Original Article

Foliar idioblasts in different-aged leaves of a medicinal plant (*Annona muricata* L.)

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Abstract

Soursop (*Annona muricata* L.) is a well-known species for indigenous use of medicines. This work aimed to determine the selected features including idioblasts in different-aged leaves of *A. muricata*. Leaf samples were collected and prepared for light microscopy. Numerous non-glandular trichomes are restricted to only the abaxial surface of hypostomatic and dorsiventral leaves. The 1st leaf is slightly different in histological structures than those of the 3rd and 8th leaves. Two kinds of foliar idioblasts were observed: subspherical idioblasts and calcium oxalate crystal-containing idioblasts. Carbohydrate and fat were detected in all leaf stages but more were observed in the 1st leaf. No protein was detected in any of the leaves and the 8th leaf exhibited the highest pigment contents. Further studies of subspherical idioblasts relating acetogenins will be necessary to determine and confirm the localization in all plant parts.

Keywords: *Annona muricata*, calcium oxalate crystal, histochemistry, pigment content, subspherical idioblast

1. Introduction

Annona muricata L. (soursop) is a well-known medicinal plant. It is one of the most economically important genera of the family Annonaceae due to its medicinal properties having the most promising activities for anticancer, antiparasitic, and insecticidal activity (Dutra *et al.,* 2012; Moghadamtousi *et al.,* 2014; Moghadamtousi *et al.,* 2015). This plant species is believed to be effective in the treatment of various ailments because of its anticonvulsant, anti-arthritic, antimalarial, hepatoprotective, and antidiabetic activities. It is also known to have cytotoxic, antileishmanial, wound healing, anti-microbial, and genotoxic properties (Gajalakshmi, Vijayalakshmi, & Devi, 2012; Moghadamtousi *et al.,* 2015; Yamthe *et al.,* 2015). *Annona* species, including *A. squamosa* were also reported to have annonaceous acetogenins, the major bioactive compounds which have a strong antitumor activity (Chen *et al.,* 2012; Wang *et al.,* 2002). Currently, the studies were mostly focused on plant parts, i.e. leaf, stem and

seed, for the discovery of a novel set of phytochemical contents and the development of new promising drugs (Chang *et al.,* 2003; Liaw *et al.,* 2002). Adewole and Ojewole (2009) reported that all plant parts, including the bark, fruit, seed, leaf, and root, are used in natural medicines in the tropics. However, the prolonged application of *A. muricata* extract at high dosages could affect the function of kidney cells leading to renal failure because of an excessive accumulation of plant calcium oxalate (CaOx) crystals and free calcium at high levels might have a toxic effect on cells (Dayeef, Karyono, & Sujuti, 2013; Nakata, 2012). Hence, CaOx crystals form to remove the excess oxalate or calcium. Nakata (2012) also revealed that high dietary oxalate consumption often results in an increase in urinary oxalate excretion and oxalate can precipitate with calcium or other substances leading to CaOx kidney stone formation.

In plants, CaOx crystal formation is generally intracellular and the crystals originate inside the vacuoles of specialized cells called crystal idioblasts (crystal-forming cell) and in the cell wall of angiosperms and gymnosperms. Based on a variation in crystal morphology, the CaOx crystals located in specific tissues or distributed in almost all plant parts have been classified into 5 groups: prism (prismatic), druse,

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styloid, raphide, and crystal sand. Moreover, large and subspherical idioblasts exhibiting a lipophilic nature (essential oils and lipids) can be found mainly in the subepidermal area of leaves. The leaf essential oil containing monoterpines, which is detected by GC-MS analysis, may be related to both the antimicrobial and antioxidant activities. This idioblast type found in the endosperm of *A. macroprophyllata* seeds was also reported to have acetogenins and probably alkaloids. These acetogenins, along with their secondary metabolic functions, were reported to be pharmacologically important and their bioactive compounds have antitumoural, antiparasitic, antimalarial, insecticidal, antimicrobial, antifungal, and antibacterial properties (Brechú-Franco, Laguna-Hernández, De la Cruz-Chacón, & González-Esquinca, 2016; Hernández, Franco, De-la-Cruz-Chacón, & González-Esquinca, 2015). Consequently, this present research aimed to provide a better understanding of some of the selected foliar characteristics in different aged-leaves of *A. muricata*, especially the presence of CaOx crystal-containing idioblasts, which result in renal failure in humans, and subspherical idioblasts that are related to acetogenins.

