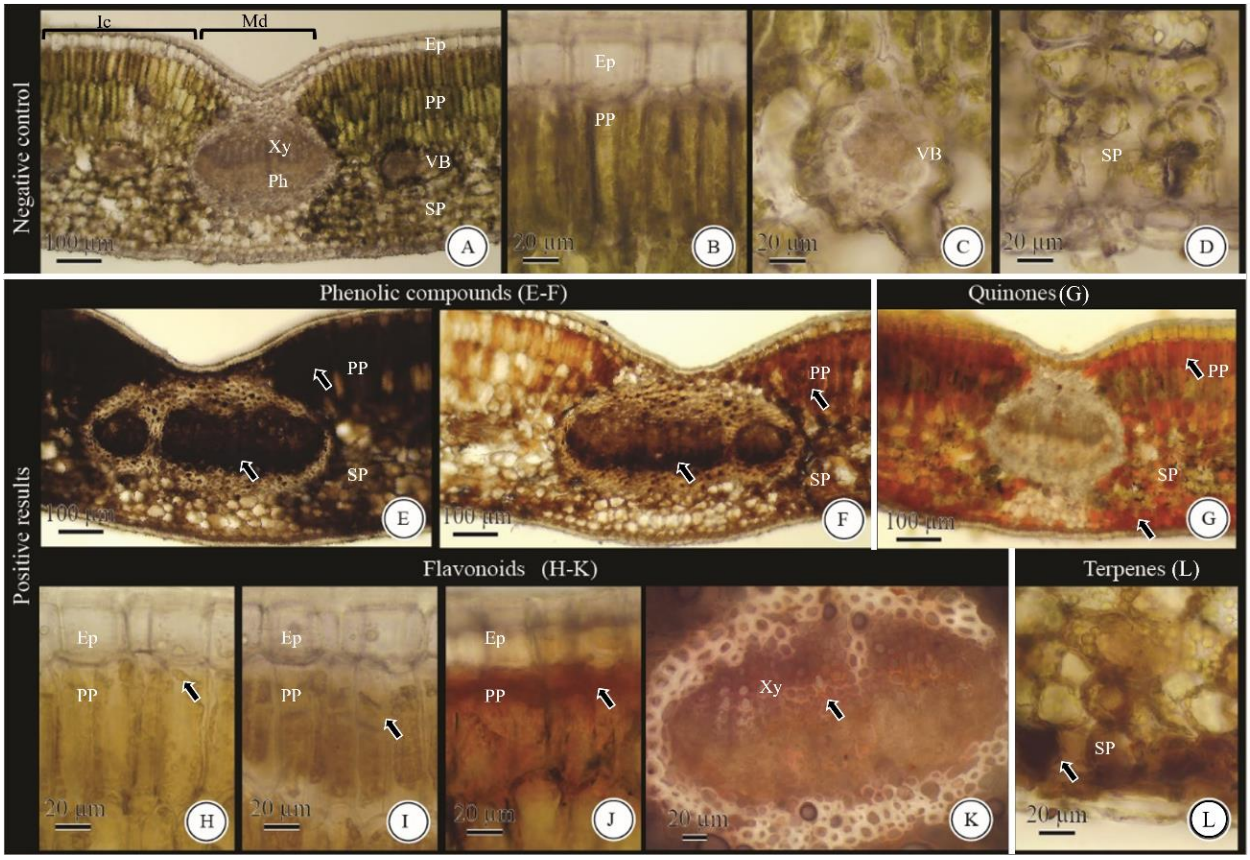

Figure 1. Histochemical characteristic of *V. varingiaefolium.* (A-D) Cross-section as a negative control. (E-F) Phenolic compounds (E) dark brown with ferric chloride (F) brown with potassium dichromate. (G) Quinones stained red with potassium hydroxide. (H-K) Flavonoids stained (H-J) yellow with (H) aluminum chloride (I) citroboric acid (J) sodium hydroxide (K) pink with vanillin-HCl. (L) Terpenes stained brown with cupric acetate. Ep, epidermis; Ic, intercostal; Md, midrib; Ph, phloem; PP, palisade parenchyma; SP, spongy parenchyma; VB, vascular bundle; Xy, xylem. Arrows indicate idioblasts with chemical compounds.

