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Microscopic study of plants containing cardiac glycoside in Apocynaceae

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Abstract. Three species of plants: *Nerium oleander* L., *Thevetia peruviana* (Pers.) K. Schum, and *Cerbera odollam* Gaertn., in the family Apocynaceae containing cardiac glycoside were studied microscopically using free-handed thin section and peeling methods for fresh samples. Leaves and flowers of each plant were artificially digested with chemicals and controlled temperature. After the digestion, the samples were collected at a specific time point (30 min, 1-5, 24 and 48 hrs) and comparing anatomically with the undigested fresh samples. The results showed that distinguishable and specific microscopic structures were found in each species. The important microscopic features in leaves are the types of stomatal complex, trichomes, calcium oxalate crystals, the number of hypodermal layer and palisade mesophyll. For the flowers, the specific characteristics are the types of trichomes, shape and the aperture of pollen grains. Comparison between the undigested and digested samples using the significant anatomical structures had been done. The results show that the digested plant material can be identified microscopically. Moreover, the significant anatomical structures in the digested samples show unchanged features throughout the time of digestion. The anatomical structures had been established as the identification parameters which can be employed for plant materials found in gastric contents of forensic cases.

Keywords: Apocynaceae, artificial digestion, cardiac glycoside, microscopic study

Introduction

Stomach contents can be collected during forensic medical examination. The digested food in the decease stomach can be promising evidence. Valuable information can be obtained from the contents: (1) whether the victim had a meal prior to death, (2) what type of food was last eaten, (3) time that the food had been eaten before death, and, perhaps, (4) the location where the food was prepared (Ernst, 1990). Parts or fragments of plant obtaining from gastric contents in autopsy cases can be evidence for cause of death. Identification of medicinal

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herbal species, natural narcotic drugs and spices using the specific anatomical structures are commonly practiced in pharmaceutical field (Jackson & Snowdon, 2000; Lakusic *et al.*, 2007; Jayeola, 2009). The laboratory manual for the identification of plant food cells in gastric contents for forensic investigations had been reported (Bock *et al*., 1988; Ernst, 1990). Family Apocynaceae, the tropical plants with beautiful flowers, commonly used as ornamental plants. It had been reported that several species *i.e. Nerium oleander* L., *Thevetia peruviana* (Pers.) K. Schum and *Cerbera odollam* Geartn. contain cardiac glycoside and found in fatal cases (Haynes *et al.*, 1985; Eddleston *et al*., 1999; Gaillallard, *et al.*, 2004; Wasfi *et al*., 2008; Bandara *et al.*, 2010; Pongpijid, *et al.*, 2011). In the past, several anatomical studies of *N. oleander, T. peruviana* and *C. odollam* had been done as they are the interested species of the Apocynaceae, mainly for the sunken stomata feature of *N. oleander* and being presented with the deathly cardiac glycoside (Mahran *et al.*, 1974; Mauseth, 2008; Rai & Tiwari, 2012). In Thailand, the anatomy of the dried samples and also the TLC (Thin Layer Chromatography) of the leaves, flowers, fruits and seeds of five species in Apocynaceae had been done, however, the fresh and digested samples, still, had not been observed (Pongpijid *et al.*, 2011). According to the literature reviews, the most effective identification method for cardiac glycoside at the present time is the TLC. However, the method focuses on the presence or type of the cardiac glycoside not the anatomical structures (Gaillallard *et al.*, 2004). Therefore, in the present study, the fresh samples of the three cardiac glycoside containing plants (*N. oleander*, *T. peruviana* and *C. odollam*) were used to study for the specific anatomical features. The important features, then, were set up as the identification criteria for each species, and compared with the artificially digested samples in order to establish the identification criteria for forensic cases.

MATERIALS AND METHODS

1. Plant materials–Plant samples (*N. oleander*, *T. peruviana* and *C. odollam*) were collected from areas in Bangkok and Nakhon Pathom, Thailand. Herbarium specimens were also made using standard procedures for herbarium materials (Bridson & Forman, 1999). Voucher specimens were kept at the Bangkok Herbarium (BK) (Table 1). Identification of the collected specimens was based on the Flora of Thailand (Middleton, 1999).

