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Research Article

Seasonal changes in the micromorphology, ultrastructure, and histochemistry of *Carissa macrocarpa* leaves

Reshika RAMASAR[1], Yougasphree NAIDOO[1], Yaser Hassan DEWIR[2]*, Thobayet AL-SHAHRANI[2], Nóra MENDLER-DRIENYOVSZKI[3]

[1]*University of KwaZulu-Natal, School of Life Sciences, Westville Campus, Private Bag X54001, Durban 4000, South Africa; reshikaramasar@gmail.com (R.R.); naidooy1@ukzn.ac.za (Y.N.)*
[2]*King Saud University, College of Food and Agricultural Sciences, Department of Plant Production, P.O. Box 2460, Riyadh 11451, Saudi Arabia; ydewir@ksu.edu.sa (*corresponding author); talshahrani@ksu.edu.sa (T.A.)*
[3]*University of Debrecen, Research Institute of Nyíregyháza, Institutes for Agricultural Research and Educational Farm (IAREF), P.O. Box 12, 4400 Nyíregyháza, Hungary; mendlerne@agr.unideb.hu (N.M-D)*

Abstract

Carissa macrocarpa (Eckl.) A. DC., is a woody shrub of the family Apocynaceae used in traditional medicine. This study aimed to investigate the seasonal variations in micromorphology, ultrastructure, and histochemistry of *C. macrocarpa* leaves using light and electron microscopy and histochemical techniques. This novel micromorphological analysis revealed the presence of glandular trichomes consisting of a short stalk and multicellular head, located on the lower surface of the leaf. The leaf was characterized as hypostomatic, containing stomata only on its lower surface. Nonarticulated laticifers were interspersed in the leaf cortex and spongy parenchyma. Transmission electron microscopy of *C. macrocarpa* leaf sections showed the presence of mitochondria, vesicles, vacuoles, and chloroplasts containing starch grains and plastoglobuli. Histochemical analysis revealed a variety of phytochemicals such as proteins, alkaloids, phenols, resin acids, lipids, polyphenols, mucilage, pectin, lignin, and cutin in *C. macrocarpa* leaves. The chemical compounds found in the latex of its laticifers likely play a vital role in herbivory prevention. Although leaves can also be used for medicinal purposes due to the presence of many pharmacologically active metabolites, future toxicology studies of *C. macrocarpa* leaves are recommended to ensure their safety for medicinal use. This study is the first to describe the ultrastructure and histochemistry of *C. macrocarpa* leaves. Given the knowledge gap regarding this species, the present research provides a foundation for the future harvest and medicinal applications of *C. macrocarpa*.

Keywords: hypostomatic; glandular trichomes; micromorphology; nonarticulated laticifers; phytochemical compounds

Introduction

Apocynaceae is a flowering plant family consisting of trees, herbs, shrubs and stem succulents (Li *et al.*, 1995). Its genus *Carissa* contains approximately 35 species native to tropical and subtropical regions of Africa,

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Asia, and Oceania (Endress and Bruyns, 2000; Li *et al*., 1995; Sennblad and Bremer, 2002; Evans, 2009). Most *Carissa* species have been used in traditional medicine to treat various illnesses, such as headaches, chest pains, rheumatism, edema, gonorrhoea, syphilis, and rabies (Kaunda and Zhang, 2017). *Carissa macrocarpa* (Eckl.) A. DC. (syn. *C. grandiflora* (E. Mey) A. DC.) *or* Natal Plum is a drought tolerant plant that is native to South Africa. *C. macrocarpa* grows from Kenya to Southern Africa (Congo, Kenya, Zambia, Zimbabwe, Mozambique, South Africa) and thrives in tropical and subtropical regions across the world (Leeuwenberg, 2001). It is found among bushes, near the sea and on sand dunes; and grows up to five meters (Allam *et al*., 2016). It has glossy foliage with fragrant, white, star-shaped flowers, which bloom from spring to midsummer (Leeuwenberg, 2001, Kaunda and Zhang, 2017). Its red, ripe fruits which are edible have been reported to contain high levels of vitamin C, calcium, and magnesium (Gaber *et al.,* 2015; Lim, 2012; Moodley *et al*., 2011; Moodley *et al*., 2012). *Carissa macrocarpa* plants are great for hedges because of their large thorns. The leaves of *C. macrocarpa* are used in traditional medicine to treat diarrhea in livestock. Its leaves are also used to treat cough and venereal diseases in humans (Moodley *et al*., 2011, Souilem *et al*., 2019). Its fruits have immune-boosting properties due to their triterpene content, and in South Africa they are believed to help prevent human immunodeficiency virus and hepatitis infection (Dhatwalia *et al*., 2021; Moodley *et al*., 2011). Furthermore, phytochemical studies on *C. macrocarpa* plant parts have revealed the presence of several bioactive compounds (phytochemicals) that are of therapeutic value (Moodley *et al*., 2011; Abbas *et al*., 2014; Khalil *et al*., 2015). *C. macrocarpa* leaves contain high latex concentrations potentially due to the presence of secretory structures such as laticifers (Gabr *et al*., 2015), Secretory structures are simple or complex structures that produce secretions within or on the plant surface. Secretory systems are classified based on their location in the plant and the substance exuded (Evert, 2006; Beck, 2010; Huchelmann *et al*., 2017). Laticifers are internal secretory structures, and they can be found within xylem, phloem, cortical, and pith parenchyma tissues (Evert, 2006; Beck, 2010; Huchelmann *et al*., 2017). Furthermore, nonarticulated laticifers have been reported to be an ancestral trait of the family Apocynaceae (Demarco and Castro, 2008; Castelblanque *et al*., 2016; Castelblanque *et al*., 2017).

Seasonal change directly influences key environmental factors such as temperature, light intensity and water availability. These factors play a crucial role in the structural (leaf anatomy) and functional (photosynthesis, phytochemical production) characteristics of plants- essential for plant survival (Chaves *et al.,* 2002; Larcher, 2003). Despite the ecological, horticultural, and medicinal importance of *C. macrocarpa*, there has been limited investigation into how its leaf structure and chemistry adapt to seasonal fluctuations. Investigating these changes can provide vital insights into the plant's physiological resilience, allowing for improved conservation methods, enhanced production and the possible exploitation of its bioactive chemicals in various industries (nutraceuticals and pharmacology) (Bohnert *et al.,* 1995; Lambers *et al.,* 2008). Therefore, this study aimed to investigate the seasonal variations in micromorphology, ultrastructure, and histochemical properties of *C. macrocarpa* leaves. The hypothesis states that seasonal variations influence the micromorphology, ultrastructure and histochemical composition of *C. macrocarpa* leaves, reflecting adaptive responses to environmental changes.