2. Materials and Methods

2.1 Plant materials

Leaf samples of different aged-leaves, specifically the 1st leaf at 3-4 cm in length, and the 3rd and 8th leaves from the apex, of the *A. muricata* L. were collected from three individual plants in Namom District of Songkhla Province, Thailand. The leaves with midrib were cut (5×5 mm^2) from the center of the lamina with a sharp razor blade for a transverse section. All samples (three replicates/leaf) were prepared for comparative histological and histochemical features using the standard paraffin method (Ruzin, 1999). Stomatal data including stomatal length, stomatal density (SD) and stomatal index (SI) were taken from a representative leaf (3rd leaf). Pigment contents of all leaves each with six replicates were also examined.

2.2 Histological observation and measurements

Samples were fixed in FAA II (formaldehyde, glacial acetic acid, 70% ethyl alcohol, 5:5:90 v/v/v) for 48 h at room temperature, dehydrated gradually through a graded ethanol-tertiary-butyl alcohol series, infiltrated and embedded in Histoplast. Serial sections of 10-12 μm thick were cut on a rotary microtome, affixed on a slide for safranin and fast green staining. All sections were observed under an Olympus BX 51 light microscope fitted with an Olympus DP72 digital camera.

Three sections taken from each of the three individual plants (replicates) of each different-aged leaves (the 1st, 3rd, and 8th leaves) were quantitatively measured for thickness of the mesophyll, as well as the upper and lower epidermis. All measurements were repeated three times to maintain the typical size of the characters measured.

The stomatal length, SD (number of stomata/mm^2), and SI taken from the 3rd leaf of each of the three individual plants were also determined. The SI was assessed using the following formula:

$$SI = [S / (S + E)] \times 100$$

where SI = stomatal index, S = number of stomata/mm^2, and E = number of ordinary epidermal cells/mm^2.

2.3 Histochemical observation

The transverse paraffin-embedded sections of the 1st, 3rd, and 8th leaves were examined for the accumulation of carbohydrate (periodic acid-Schiff staining), fat (Oil Red O staining), and protein (ninhydrin staining). Fresh samples were also cut using a Leica cryostat and determined for fat (Oil Red O staining) and protein (Amido Black staining) (Regan & Moffatt, 1990).

2.4 Chlorophyll (chl) and carotenoid determination

Leaf samples (approximately 0.1 g in fresh weight [FW]) of the 1st, 3rd, and 8th leaves were crushed in 5 mL of 80% chilled acetone at room temperature and extracted three times by centrifugation at 3000 rpm for 10 min. The supernatant was stored in a dark cool container and the absorbance was measured at 480, 510, 645, and 663 nm using an HP-8453E UV-visible spectroscopy system. The amounts of chl *a*, chl *b*, total chl, and carotenoid contents were calculated in mg.g^{-1} FW by the following formulae (Mistra, Toppo, Gupta, Chakrabarty, & Tuli, 2010):

$$\text{Chl } a = [12.7(A_{663}) + 2.63(A_{645})] \times V/1000 \times FW$$
$$\text{Chl } b = [22.9(A_{645}) - 4.68(A_{663})] \times V/1000 \times FW$$
$$\text{Total chl} = [20.2(A_{645}) + 8.02(A_{663})] \times V/1000 \times FW$$
$$\text{Carotenoid} = [7.6(A_{480}) - 2.63(A_{510})] \times V/1000 \times FW$$

where, A_{480}, A_{645}, A_{510}, and A_{663} are the absorbance values at 480 nm, 645 nm, 510 nm, and 663 nm, respectively. V is the volume of the extraction solution (mL) and FW is the fresh weight of the leaf sample (g).