In *D. kaki* leaves, the unstained section as the negative control is shown in Figure 2A-E. The histochemical tests showed phenolic compounds in the midrib and intercostal area (Figure 2F; 2I) and the external secretory structure as glandular trichomes (Figure 2G-H; 2J-K). Flavonoid compounds were in idioblast cells of the epidermis and parenchyma in the intercostal (Figure 2L-N) and midrib areas (Figure 2O). The terpenoid compounds were detected in the glandular trichomes (Figure 2P-Q). These histochemical results align with the previous results based on phytochemical screening conducted by Zreen *et al.* (2019). Both tests showed tannins (phenolic compounds), flavonoids, and terpenoids.

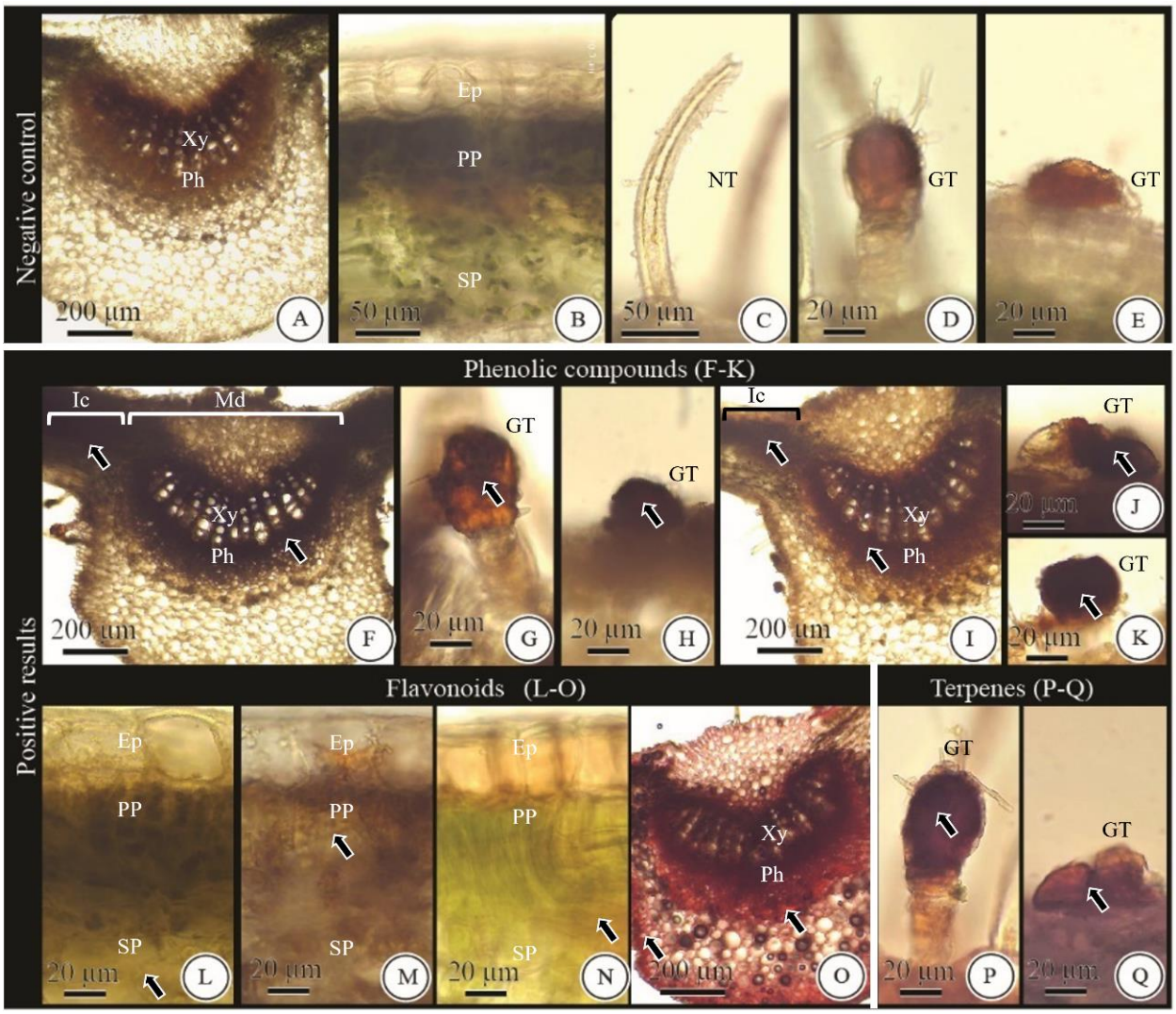

Figure 2. Histochemical characteristic of *D. kaki* (A-E) Cross-section as negative control. (F-K) Phenolic compounds (F-H) dark brown with ferric chloride (I-K) brown with potassium dichromate. (L-O) Flavonoids stained (L-N) yellow with (L) aluminum chloride (M) citroboric acid (N) sodium hydroxide (O) pink with vanillin-HCl. (P-Q) Terpenes stained brown with cupric acetate. Ep, epidermis; GT, glandular trichome; Ic, intercostal; Md, midrib; NT, non-glandular trichome; Ph, phloem; PP, palisade parenchyma; SP, spongy parenchyma; Xy, xylem. Arrows indicate idioblasts with chemical compounds.

C. cainito presented transverse sections of leaves before adding the reagent (Figure 3A-E). Phenolic compounds were detected in the intercostal area (Figure 3H-I) and the external secretory structure as non-glandular trichomes (Figure 3F-G; 3J-K). Flavonoid was observed in idioblast cells of the

epidermis, parenchyma in the intercostal (Fig. 3L-N), and midrib area (Figure 3O). The terpenoid compounds were in the parenchyma and spongy parenchyma of the intercostal area (Figure 3P). Phytochemical screening (Zuhro *et al.* (2016) exhibited saponin, triterpenoid, flavonoid, and phenolic compounds in *C. cainito* leaves. It is similar with our findings which showed that through histochemical examination, C. *cainito* leaves contained flavonoids, phenolic compounds, terpenoids and not contained alkaloids.