Materials and Methods

Plant material collection

Fresh *Carissa macrocarpa* (Eckl.) A. DC. leaves were harvested in summer (January 2021) and winter (July 2021) from the University of KwaZulu-Natal Westville campus (29.817°S 30.940°E), Durban, South Africa. After being identified by Dr. Ramdhani (botanist), a voucher specimen was deposited at the Bews

Herbarium, University of KwaZulu-Natal Pietermaritzburg campus, with accession number 92175 (voucher number 04). The leaves were sampled in triplicates.

Stereomicroscopy

Freshly harvested leaves were used, and adaxial and abaxial surfaces were examined. Images were captured with a Nikon AZ100 stereomicroscope equipped with a Nikon fiber Illuminator, using NIS-Elements D software (Nikon, Tokyo, Japan).

Scanning electron microscopy

Chemical fixation

Chemical fixation was conducted as per the procedure of Naidoo *et al*. (2012). *C. macrocarpa* leaves (summer and winter) were manually sectioned and preserved in 2.5% glutaraldehyde for 24 h. The sections were rinsed in 0.1 M phosphate buffer (pH 7.2) (3 times for 5 min each), before being postfixed for 1 h in 0.5% osmium tetroxide. Due to the light sensitivity of osmium tetroxide, sections were placed in the dark. Thereafter, samples were rinsed with distilled water (3 times for 5 min each) and dehydrated with a graded ethanol series (30%, 50%, 75%, and 100%) and then critical point dried with a Quorum K850 Critical Point Dryer. The leaf sections were secured on aluminum stubs with double-sided carbon tape and sputter coated with gold in a Quorum Q150 RES sputter coater. Samples were examined using a Zeiss LEO 1450 scanning electron microscope with a working distance of 14-17 mm (5.00 kV). SmartSEM imaging software was used for image capturing.

Freeze-fracture

Fresh leaves were cut (5 mm^2) and cooled in liquid nitrogen slush (−210 °C) as per Naidoo *et al*. (2009) method. Afterward, sections were manually fractured with a blade and freeze-dried for 72 h using an Edward's Modulyo EPTD3 freeze-drier. Freeze-dried samples were placed on aluminum stubs with carbon adhesive tape and sputter coated with gold in a Quorum 150 RES sputter coater. Samples were subsequently analyzed using a LEO 1450 (Zeiss) scanning electron microscope with Smart SEM imaging software at a working distance of 14-17 mm (5.00 kV).

Transmission electron microscopy

For 24 h, fresh leaf sections (5 mm^2) (summer and winter) were fixed in 2.5% glutaraldehyde. Sections were then rinsed three times in 0.1 M phosphate buffer (pH 7.2) for 5 min each time, and postfixed for 1 h with 0.5% osmium tetroxide. After this, samples were washed three times in 0.1 M phosphate buffer for 5 min each time and then dehydrated in 30%, 50%, 75%, and then 100% acetone. For 12 h, the dehydrated samples were infiltrated with 25%, 50%, and then 75% of a Spurr's resin and acetone mixture. Afterward, samples were infiltrated with 100% resin twice for 12 h each time, followed by embedding in 100% resin using silicone molds and polymerization in an oven at 70 °C for 8 h. The Leica EM UC7 ultramicrotome was fitted with a glass knife processed with a Glass Knife Maker LKB 7801A to section the resin block samples containing the leaf sections. Survey sections (0.5–1 μm) were mounted on slides, stained with toluidine blue, and examined under a Nikon Eclipse 80i light compound microscope (Nikon, Japan) using NIS-Elements D imaging software. After observing the areas of interest using the survey sections, ultrathin leaf sections (100 nm) were cut and picked up with copper grids and stained with 2.5% uranyl acetate for 10 min at 23 °C before being rinsed with distilled water. Thereafter, in a closed glass Petri dish with dry sodium hydroxide (NaOH) pellets, the copper grids were placed on top of lead citrate droplets to prevent the build-up of moisture, which can result in precipitation of the stain. After staining for 10 min, the copper grids were washed with distilled water and dried on filter paper. A 100 kV JEOL 1010 TEM equipped with iTEM software (JEOL, Peabody, MA, USA) was used to examine sections and obtain images.

Histochemistry

For the histochemical analysis, fresh leaves were segmented into semithin sections (80-100 µm). To detect the presence of chemical components, the material was manually sectioned with a blade for staining with mercuric bromophenol blue (Mazia *et al*., 1953) and Coomassie blue for proteins (Fisher, 1968), Sudan Black B (Demarco, 2017), and Nile Blue and Sudan III/IV for lipids or cutin (Buda *et al*., 2009), -phloroglucinol for lignins (Demarco, 2017), acridine orange for cell viability (Gupta and De, 1983), ferric chloride for phenolics (Demarco, 2017), toluidine blue for polyphenols (O'Brien *et al*., 1964), NADI reagent for resin acids (Demarco, 2017), Dittmar's reagent for alkaloids (Furr and Mahlberg, 1981), and ruthenium red for acidic mucilages and pectins (Demarco, 2017). The stained sections were examined and imaged using a Nikon Eclipse 80i fitted with a Nikon DS-Fi1 camera head (Nikon, Japan) and NIS-Elements D imaging software. Three leaf samples were analyzed for each stain.

Results and Discussion

The stereomicrographs revealed that *C. macrocarpa* plants have simple and opposite, decussate leaves with an ovate to elliptical shape and an entire margin (Figure 1). The adaxial surface of the leaf is dark green (Figure 1B and 1D) and the abaxial leaf surface is a lighter green colour (Figure 1A and 1C). The leaves are thick, leathery and odourless. The leaf has a mucronate apex, an obtuse base, and pinnate reticulate venation (Figure 1). Additionally, *C. macrocarpa* leaf venation can be described as brochidodromous because the secondary veins do not end at the edge of the leaf. Instead, its secondary veins join, creating arches. The secondary veins branch into smaller tertiary veins, leading to enclosed small areolas between them (Figure 1E). The characteristics observed are mostly congruent with those of other studies conducted by Khalil *et al*. (2015), Alsudani and Altameme (2021), Allam *et al*. (2016) and Khalaf (2021). However, Khalil *et al*. (2015) described the leaf apex as acuminate.