2.5 Statistical analyses

Pigment determination was conducted using the completely randomized design with three replicates and repeated twice. Leaf blade thicknesses, including the upper epidermis, the mesophyll, and the lower epidermis of different aged-leaves, were measured from three replicates (individual plant) each with three sections (sample). Data were statistically analyzed using one-way ANOVA and followed by Duncan's multiple range test (DMRT) at $P \leq 0.05$.

3. Results and Discussion

3.1 General leaf characteristics

The *Annona muricata* L. leaf blade consists of three main layers, namely the upper (adaxial) epidermis, the mesophyll, and the lower (abaxial) epidermis. The epidermal cells are polygonal and tightly arranged. Both the adaxial and abaxial epidermal layers, being made of different sizes, are uni-

seriate and covered with a cuticle layer that prevents the plant from excessive loss of water via evaporation (Graham *et al.,* 2006). The presence of an epidermis with multiple layers is associated with xerophytic species as an ecological adaptation to prevent water loss, while an epidermis of a single layer is considered to be the common and normal type in mesophytic species and is typically regarded as normal in vascular plants (Dickison, 2000). Adaxial epidermal cells (Figure 1A, UE) are relatively larger and longer than those of the abaxial side (Figure 1A, LE). The upper epidermal cells are larger at the lamina region and are slightly reduced at the midrib region. Cuticles (Figure 1A, arrows) are found to cover both epidermal layers. The vertically elongated, double layered compactly arranged palisade parenchyma of the dorsiventral leaf is rich in chloroplasts. The spongy parenchyma, major portion of mesophyll, consisted of 6-7 layers of loosely or compactly arranged cells with large intercellular spaces. Many species of *Annona* were mostly reported to have dorsiventral mesophyll (Bakkar & Gerritsen, 1992). The non-glandular trichome (Figure 1B), which consisted of a row of 2-3 cells having elongated pointed-tip, was found only on the abaxial leaf surface. Similar non-glandular trichomes were reported in almost all genera of Annonaceae in neotropical regions (Bakker & Gerristen,

1992). Uniseriate and non-glandular trichomes were reported by several authors to be common types in Annonaceae and thus considered almost certainly primitive. The presence of epidermal foliar appendages assisted the plants over many abiotic and biotic stresses (Amme *et al.,* 2005). Thus, trichomes help control the rate of transpiration in leaves thus preventing water loss due to the microclimate created by the extension of appendages from the leaf on the surfaces (Ehleringer, 1985).

Additionally, the epidermal anticlinal walls exhibiting a highly sinuous structure are parallel and perpendicular to the epidermal surfaces (Figure 1C, arrow heads). Olowokudejo (1990) revealed that the sinuous anticlinal walls were also found in epidermal cells of *A. squamosa* while those of *A. muricata* presented curved anticlinal walls. According to Gifford and Foster (1989), epidermal wall shapes are affected by the habitat and environment. For environmental adaptation, the anticlinal walls of species in mesophytic habitats are considered to be sinuous while species of xerophytic habitats are associated with straight anticlinal walls. The *A. muricata* exhibited a hypostomatic leaf with paracytic stomatal apparatus (Figure 1C) which agreed with the findings in *A. squamosa* (Agrawal *et al.,* 2011; Olowokudejo, 1990), *A. glabra*, *A. chrysophylla* (Olowokudejo, 1990), *A. senegalensis* (Abdulrahaman *et al.,* 2013; Olowokudejo, 1990), and *A. reticulate* (Jani, Harisha, & Behzad, 2012). The stomata were seen to be frequent, regularly distributed, and confined only to the abaxial side due to water loss adaptations. SD (145.78±5.62) and SI (12.49±0.51%) (Table 1), a reflection of physiological responses to environmental conditions, are known to affect CO_2 diffusion into the leaf and thus the photosynthetic rate (Vráblová, Vrábl, Hronková, Kubásek, & Šantrůček, 2017).