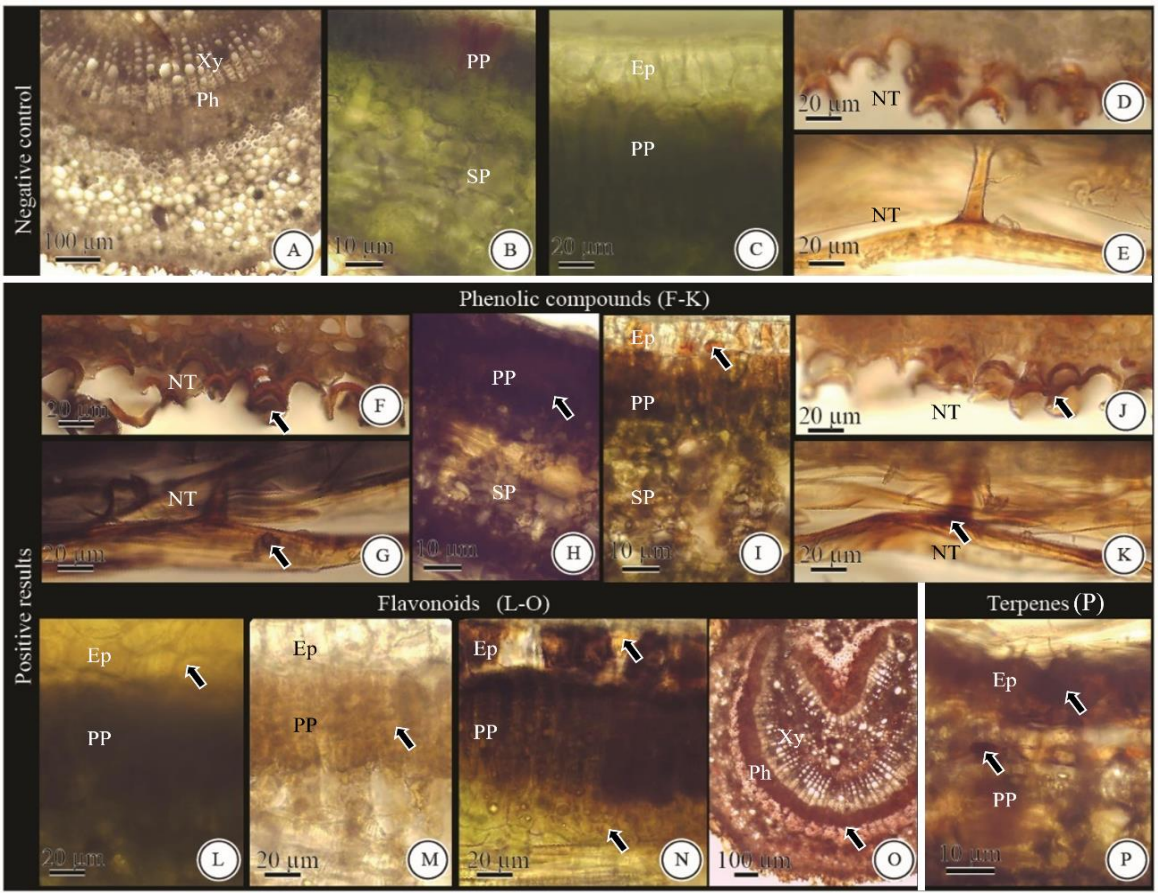

Figure 3. Histochemical characteristic of *C. cainito* (A-E) Cross-section as a negative control. (F-K) Phenolic compounds (F-H) dark brown with ferric chloride (I-K) brown with potassium dichromate. (L-O) Flavonoids stained (L-N) yellow with (L) aluminum chloride (M) citroboric acid (N) sodium hydroxide (O) pink with vanillin-HCl. (P) Terpenes stained brown with cupric acetate. Ep, epidermis; Ic, intercostal; Md, midrib; NT, non-glandular trichome; Ph, phloem; PP, palisade parenchyma; SP, spongy parenchyma; Xy, xylem. Arrows indicate idioblasts with chemical compounds.

In the transverse sections of *M. zapota* leaves (Figure 4A-D), without chemical treatment (negative control) was not possible to detect any chemical compounds. The histochemical tests showed that phenolic compounds are widely distributed in idioblast cells of intercostal (Figure 4E-F). The terpenes compounds were detected in the epidermal cells (Figure 4G) and the midrib (Fig. 4H). Flavonoids are located in idioblast cells of parenchyma, sponges in the midrib (Figure 4I), and intercostal area (Figure 4J-L). Islam *et al.* (2013) reported that flavonoids, tannins, saponins, and alkaloids were found in *M. zapota* leaves with phytochemical screening. The histochemical and phytochemical results of the alkaloids were different due to where the growing location of the plant. Previously, Moura et al. (2019) identified histochemical tests in the leaflets of *M. zapota* for phenolic compounds, and their results were similar to ours. These authors obtained positive tests with

10% potassium dichromate for phenolic compounds, vanillin hydrochloric acid for tannins, and antimony trichloride for triterpenes and steroids. Although this research used a different reagent for triterpenes and steroids, it showed positive results with similar localization. Phenolic compounds were identified using the same reagents with the same results. In this journal, there are different terms wherein vanillin is mentioned to detect tannins. According to Mace and Howell (1974), vanillin hydrochloric acid can identify tannin precursors, particularly gallocatechin and catechin, as flavan-3-ol compounds. A study in the journal Guerin et al. (1971) also showed that vanillin hydrochloric acid could detect flavanol and flavane-diol compounds. Catechins were previously classified as tannins because they have similar ingredients (85% of them are the same). However, there are some differences, like the plants containing tannin can be used for tanning leather, but catechin does not have that function. Moreover, it is known that catechin and epicatechin polymerize tannins. (Rauf *et al.* 2019).