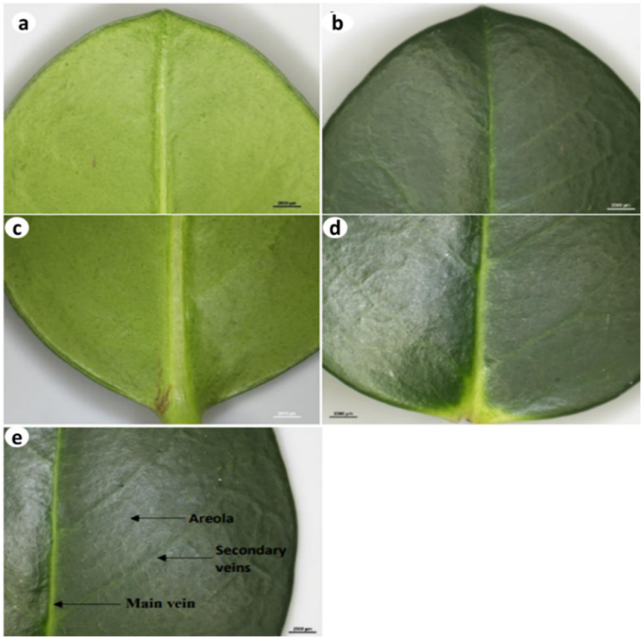

Figure 1. Stereo micrographs showing the general morphology of *C. macrocarpa* leaf. (a and c) abaxial surface, (b and d) adaxial surface, (e) adaxial surface showing venation pattern

Scanning electron micrographs revealed the presence of stomata on the lower surface of the leaf (Figures 2 and 3). The stomata were round to oval in shape and anomocytic (3 to 5 subsidiary cells surrounds the guard cells). Leaves were found to be hypostomatic, with stomata present only on their lower surface and absent from their upper surface (Figures 2 and 3). This may be a strategy to prevent excess water loss through transpiration, as stomata on the lower surface of leaves are less exposed to sunlight. The analysis of *C. macrocarpa* plants grown in Egypt (Allam *et al*., 2016) reported similar observations. Scanning electron micrographs also revealed the presence of glandular trichomes (peltate trichomes), which had short stalks and large multicellular heads (Figures 2 and 3). Peltate trichomes were mainly found on the midrib and leaf lamina. These trichomes were only present on the lower surface of the leaf. Glandular trichomes can manufacture, store, and release various phytochemicals (Fahn, 2000; Schilmiller *et al*., 2008). Key phytochemicals identified in trichomes include terpenoids, phenylpropenes, flavonoids, methyl ketones, acyl sugars, and defensive proteins (Fridman *et al*., 2005; Gang *et al*., 2001; Gershenzon and Dudareva, 2007; Kroumova and Wagner, 2003; Shepherd *et al*., 2005; Treutter, 2006). While chemical compounds play a role in the plants' defence mechanism, glandular trichomes may have other functions. These functions include protecting the plant against UV, attracting pollinators, regulating temperature, and reducing water loss (Ehleringer, 1984; Glas *et al*., 2012; Karabourniotis *et al*., 1995; Karabourniotis *et al*., 1998; Werker, 2000). Furthermore, phytochemicals found in glandular trichomes are commercially important as biological pesticides, food additives, and pharmaceuticals (Duke *et al*., 2000; Aharoni *et al*., 2006). There were no differences in the surface characteristics of summer and winter *C. macrocarpa* leaves (Figures 2 and 3). Other *Carissa* species such as *C. spinarum* have been reported to have glandular trichomes (Gabr *et al*., 2015).

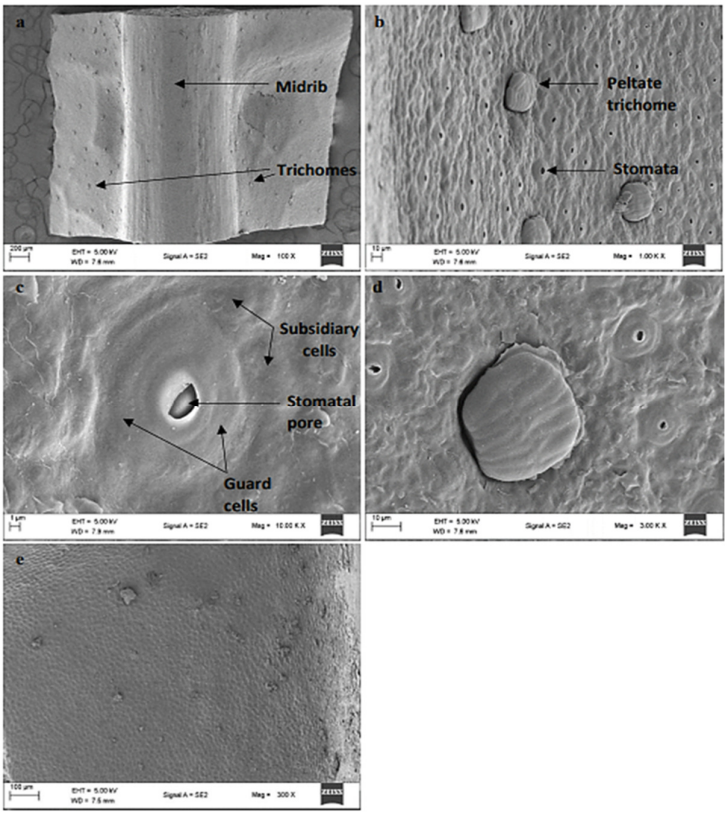

Figure 2. Scanning electron micrographs depicting the surface of *C. macrocarpa* leaves in summer. (a–d) Abaxial surface showing stomata and peltate trichomes. (e) Adaxial surface devoid of stomata and trichomes