Table 1.   Stomatal characteristics of *Annona muricata* L.

Stomatal parameters	Mean±SE
Stomatal length (µm)	30.97±0.73
Stomatal density (stomata/mm^2)	145.78±5.62
Stomatal index (%)	12.49±0.51

SE, standard error; data were taken from the 3rd leaf (the representative leaf sample).

Two kinds of idioblasts were observed: subspherical idioblast and calcium oxalate (CaOx) crystal-containing idioblast. Subspherical idioblasts (Figure 1A, asterisk marks) are roughly spherical to oblong blocky shaped cells which occur mostly in the palisade mesophyll of the leaf lamina and are usually observed in the subepidermal area, while only a few occur in the spongy regions. Idioblasts containing druses (Figure 1D) and prismatic crystals (Figure 1E and 1F) are seen in the midrib veins and mesophyll region of the lamina blade, respectively. Druses are numerous and occur exclusively in vascular bundles (midvein and conducting tissues in the lamina). Prismatic crystals are found solitary within a cell and seen largely lying in the mesophyll region. These elongated prismatic crystals either have pointed tapered ends (Figure 1E) or squared ends towards the tip (Figure 1F). The presence of prismatic crystals in the lamina was also reported in the leaves of *A. reticulate* (Jani *et al.,* 2012; Pathak & Zaman, 2013; Zaman & Pathak, 2013) and *A. squamosa* (Agrawal *et al.,* 2011; Jani, *et al.,* 2012). However, sphaeraphides and microrosette
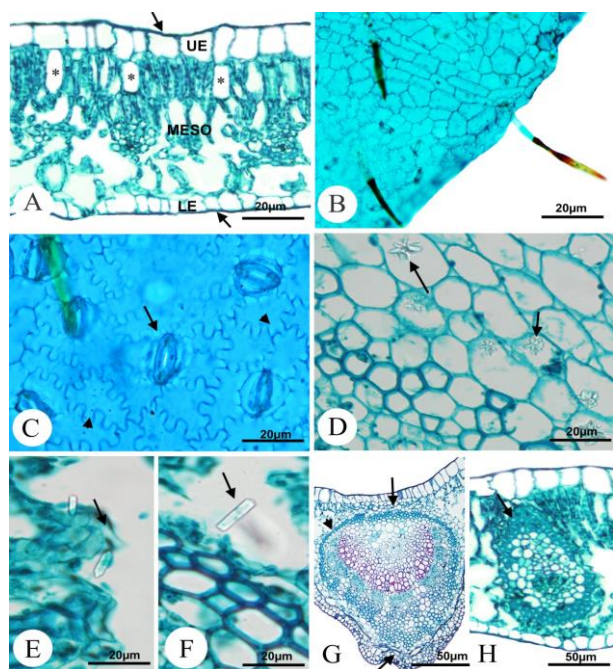

Figure 1.   General features of the 3rd leaf of *Annona muricata* L. (A) Transverse section of the leaf blade showing three layers; upper epidermis (UE), mesophyll (MESO) and lower epidermis (LE). Cuticular layers (arrows) on both adaxial and abaxial surfaces. Subspherical idioblasts (*) appearing in palisade mesophyll (B) Non-glandular trichome at lower epidermis. (C) Epidermal cells exhibiting sinuous anticlinal wall (arrow heads) with paracytic stomatal apparatus (arrow). D. Druses (arrows) in midrib cells. (E-F) Prismatic crystals in mesophyll with (E) tapered ends (arrow) and (F) squared ends (arrow). (G) Midrib showing collenchyma tissues (arrows) at adaxial and abaxial sides. Sclerenchyma sheaths (arrow head) surround the nervure of midrib and (H) the lateral vein of lamina.