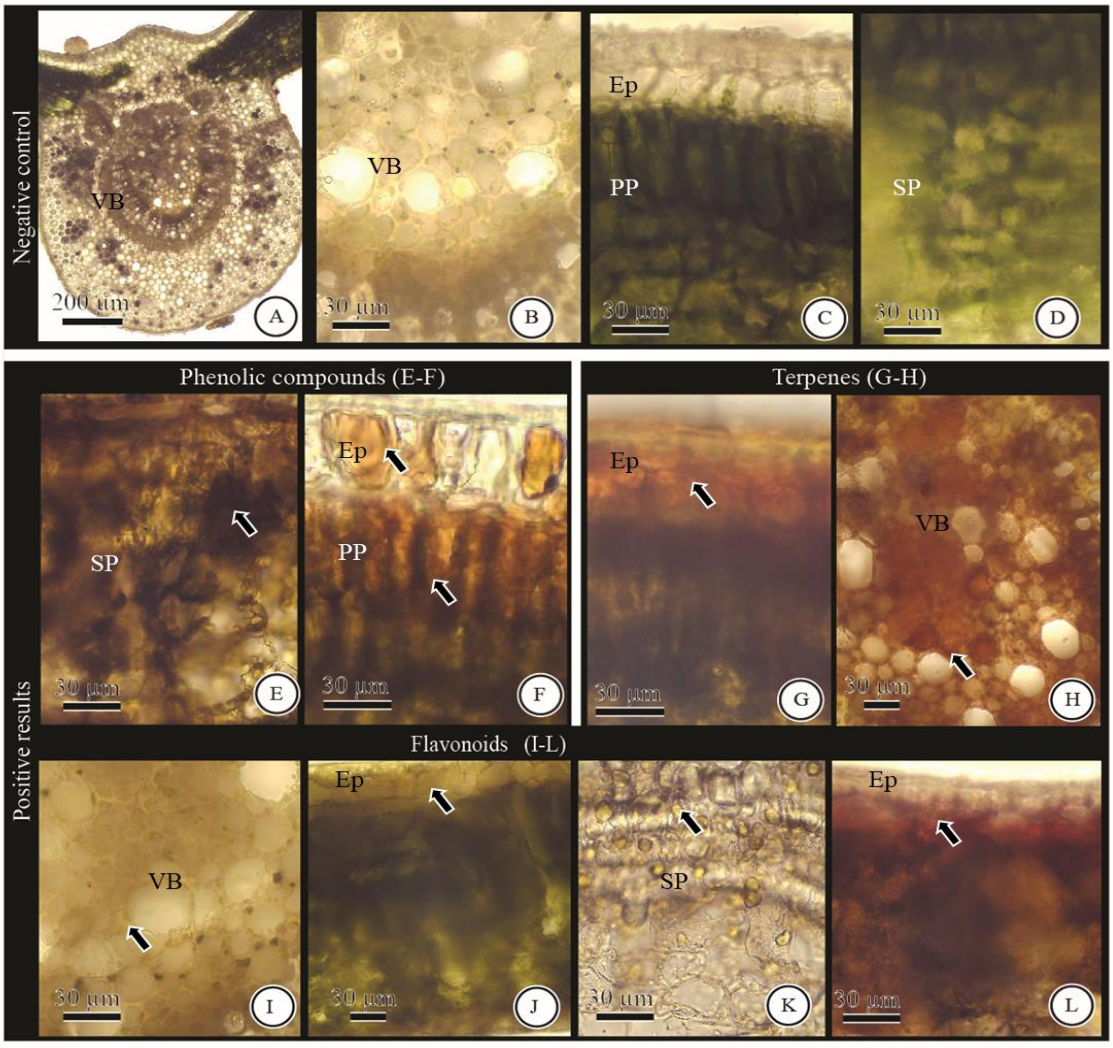

Figure 4. Histochemical characteristic of *M. zapota* (A-D) Cross-section as a negative control. (E-F) Phenolic compounds (E) dark brown with ferric chloride (F) brown with potassium dichromate. (G-H) Terpenes stained brown with cupric acetate. (I-L) Flavonoids stained (I-K) yellow with (I) aluminum chloride (J) citroboric acid (K) sodium hydroxide (L) pink with vanillin-HCl. Ep, epidermis; PP, palisade parenchyma; SP, spongy parenchyma; VB, vascular bundle. Arrows indicate idioblasts with chemical compounds.