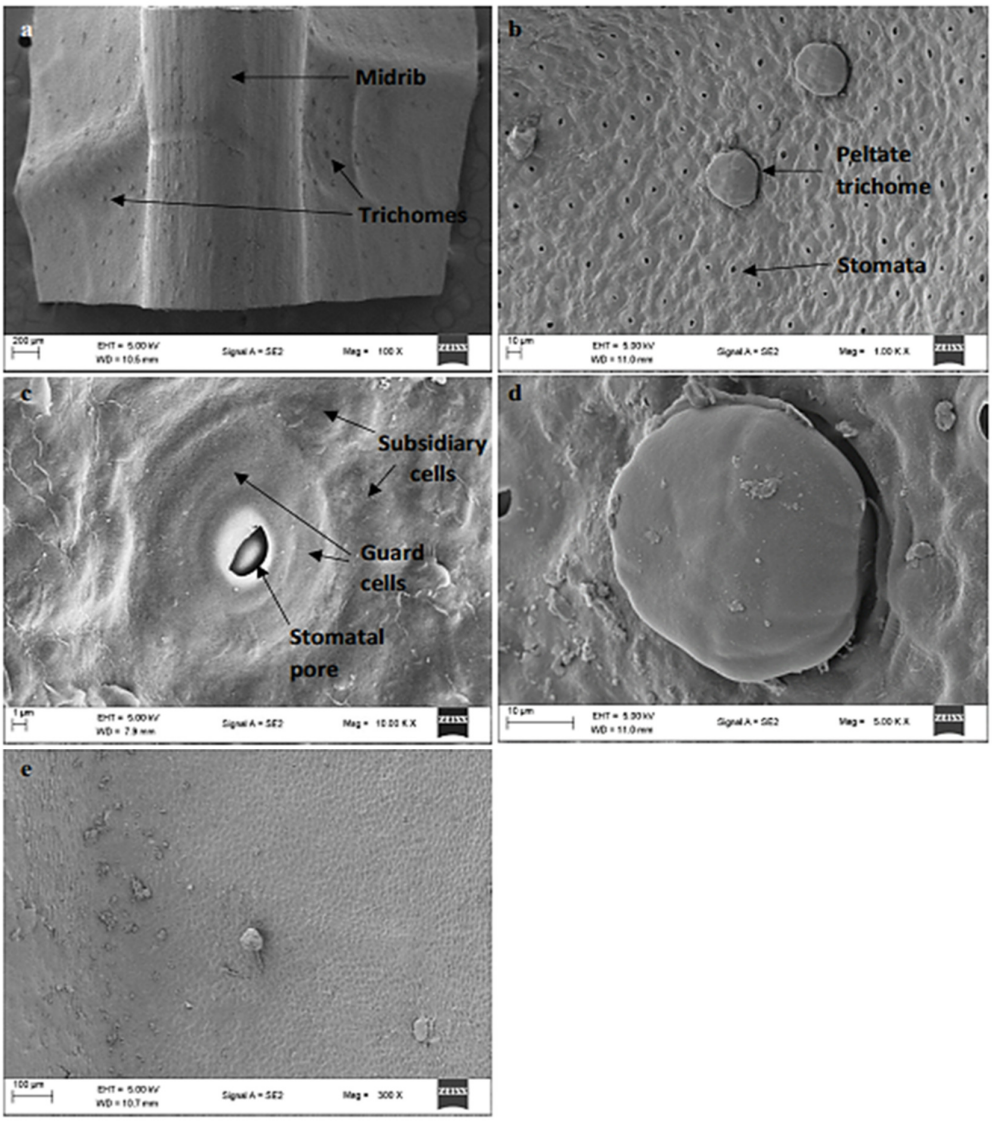

Figure 3. Scanning electron micrographs showing the surface of *C. macrocarpa* leaves in winter. (a–d) Abaxial surface showing stomata and peltate trichomes. (e) Adaxial surface devoid of stomata and trichomes

The transverse section of the *C. macrocarpa* leaf midrib revealed that the midrib region is depressed on the upper side of the leaf, white its protruding on lower side of the leaf. (Figure 4A and 4B). The upper and lower epidermis consists of a single layer of polygonal to square cells. The outer wall of the epidermis is full of cutin. A thick cuticle protects the leaf from water loss (Figures 4A, 4B, and 5). A collateral type of vascular bundle was observed, although there were also a few perimedullary phloem (Figure 4A and 4B). On the lower side of the leaf, collenchyma tissue appeared to be two to three layers thick (Figure 4A and 4B). The vascular bundle was surrounded by round parenchyma cells that have thin cellulose walls. Ducts resembling laticifer cells (with or without latex) were observed in the parenchyma cells (Figure 4A and 4B). Laticifers are thought to produce the white, milky fluid that is released when leaves are broken. The milky latex of *C. macrocarpa* is commonly found in many Apocynaceae species (Pickard, 2008; Kumar *et al*., 2011; Nazar *et al*., 2013; Rapini *et al*, 2003). Small vascular bundles of veinlets, also known as lateral veins, are found within the spongy tissue.

The upper side of the leaf consists of palisade mesophyll composed of two or three layers of columnar cells. These cells are densely packed. In contrast, the lower side consists of spongy mesophyll cells that are irregular in shape and have air spaces between them (Figure 4A and 4B). The characteristics observed in the transverse sections of summer and winter *C. macrocarpa* leaves were similar (Figure 4A and 4B) to those observed in previous studies (Khalil *et al*., 2015; Allam *et al*., 2016; Souilem *et al*., 2018; Alsudani and Altameme, 2021).

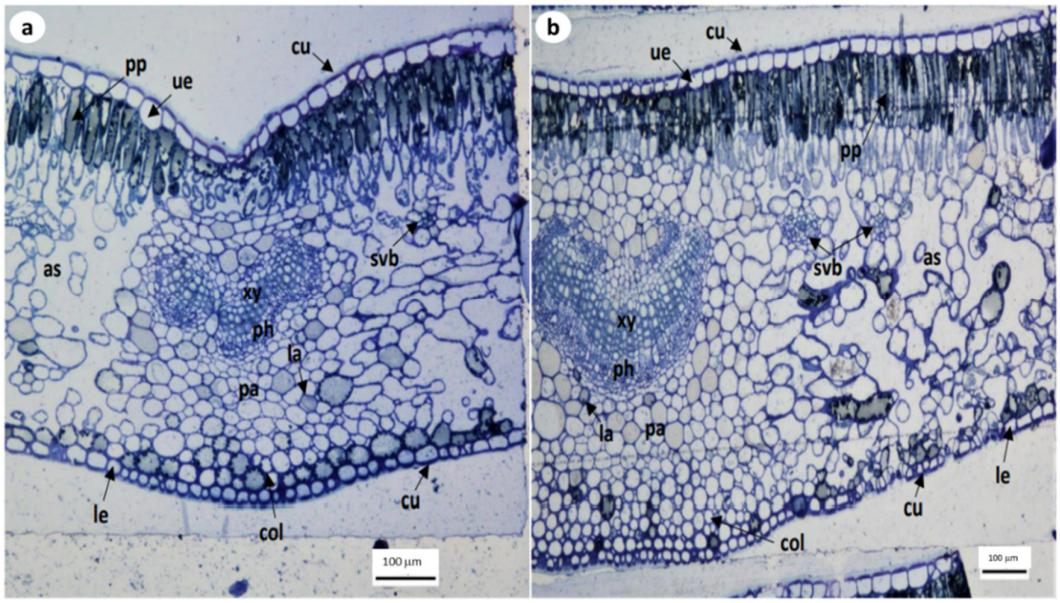

Figure 4. Light micrographs showing toluidine blue staining of *C. macrocarpa* leaves. (a) Summer and (b) Winter *C. macrocarpa* leaves stained with toluidine blue. Abbreviations: cu: cuticle, ue: upper epidermis, pp: palisade parenchyma, xy: xylem, ph: phloem, svb: small vascular bundle, as: air space, pa: parenchyma, col: collenchyma, la: laticifer cell, le: lower epidermis

The spongy parenchyma cells contain nonarticulated laticifers and druse crystals of calcium oxalate (Figure 5). Nonarticulated laticifers develop from a single cell and can grow to considerable lengths. Some Apocynaceae species in which nonarticulated laticifers have been detected are *Nerium oleander, Vallaris solanacea*, *Allamanda violacea*, *Calotropis gigantea, Mandevilla ilustris*, *Mandevilla velutina*, *Vinca sardoa*, and *Asclepias speciosa* (Lopes *et al*., 2009). Laticifers contain latex, which is a combination of phytochemicals such as carbohydrates, organic acids, and alkaloids, along with rubber particles, resins, proteins, mucilage, and essential oils (Souilem *et al*., 2018). The laticifer type found in *C. macrocarpa* leaves in this study is consistent with that described by (Souilem *et al*., 2018).