crystals were also observed in the leaves of *A. reticulata* (Jani *et al.,* 2012; Pathak & Zaman, 2013). The CaOx crystal forms endogenously from oxalic acid and calcium is considered a normal physiological process as a potential defense mechanism (Chairiyah, Harijati, & Mastuti, 2013; Faheed, Mazen, & Elmohsen, 2013). The position, size and concentration gradients of CaOx crystals were reported to partly reflect the functions of the cell types in which the crystal is formed and are related to the amount of calcium uptake for the specific growth point by the plant and change in environmental conditions which governs transpiration rates (Mazen, Zhang, & Franceschi, 2003; Prychid, Jabaily, & Rudall, 2008). Not only for the taxonomic value, crystals in medicinal plants have definite biological functions and are of immense importance as diagnostic tools for crude drugs and the detection of adulterants in herbal drugs.

A transverse section of the midrib exhibited a concave and a convex part at the adaxial and the abaxial sides, respectively (Figure 1G). At the adaxial surface, the epidermal cells of the midrib were smaller than the epidermal cells of the lamina. In both the adaxial and abaxial sides, the collenchyma, as a supporting tissue, was represented by 3-7 layers of cells (Figure 1G, arrows). The collateral vasculature is surrounded by parenchymatous cells and a continuous sclerenchyma cap (Figure 1G, arrow head). Terminal collateral veins in the leaf blade were often covered with sclerenchymatic caps or sheaths (Figure 1H, arrow). The nervures, being collaterally sheathed by sclerenchyma, were reported in the species of *Annona* (Bakkar & Gerritsen, 1992). A similar presence of sclerenchymatous sheaths in the veins and supporting tissues of collenchyma cells in the midrib were also reported in *A. reticulata* (Pathak & Zaman, 2013; Zaman & Pathak, 2013).

3.2 Features of different-aged leaves

All three different-aged leaves exhibited dorsiventral mesophyll with CaOx crystal- (prismatic and druse) containing idioblasts and subspherical idioblasts. The thickness of the adaxial (upper) epidermis of the 1st leaf (43.88± 1.82 μm) was less than the 3rd (51.52±1.97 μm) and 8th (48.88±1.34 μm) leaves (Table 2). No difference was observed in the thickness of the upper epidermis of the 3rd and 8th leaves. Larger upper epidermal cells could be seen in the older leaves. The mesophyll of the 1st leaf was slightly different from the 3rd and 8 leaves. In the latter case, they were similar in the histological structure of the mesophyll. The 1st leaf exhibited a compact, overall mesophyll arrangement which indicated an immature or developing leaf (Figure 2A). Short spongy mesophyll cells comprised of 3-4 cell layers had firmly arranged compact cells with few intercellular spaces. The spongy mesophyll contained 5-7 cell layers and the lacunae could be seen in the 3rd (Figure 2B) and 8th leaves (Figure 2C). Alterations in the internal leaf structures, such as the distribution and amount of mesophyll in the respective developmental stages, likely occurred as an adaptation depending on the degree of light intensity that plants were exposed to (James *et al.,* 1999). Leaf exposure to sunlight and the availability of water are significant influential factors which affect the leaf structure and orientation. These are plant responses to avoid the incident irradiance and to tolerate adaptation for functional advantages depending upon the level of stress in the habitat (Smith *et al.,* 1998). Differences in the structures of mesophyll and performance of photosynthesis in leaves were reported to be associated with the processes of regulating internal light and carbon dioxide (James *et al.,* 1999). Similar studies with different mesophyll structures and chlorophyll content in different stages of leaves were reported in *Eucalyptus globulus* (James *et al.,* 1999). Except for the upper epidermis, there were no completely different proportions of mesophyll and lower epidermis that correlated with different ages of the leaves.

Carbohydrate accumulation was mostly seen in the midrib cells and few in the epidermal cells. Most of the reserved carbohydrates were revealed in the midrib of the 1st leaf (Figure 2D). The least amounts of stored carbohydrates

Table 2.　　Some characteristics of the different-aged leaves of *Annona muricata* L.