TABLE 1. List of voucher specimens

Species	Localities	Vouchers
Cerbera odollam Gaertn.	Bangkok	WGK 007 (BK)
	Nakhon Pathom	WGK 008 & 009 (BK)
Nerium oleander L.	Nakhon Pathom	WGK 001, 002 & 003 (BK)
Thevetia peruviana (Pers.) K. Schum	Bangkok	WGK 004 (BK)
	Nakhon Pathom	WGK 005 & 006 (BK)

2. Anatomical study–Free-handed thin section and epidermal peeling slides were prepared using thin razor blade and Safranin O staining. The sections were observed and the photographs were taken using Olympus light-microscope model CX31RTSF with DP20 BASIC photographing. The characteristics of the plants were studied, described and categorized (Evert, 2006; Hesse *et al*., 2009).

3. Artificial digestion–Ten grams of each plant samples (leaves and flowers) were ground to mimic chewing and placed into a zippered plastic bag. The simulated digestion was done with some modifications using: artificial saliva (α-amylase 2 g/L, NaCl 0.117 g/L, KCl 0.149 g/L and $NaHCO_3$ 2.1 g/L, pH 7), artificial gastric juice (4 mL hydrochloric acid in 250 mL dH_2O) and pepsin (0.5% solution; 1 g/200 mL dH_2O) (Kong & Singh, 2008; Culp, 2010). After well mixed, each bag was placed into a beaker and submerged into 37°C water bath to mimic body temperature. The bags were squeezed and shaken regularly. The digested samples were collected at 30 min, 1-5 hrs, then the temperature controller was turned off to let the digestion processed under room temperature and collected sample at 24 and 48 hrs.

4. Digested sample slide preparation–The digested samples were stained with Safranin O and smeared on glass slides. Few drops of distilled water were added to the stained sample before covering with a cover slip. The prepared slides were observed and compared for the anatomical parameters from the established identification criteria.

Results

The results showed several anatomical structures of the leaves and flowers parts of the three plants.

***Cerbera odollam*–Leaves** (cross section): thin cuticle layers were observed with the absent of hypodermis. Trichome is absent from the leaves. Palisade mesophyll presented with a single-layer and only located beneath the upper epidermis. *Surface view*: mark wavy outline upper and lower epidermal cells are present and trichomes are absent from both surfaces. The prismatic calcium oxalate crystals can be seen. Anisocytic stomata are present on the lower surface. **Flowers** (cross section): glandulous unicellular trichomes were observed on the inner epidermis. *Surface view*: stomata were found on the outer epidermis. Thin-walled upper and lower epidermal cells are present. The isopolar, 4-colporate, spheroidal pollen grains with diameter 90.7 μm were observed.

***Nerium oleander*–Leaves** (cross section): the epidermal cells and cuticle are thick on both upper and lower epidermis. The 2-3 hypodermal layers are present beneath the epidermal layer of both sides. Upper and lower surfaces are covered with the sharp end non-glandulous unicellular covering trichomes and the blunt end non-glandulous unicellular covering trichomes are present around the opening of the stomatal crypts. Palisade mesophyll arrangement shows 2-3 layers beneath the upper epidermis, while 1-2 layers of the palisade mesophyll are present on the lower side. Prismatic calcium oxalate crystals are rarely found while

abundant amount of rosette-aggregate calcium oxalate crystals are present throughout the leaves. The sunken stomata are clearly seen on the lower epidermis and have been considered to be the prominent parameter for leaf identification. *Surface view*: the entire outline of polygonal epidermal cell is present on both sides of the leaf surfaces and both upper and lower epidermal surfaces are covered with sharp end non-glandulous unicellular covering trichomes. The stomatal crypts were clearly observed on the lower surface with blunt end non-glandulous unicellular trichomes. Prismatic calcium oxalate crystals are rarely found while rosette aggregate crystals are present. **Flowers** (cross section): the smooth-surface glandulous unicellular trichomes presented on the inner surface of the petals. *Surface view*: stomata were found on the outer surface of the petals. Inner and outer epidermal cells presented with thin-walled. The isopolar, 4-porate, oblate-spheroidal pollen grains with diameter 45.43 μm were observed.