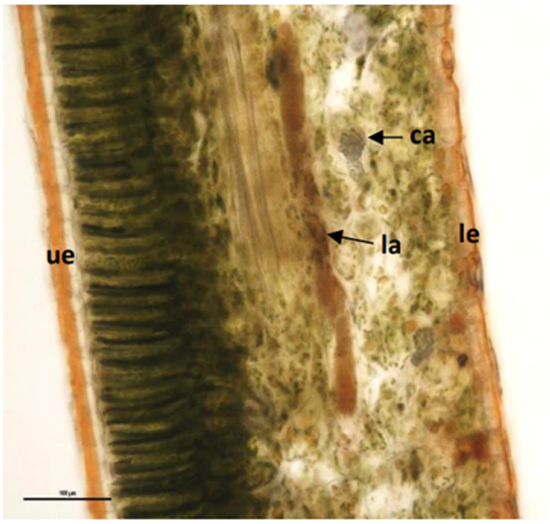

Figure 5. Light micrographs of lateral lamina showing laticifers stained with Sudan III/IV. Red-orange coloration indicating the presence of lipids and cutin. Abbreviations: up: upper epidermis, la: laticifer, ca: calcium oxalate, le: lower epidermis, pp: palisade parenchyma, sp: spongy parenchyma

Scanning electron microscopy of freeze-fractured leaf sections revealed that nonarticulated laticifers were interspersed in the cortex, surrounding the vascular bundle (Figure 7A and 7B). Additionally, laticifers were also found in the spongy mesophyll region (Figure 7F). These laticifer distribution results are congruent with those of a previous study (Souilem *et al*., 2018). Some spongy parenchyma cells contain large calcium oxalate crystals (Figure 7D). Calcium oxalate crystals can be found in plant tissues or organs. They are produced in the vacuoles of specialized cells known as crystal idioblasts (Franceschi and Horner, 1980) (Figure 7E). The production of calcium oxalate crystals in plants is crucial for key functions such as the regulation of calcium in tissues, protection from herbivores, and metal detoxification (Franceschi and Horner, 1980). The presence and distribution of calcium oxalate crystals in *C. macrocarpa* leaves observed in this study are consistent with those found in previous studies Souilem *et al.* (2018), Alsudani and Altameme (2021), Allam *et al.* (2016), Khalaf (2021) and Khalil *et al.* (2015). Due to the unavailability of resources, freeze-fracture analyses of *C. macrocarpa* summer leaves could not be conducted.

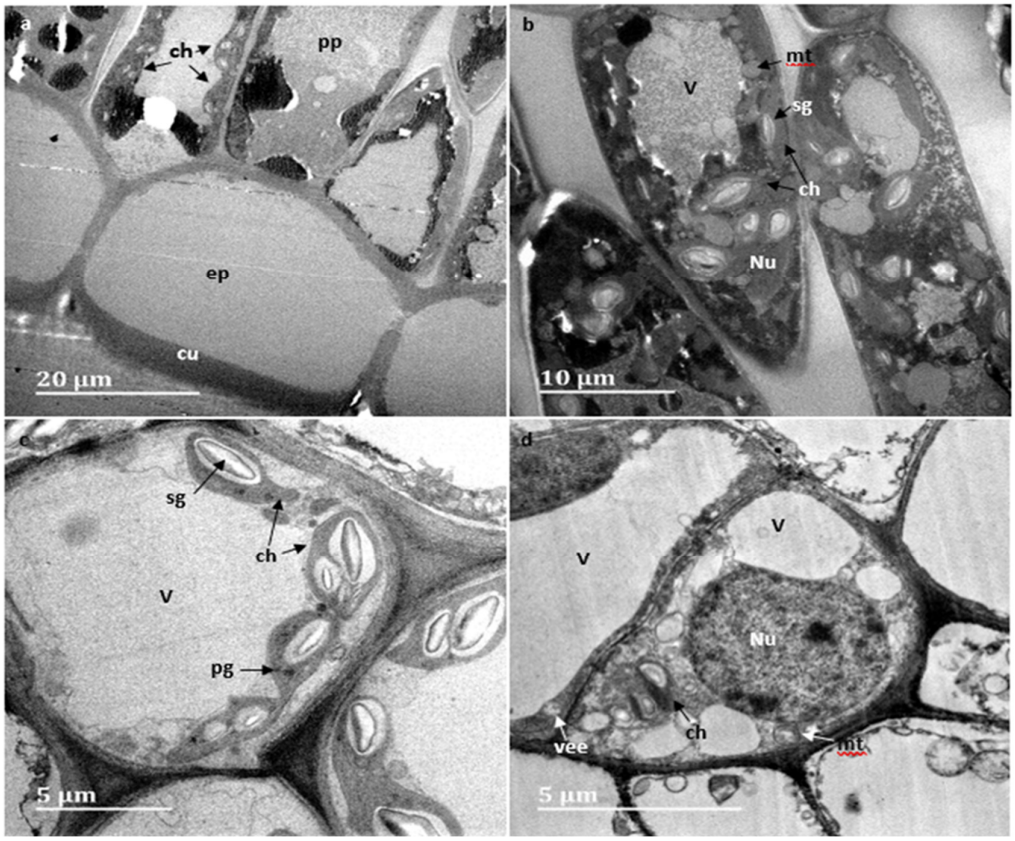

Figure 6. Transmission electron micrographs showing C. macrocarpa summer leaf sections. (a) Upper epidermis and palisade parenchyma. (b) Palisade parenchyma showing chloroplasts containing starch grains. (c) Spongy parenchyma showing chloroplasts containing large starch grains. (d) Spongy parenchyma showing mitochondria and vesicles near the cell periphery. Abbreviations: cu: cuticle, ep: epidermis, pp: palisade parenchyma, ch: chloroplast, V: vacuole, Nu: nucleus, sg: starch grain, mt: mitochondria, pg: plastoglobuli, vee: vesicle

The transmission electron micrographs revealed several metabolically active organelles, such as vesicles and vacuoles, in *C. macrocarpa* leaves (Figure 6 and 8). Vesicles were found near the plasma membrane. This could indicate the transport of larger molecules such as polysaccharides and proteins as well as other secretory compounds (Evert, 2006). Mitochondria were observed toward the cell periphery (Figure 6 and 8), indicating that the plasma membrane may be actively transporting compounds in or out of the cell (Evert, 2006). Several chloroplasts, containing plastoglobuli and starch grains, could be observed in the palisade and spongy parenchyma of the leaf (Figure 6 and 8). Starch grains may operate as storage products, accumulating starch during active photosynthesis (Evert, 2006). Plastoglobuli are lipoprotein globules whose functions involve storing lipids, controlling plant stress responses, breaking down the thylakoid in senescing tissues, and facilitating the transition of chloroplasts into chromoplasts (Kessler and Vidi, 2007). Summer leaves contained more and larger starch grains in palisade and spongy parenchyma than winter leaves (Figure 6 and 8). This may be due to summer leaves receiving more sunlight for extended periods, thus increasing photosynthesis and starch production (Kong *et al*., 2016).