Characteristics	1st leaf	3rd leaf	8th leaf
Leaf thickness (μm)			
Upper epidermis t	43.88 ± 1.82^{b}	51.52 ± 1.97^{a}	48.88 ± 1.34^{a}
Mesophyll	162.36 ± 6.10^{ns}	173.33 ± 3.79^{ns}	171.80 ± 5.08^{ns}
Lower epidermis	27.08 ± 1.43^{ns}	28.88 ± 1.16^{ns}	27.91 ± 1.90^{ns}
Substances			
Carbohydrates	+++	++	+
Fats/lipid bodies*	++	++	+
Proteins	-	-	-
Foliar idioblasts:			
1. CaOX crystal-containing idioblast			
Druses	P	P	P
Prismatic crystals	P	P	P
2. Subspherical idioblast	+++	++	++

Values with different letters within each row are significantly different at *P≤0.05* (Duncan's multiple range test); ns, no significant difference, +++ = more, ++ = Less, + = Least, − = absent/could not be detected, P = present, * = mainly in subspherical idioblast
Paraffin-embedded samples were stained for accumulation of carbohydrate (PAS staining), fat (Oil Red O staining) and protein (ninhydrin staining). Fresh samples were also stained for protein by Amido Black staining.
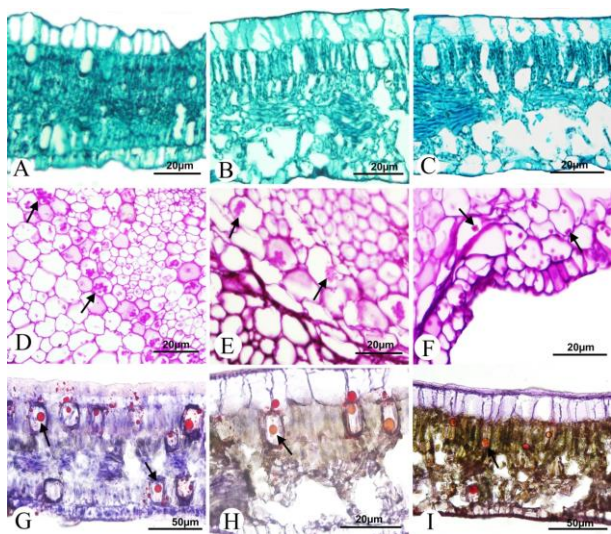
Figure 2.   Histological and histochemical features of three different-aged leaves of *Annona muricata* L. Transverse sections of the lamina and the midrib exhibiting (A-C) general structure and (D-I) the substance accumulation in the 1st (A, D, G), 3rd (B, E, H), and 8th (C, F, I) leaves. (A) The 1st leaf with a compact mesophyll arrangement indicating immature or developing leaf. (B) The mature 3rd and (C) 8th leaves as indicated by mesophyll arrangement. (D-F) Parenchymatous cells of the midrib presenting (D) numerous carbohydrate granules in the 1st leaf (arrows), (E) some granules (arrows) in the 3rd leaf and (F) few granules (arrows) in the 8th leaf. (G) Numerous reddish orange fat/lipid globules (arrows) in the subspherical idioblast of the 1st leaf. (H) Fat/lipid droplets in the upper epidermis and the subspherical idioblast (arrow) of the 3rd leaf. (I) Subspherical idioblast of the 8th leaf exhibiting pale orange droplets of fat (arrow).