***Thevetia peruviana*–Leaves** (cross section): thin cuticle layers were observed with the absent of hypodermal layer. A single layer of palisade mesophyll was observed beneath the upper epidermis. *Surface view*: sinuous polygonal epidermal cells present on the upper epidermis, while the wavy outline epidermal cells present on the lower epidermis. A great numbers of rosette-aggregate calcium oxalate crystals present throughout the mesophylls. Having a large amount of the calcium oxalate crystals, it was taken as one of the parameters for the identification. The trichome is absent on both upper and lower surfaces of the leaves. Anomocytic stomata present only on the lower surface of the leaves. **Flowers** (cross section): smooth non-glandulous unicellular trichomes were observed on the inner surface. *Surface view*, stomata were found on the outer surface of the petals. Thin-walled epidermal cells were observed in the inner and outer epidermis. The isopolar, 3-colporate, oblate or oblate-spheroidal pollen grains with diameter 75.72 μm were observed.

Five specific anatomical features from the leaves and two anatomical features from flowers of each plant species were chosen for the identification parameters which are: the types of stomatal complex, trichomes, calcium oxalate crystals, the numbers of hypodermal layers and palisade mesophyll for the leaves. For flowers, the types of trichomes, the shape and apertures of the pollen grains (Tables 2-3, Figures 1-6). When compared the digested samples with the identification parameters, almost all of the significant anatomical structures, both in the leaves and flowers, can be observed during different time of digestion (Tables 4-6, Figures 7-11). Each digested plant material can be identified using the anatomical structures listed above.

Table 2. Anatomical parameters for the leaf identification

Species	Stomata	Trichomes	Palisade mesophyll layers	Hypodermal layer	Calcium oxalate crystals
C. odollam	Anisocytic	Absent	Single layer	Absent	Prism
N. oleander	Sunken	Sharp-end non-glandular unicellular & blunt-end non-glandular unicellular	2-3 layers	2-3 layers	Rosette & prism
T. peruviana	Anomocytic	Absent	Single layer	Absent	Rosette

Table 3. Anatomical parameters for the flower identification

Species	Trichomes	Pollen grains
C. odollam	Rough-surfaced non-glandular unicellular	4-colporate spheroidal shape
N. oleander	Smooth-surfaced glandular unicellular	4-porate oblate or oblate-spheroidal shape
T. peruviana	Smooth-surfaced non-glandular unicellular	3-colporate oblate or oblate-spheroidal shape

Table 4. Anatomical structures of digested *C. odollam* (×=absent, ✓=present)

Specific anatomical structures	Digested time and plant structure							
	min			hrs				
	30	1	2	3	4	5	24	48
Digested leaves								
- Anisocytic stomata	✓	✓	✓	✓	✓	✓	✓	✓
- Single-layer palisade mesophyll	×	×	×	×	×	×	×	×
- Prism calcium oxalate crystals	×	×	×	×	×	×	✓	×
Digested flowers								
- Rough-surfaced glandular unicellular trichomes	✓	✓	✓	✓	×	×	✓	✓
- 4-colporate spheroidal shape pollen grains	✓	✓	✓	✓	✓	✓	✓	✓

TABLE 5. Anatomical structures of digested *N. oleander* (×=absent, ✓= present)