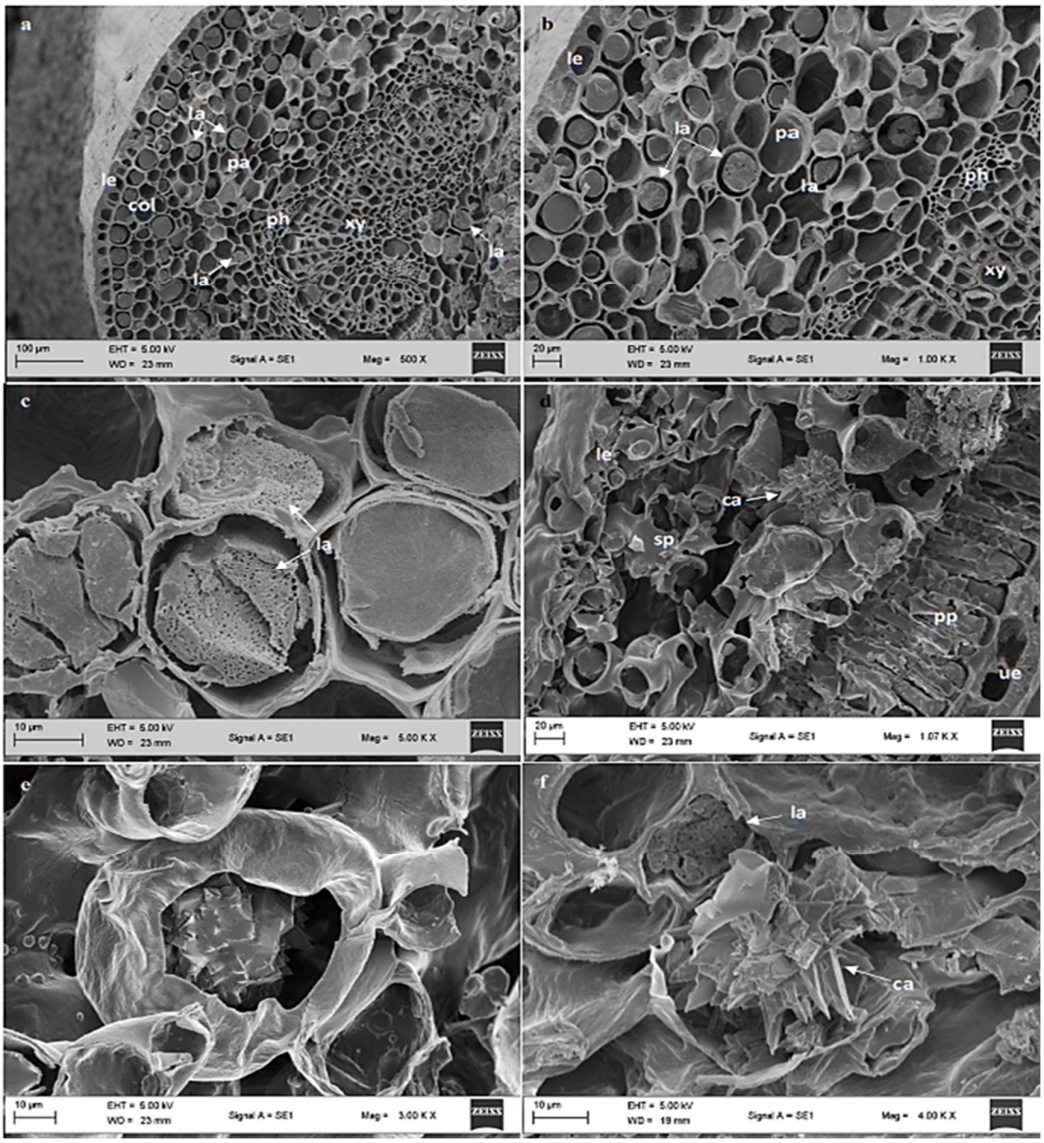

Figure 7. Scanning electron micrographs showing freeze-fractured *C. macrocarpa* leaves in winter. (a) Freeze-fracture of the midrib of a leaf. (b) Freeze-fracture showing the distribution of laticifers in the cortex of the leaf midrib. (c) Laticifer showing latex exudate from a leaf. (d) Leaf lamina showing calcium oxalate in spongy parenchyma. (e) Calcium oxalate in crystal idioblast. (f) Laticifer and calcium oxalate in spongy parenchyma. Abbreviations: ue: upper epidermis, col: collenchyma, pa: parenchyma, ph: phloem, xy: xylem, pp: palisade parenchyma, le: lower epidermis, sp: spongy parenchyma, la: laticifer, ca: calcium oxalate crystal

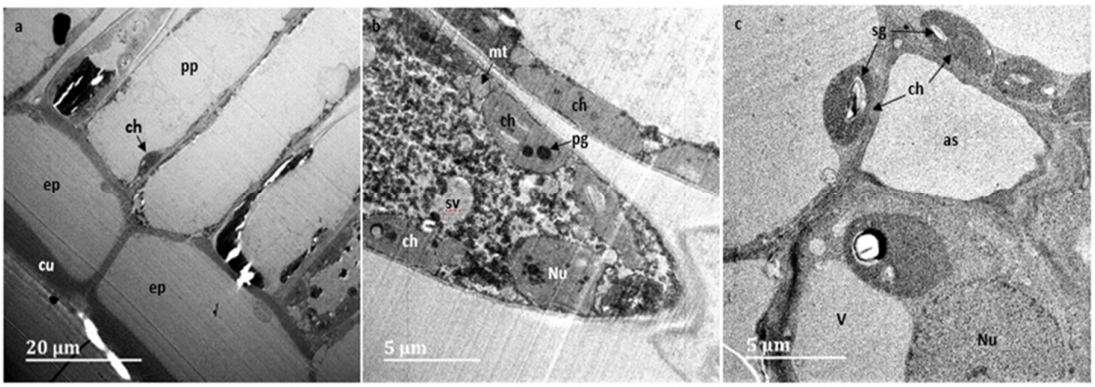

Figure 8. Transmission electron micrographs showing *C. macrocarpa* winter leaf sections. (a) Upper epidermis and palisade parenchyma. (b) Palisade parenchyma containing chloroplasts. (c) Spongy parenchyma showing chloroplasts containing starch grains. Abbreviations: cu: cuticle, ep: epidermis, pp: palisade parenchyma, ch: chloroplast, sv: small vacuole, Nu: nucleus, mt: mitochondria, pg: plastoglobuli, sg: starch grain, V: vacuole, as: air space