The natural compounds, known as primary and secondary metabolites, are synthesized by secretory cells. The secretory cells occur in a single cell (idioblasts) or multicellular structures such as epidermis, hypodermis, parenchyma, trichomes, cavities, and ducts (Fahn 2000). Therefore, plants' secreting structure is found mainly in idioblasts (Mamoucha *et al.* 2016). The idioblasts can accumulate and secrete a compound such as oil, resin, mucilage, and tannin (Fahn 1979). During the evolution, secretory tissues seem to have developed from secretory idioblasts scattered among the normal tissues. Subsequently, ducts and cavities developed, and finally secretory trichomes (Fahn 1988). The internal secretory structures present a large distribution in four leaves, and the external secretory structures only present in *D. kaki* and *C. cainito* leaves. In *V. varingiaefolium*, the trichomes decrease and do not remain in some old stems, ripe fruit, and old leaves (Sholikhah *et al.* 2015).

The histochemical tests on four leaves of Ericales plants showed that phenolic compounds were presented in the idioblasts of the internal secretory structure. They can be detected using ferric chloride and potassium dichromate reagents, as demonstrated in other studies. Matias *et al.* (2016) performed histochemical tests on species of Solanum for phenolic compounds stained brown with ferric chloride and potassium dichromate reagents. Andrade *et al.* (2017), Betim *et al.* (2020) and Ribeiro *et al.* (2021) also showed that phenolic compounds reacted positively with ferric chloride (brown color). Besides the idioblasts, *D. kaki,* and *C. cainito* leaves also have trichomes that contained phenolic compounds. Phenolic compounds and glandular trichomes on *D. kaki* leaves also contain terpenes. The glandular trichomes often produce and accumulate terpenes and phenylpropanoid oils (Wagner *et al.* 2004). Another secondary metabolite accumulated in glandular trichomes was investigated histochemically as demonstrated by Liu and Liu (2012) on the leaves of *Isodon rubescens* (Lamiaceae) that contained terpenoids, flavonoids, carbohydrates, phenolics, and alkaloids. In the *C. cainito* leaves, the trichomes showed the location of phenolic compounds in non-glandular trichomes. The non-glandular trichomes, in this case, established the association with chemical reactions in the plants. Tozin *et al.* (2016) also found that the non-glandular trichomes from Lamiaceae and Verbenaceae participate in the chemical interaction of the plants with the environment, supplementing the work of the typically glandular trichomes. It indicates that non-glandular trichomes functioned as physical and chemical protection in plants.

The flavonoid compounds were detected in the epidermis and parenchyma using aluminum chloride reagents, citroboric acid, and sodium hydroxide. Kuster and Vale (2016) studied four leaves of medicinal species from Cerrado and found the flavonoid location in parenchyma cells, especially in the palisade and spongy parenchyma, with histochemical tests. Different location of flavonoid in this study was shown with a vanillin-hydrochloric acid reagent which only showed positive reactions in idioblast cells of the midrib and intercostal area due to the limited detection capability of vanillin-hydrochloric acid reagent to detect the presence of flavanol and flavandiol in plant tissues (Mace and Howell 1974, Guerin *et al.* 1971). In other cases, the aluminum chloride reagent detects flavonoids with free o-dihydroxy groups, while the citroboric acid and sodium hydroxide detect the presence of flavonoids in general (Pedro *et al*. 1990, Robinson 1983). According to Mulyani and Laksana (2011), the histochemical method can be used to analyze the presence of flavonoids in the test material, indicated by the yellow color with ammonia, NaOH, and AlCl3, citroboric acid reagents. Mamoucha *et al.* (2016) used vanillin-HCl to observe flavonoid compounds that showed pink color in the leaf of *Ficus carica*. These two studies have the same occurred in our study.

CONCLUSION

The histochemical test is a useful technique for detecting bioactive substances in secretory medicinal plants. In contrast to the conventional phytochemical screening procedure, only a cross-section sample and a few drops of the reagent are required. The four leaves contained phenolic compounds, flavonoids, and terpenes, while quinones were only detected in cantigi leaves. Internal secretory structures were observed in the midrib (leaf bone) and intercostal (part of the leaf bone and leaf edges), especially as idioblast cells. The idioblast cells can be seen on the epidermis, parenchyma, and vascular tissues. Meanwhile, the

external secretory structures in trichomes containing phenolic compounds were only found in *D. kaki,* and *C. cainito* leaves. Besides phenolic compounds, the glandular trichomes in *D. kaki* leaves also had terpenes.

ACKNOWLEDGMENTS

None. No funding to declare

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