Specific anatomical structures	Digested time and plant structure							
	min				hrs			
	30	1	2	3	4	5	24	48
Digested leaves								
- Sunken stomata	✓	✓	✓	✓	✓	✓	✓	✓
- Sharp-end non-glandular unicellular trichomes	✓	✓	✓	✓	✓	✓	✓	✓
- Blunt-end non-glandular unicellular trichomes	✓	✓	✓	✓	✓	✓	✓	✓
- 2-3 layers palisade mesophyll	×	×	×	×	×	×	×	×
- Rosette aggregate calcium oxalate crystals	✓	✓	✓	✓	✓	✓	✓	✓
- Prismatic calcium oxalate crystals	×	×	×	×	×	✓	×	×
Digested flowers								
- Smooth-surfaced glandular unicellular trichomes	✓	✓	✓	✓	✓	✓	✓	✓
- 4-porate oblate or oblate-spheroidal shape pollen grains	✓	✓	✓	✓	✓	✓	✓	✓

TABLE 6. Anatomical structures of digested *T. peruviana* (×=absent, ✓= present)

Specific anatomical structures	Digested time and plant structure							
	min				hrs			
	30	1	2	3	4	5	24	48
Digested leaves								
- Anomocytic stomata	✓	✓	✓	✓	✓	✓	✓	✓
- Single-layer palisade mesophyll	×	×	×	×	×	×	×	×
- Rosette aggregate calcium oxalate crystals	✓	✓	✓	✓	✓	✓	✓	✓
Digested flowers								
- Smooth-surfaced non-glandular unicellular trichomes	✓	✓	✓	✓	✓	×	✓	✓
- 3-colporate oblate-spheroidal shape pollen grains	✓	✓	✓	✓	×	×	✓	✓

DISCUSSIONS

For the identification of the leaves, only the types of stomatal complex can be used for the identification among the three species. However, the types of trichomes in leaves and calcium oxalate crystals should be observed to support the identification. When observed the digested samples for the identification parameters, the results showed that almost all significant anatomical structures can be found in the digested sample at various time points.

For the unobserved digested anatomical features which are: the 2-3 layers of palisade

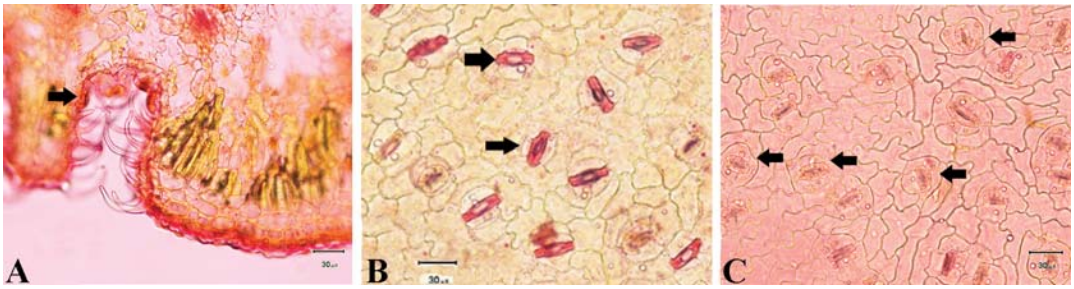

Figure 1. Stomatal types (arrows): A. sunken stomata of *N. oleander*'s leaf; B. anomocytic stomata of *T. peruviana*'s leaf; C. anisocytic stomata of *C. odollam*'s leaf.

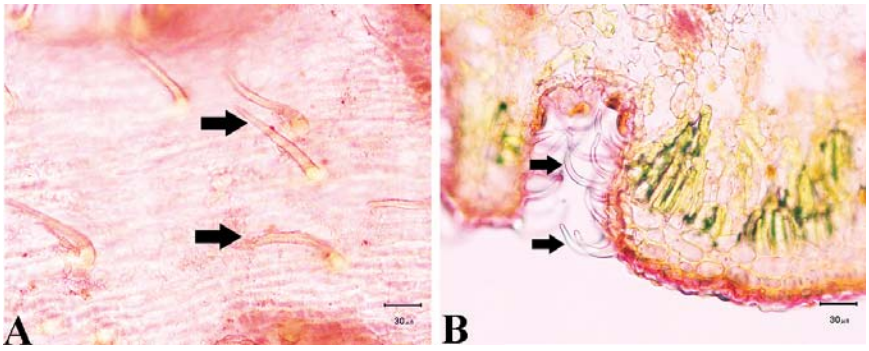

Figure 2. Trichomes of *N. oleander*'s leaf (arrows): A. sharp-end non-glandular unicellular trichomes; B. blunt-end non-glandular unicellular trichomes.