The histochemical analysis revealed the presence of various important phytochemicals in *C. macrocarpa* leaves, including proteins, alkaloids, phenols, resin acids, lipids, polyphenols, mucilage, pectin, lignin, and cutin (Figures 9 and 10). Furthermore, for the first time in *C. macrocarpa*, the histochemical analysis of its leaves identified the presence of laticifers and latex containing proteins, alkaloids, phenols, resin acids, lipids, polyphenols, mucilage, pectin, lignin, and cutin (Figures 9 and 10). Mucilage is a common product of plant cellular metabolism. It is used in medicine for its anti-inflammatory and anti-irritant activity (Geetha *et al*., 2009). Polyphenols, such as flavonoids, possess anti-inflammatory and antimicrobial properties (Hodek *et al*., 2002; Killedar and More, 2010). The presence of polyphenols in *C. macrocarpa* leaves has led to their use in wound treatment. Moreover, the presence of mucilage and flavonoids in *C. macrocarpa* leaves (Figures 9 and 10) may substantiate their use in traditional medicine for treating coughs and venereal diseases (National Research, 2008; Ibrahim *et al*., 1999; Alsudani and Altameme, 2021). The polymerization of phenolic compounds can result in the formation of glue-like substances that stick insects to the leaf surface (Yu *et al*., 1992). Lignin is a cell wall polymer that is responsible for the mechanical support of plant organs. Lignin protects plants against infection and herbivory (Boudet, 2000; Figueiredo *et al*., 2008; Moura *et al*., 2010). Proteins such as proteinase inhibitors can accumulate in plant tissues after injury (Schilmiller *et al*., 2008). It inhibits the digestive proteins of insects or animals after they have consumed the plant, and interferes with their physiology (Schilmiller *et al*., 2008). A potential association between the phytochemicals of latex and defence mechanisms against herbivores and pathogens has been reported (Demarco and Castro, 2008; Fahn, 2002; Farrell *et al*., 1991; Hagel *et al*., 2008; Konno, 2011; Mahlberg *et al*., 1987; Pickard, 2008). Therefore, the latex in *C. macrocarpa* laticifers contains chemical compounds that can be lethal or deployed to prevent herbivory and pathogenesis (Hagel *et al*., 2008; Pickard, 2008; Konno, 2011). All the chemical compounds tested for were present in both summer and winter *C. macrocarpa* leaves (Figures 9 and 10).

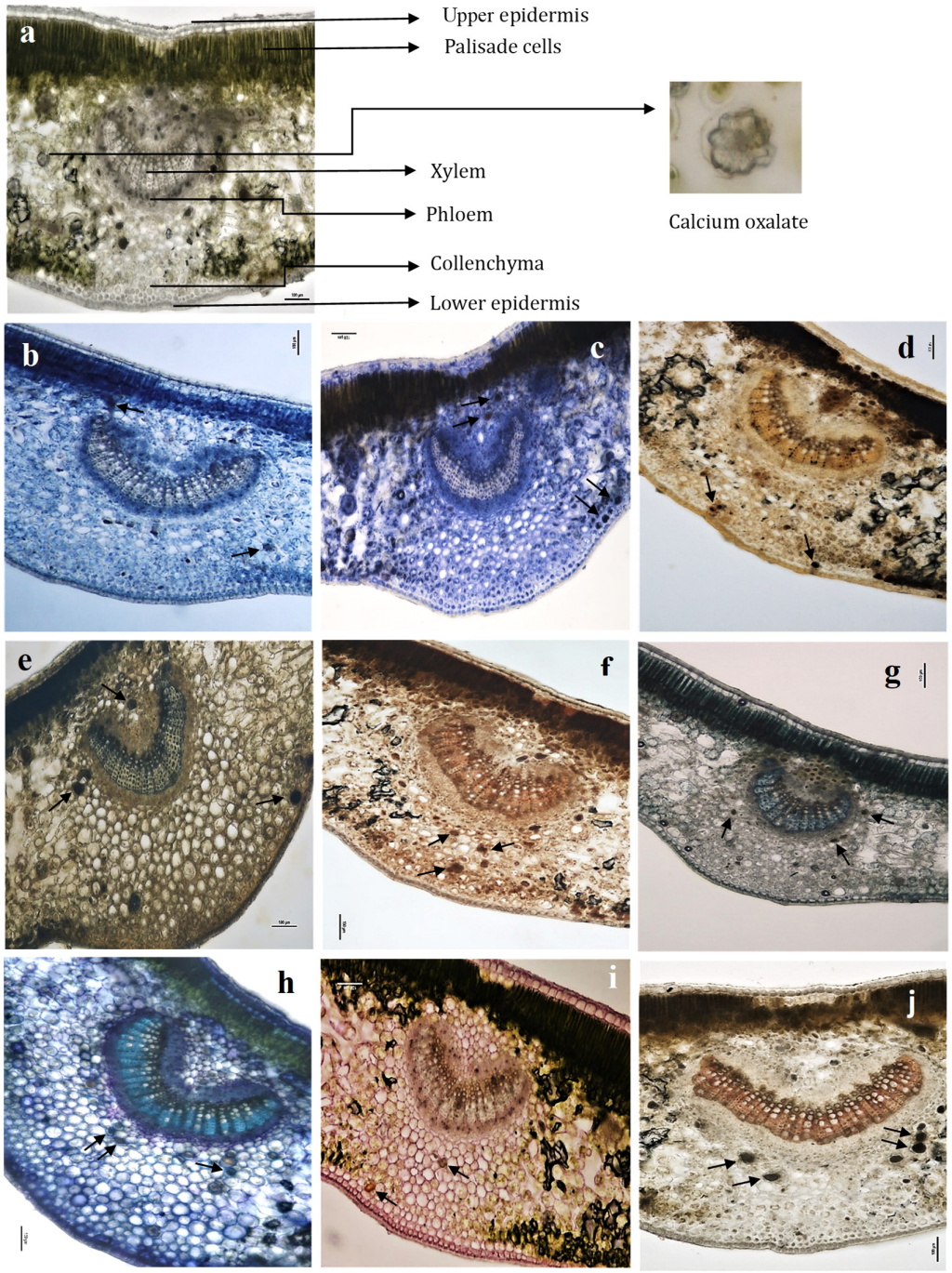

Figure 9. Histochemical observations of a transverse section of a *C. macrocarpa* summer leaf midrib. Colour change was indicative of a positive stain. (a) Unstained section showing the presence of calcium oxalate crystals. (b) Proteins-stained blue using bromophenol blue. (c) Proteins-stained blue using Coomassie. (d) Alkaloids-stained orange/brown using Dittmar's reagent. (e) Phenols stained brown/black using ferric trichloride. (f) Resin acids stained red using the NADI reagent. (g) Lipids stained blue/black using Sudan Black (h) Polyphenols stained green/blue using toluidine blue. (i) Mucilage and pectin-stained pink/red using ruthenium red. (j) Lignin aldehydes stained red using phloroglucinol. Arrows refer to laticifer.