were observed in the 3rd (Figure 2E) and the 8th leaves (Figure 2F). Comparative studies of the levels of carbohydrate accumulation in different-aged leaves of *A. muricata* showed that the pattern was 1st > 3rd > 8th leaves. This pattern coincided with greater amounts of carbohydrate in the young leaves of *Cleome chellidonii* and *C. speciosa* than in mature and senescent leaves reported by Aparadh & Karadge (Aparadh & Karadge, 2012a; Aparadh & Karadge, 2012b). Carbohydrates are the direct products of photosynthetic carbon fixation and are the main source of energy for plant growth and development. Carbohydrates also serve as precursors for the respiretory process. Positive reactions with Oil Red O were observed in subspherical idioblasts having fat/lipid bodies of all different-aged leaves. Similarly, the 1st leaf also showed the strongest response with distinct, deeply stained red fat/lipid bodies (Figure 2G) while the 3rd (Figure 2H) and 8th leaves (Figure 2I) exhibited light red to pale orange bodies. Interestingly, these reserved lipid bodies mostly occurred in the subspherical idioblasts of palisade mesophyll and few of them appeared to be scattered through other mesophyll and epidermal cells. Consequently, Oil Red O, which is similar to fat/lipid detection using Sudan III, caused a reddish orange droplet of lipid/fat nature which indirectly indicated the presence of bioactive acetogenins (cancer-killing phytochemicals) in subspherical idioblasts in all leaves that were examined (Bre-

chú-Franco *et al.,* 2016; Hernández *et al.,* 2015). Protein could not be detected in any of the leaves examined. Therefore, this reaction should be confirmed by further histochemical determination.

3.3 Determination of chlorophyll and carotenoid contents

Significant differences in all pigment contents were found among the different-aged leaves. The 1st and 3rd leaves had lower contents of total *chl* and carotenoid than the 8th leaf (Figure 3). Pigment contents were the highest in the 8th leaf and *chl a* was the dominant pigment in all leaves. Similar observations were made in mature leaves of *Cleome gynandra* and *C. speciosa* (Aparadh & Karadge, 2012a; Aparadh & Karadge, 2012b). Aging causes a change in the chlorophyll level which ultimately increases from tender to mature leaves. The level and state of leaf chlorophyll contents are important factors to determine the overall photosynthetic efficacy of a plant (Aparadh & Karadge, 2012a; Aparadh & Karadge, 2012b).
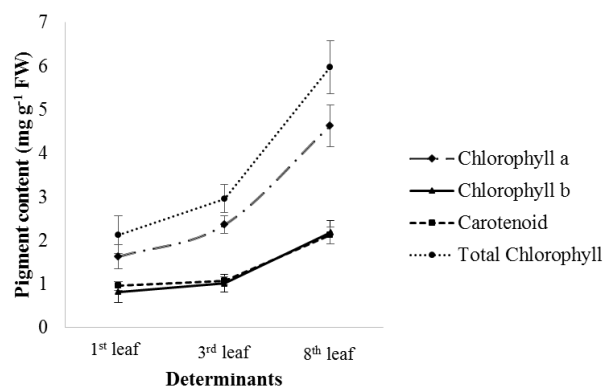

Figure 3.   Pigment contents in different-aged leaves of *Annona muricata* L. The hightest contents of *chl a* appearing in all leaves examined. Bars display standard error.

4. Conclusions

Annona muricata presents a hypostomatic and dorsiventral leaf with non-glandular trichomes which are restricted to only the abaxial surface. Foliar idioblasts, as subspherical idioblasts having fat, were found mainly at the palisade mesophyll and CaOx crystal-containing idioblasts were observed. In the latter case, druse and prismatic crystals were found in the midrib and mesophyll, respectively. Carbohydrate and fat were present in all leaf stages but observed to be greater in the 1st leaf. Unfortunately, none of the different-aged leaves exhibited any protein. Mass contents of the ergastic substances in the 1st leaf and the highest pigment contents (chlorophyll and carotenoid) in the 8th leaf can also be of importance for use of different-aged leaves. This work gives insights of knowledge on the use of different-aged leaves in various fields for future research. For additional insights into the aspects of CaOx crystal formation and annonaceous acetogenins in different types of idioblasts located in the same plant part, further studies on the histological localization of idioblasts related to acetogenins in all plant parts and the impact of plant oxalates on human health need to be examined in detail.

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