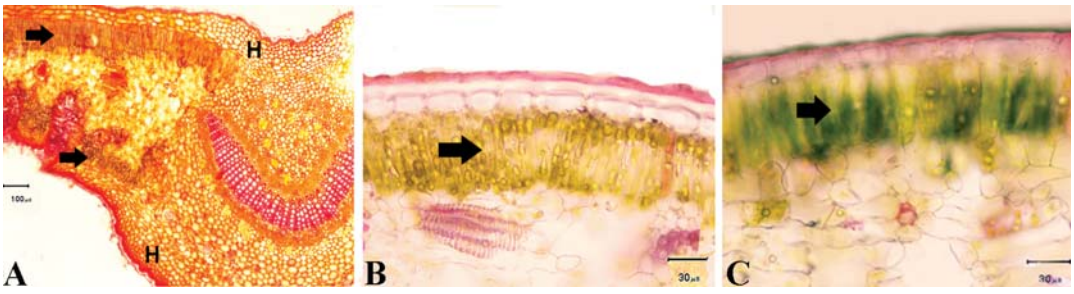

Figure 3. Palisade mesophyll (arrows): A. 2-3 layers of palisade mesophyll of *N. oleander*, H=hypodermal layers; B. single-layer of palisade mesophyll of *T. peruviana*; C. single-layer of palisade mesophyll of *C. odollam*.

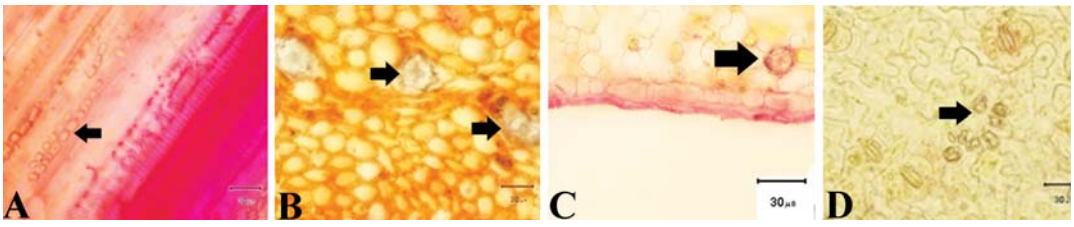

Figure 4. Calcium oxalate crystals types (arrows): A. prismatic crystals of *N. oleander*; B. rosette-aggregate crystals of *N. oleander*; C. rosette-aggregate crystals of *T. peruviana*; D. prismatic crystals of *C. odollam.*

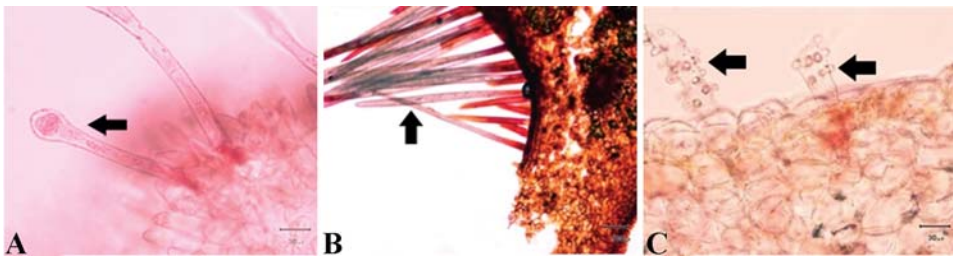

FIGURE 5. Trichomes in flowers (arrows): A. *N. oleander*; B. *T. peruviana*; C. *C. odollam*