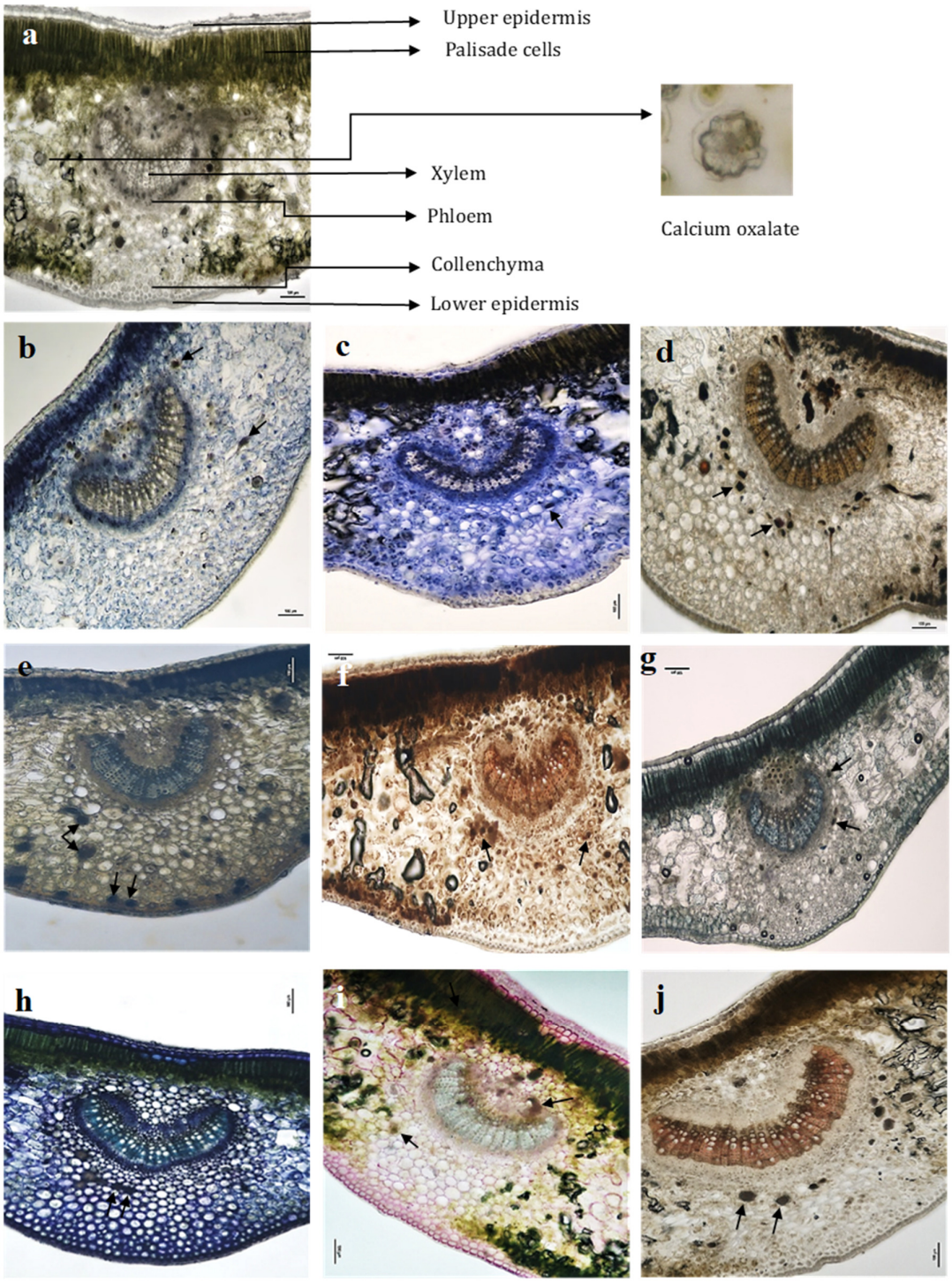

Figure 10. Histochemical observations of a transverse section of a *C. macrocarpa* winter leaf midrib. Colour change was indicative of a positive stain. (a) Unstained section showing the presence of calcium oxalate crystals. (b) Proteins-stained blue using bromophenol blue. (c) Proteins-stained blue using Coomassie. (d) Alkaloids-stained orange/brown using Dittmar's reagent. (e) Phenols stained brown/black using ferric trichloride. (f) Resin acids stained red using the NADI reagent. (g) Lipids stained blue/black using Sudan Black (h) Polyphenols stained green/blue using toluidine blue. (i) Mucilage and pectin-stained pink/red using ruthenium red. (j) Lignin aldehydes stained red using phloroglucinol. Arrows refer to laticifer.

Conclusions

Scanning electron microscopy revealed the presence of peltate trichomes and stomata only on the lower surface of the *C. macrocarpa* leaf. Nonarticulated laticifers were interspersed in the cortex and spongy parenchyma, surrounding the vascular bundles of the studied leaves. Calcium oxalate crystals were found in leaf tissues. Transmission electron microscopy revealed the presence of mitochondria, vesicles, vacuoles, and chloroplasts containing starch grains and plastoglobuli in *C. macrocarpa* leaf cells. This was the first histochemical study to report the presence of pharmacologically relevant phytochemicals (secondary metabolites) in *C. macrocarpa* leaves. Considering the chemical nature of the latex found in laticifers, it may serve as a defence mechanism against herbivory. Thus, *in vivo* toxicology testing of *C. macrocarpa* leaves is recommended to ensure the safety of the plant for medicinal use. There were little to no differences in the surface morphology and histochemistry of summer and winter leaves of *C. macrocarpa*. Regarding leaf histochemistry, both seasons seem suitable for the harvesting of this plant for medicinal use. However, transmission electron microscopy revealed that summer leaves contained more and larger starch grains, which may be due to increased exposure to sunlight leading to increased photosynthesis. *C. macrocarpa* has the ability to thrive in many habitats and has an efficient bio-metabolic system that allows it to tolerate environmental stresses. These factors can be considered in the future for the harvest and medicinal applications of *C. macrocarpa*.

Authors' Contributions

Conceptualization, R.R and Y.N.; methodology R.R and Y.N.; software, R.R.; validation, Y.H.D., T.A. and N.M.D.; formal analysis, R.R and Y.N.; investigation, R.R and Y.N.; resources, Y.N. and Y.H.D.; data curation, R.R. and Y.N.; writing—original draft preparation, R.R and Y.N.; writing—review and editing, Y.N., Y.H.D., T.A. and N.M.D.; visualization, T.A. and N.M.D.; supervision, Y.N. and Y.H.D.; project administration, Y.N.; funding acquisition, Y.H.D. All authors have read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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