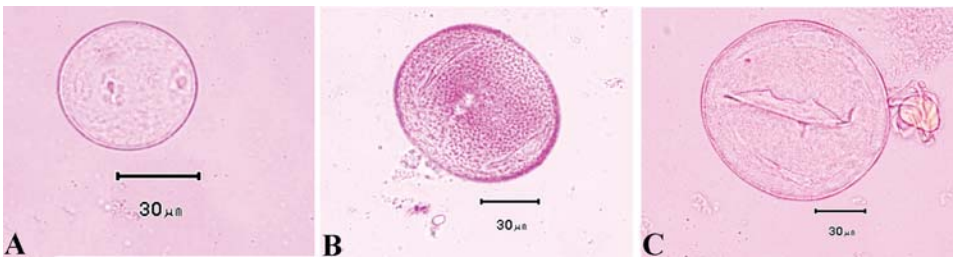

FIGURE 6. A. 4-porate oblate-spheroidal shaped pollen grian of *N. oleander*; B. 3-colporate oblate or oblate-spheroidal shaped pollen grain of *T. peruviana*; C. 4-colporate spheroidal shaped pollen grain of *C. odollam*

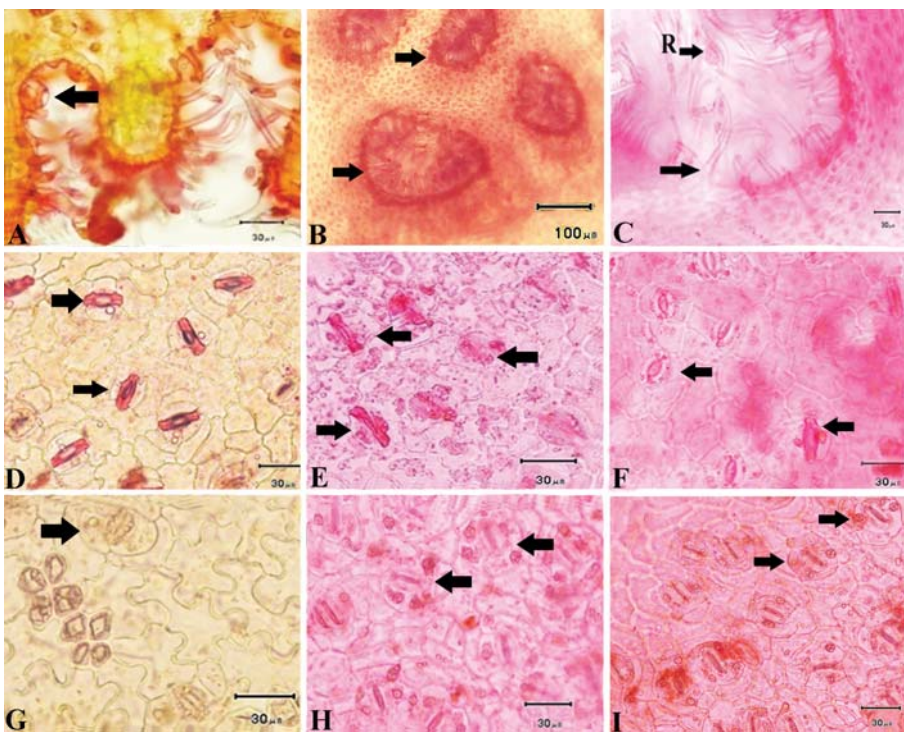

FIGURE 7. Stomata of undigested and digested found in leaves (arrows): A. undigested stomata of *N. oleander*; B. digested *N. oleander* stomata at 5 hrs; C. digested *N. oleander* stomata at 48 hrs, R=rosette crystal; D. undigested stomata of *T. peruviana*; E. digested *T. peruviana* stomata at 5 hrs; F. digested *T. peruviana* stomata at 48 hrs; G. undigested stomata of *C. odollam*; H. digested *C. odollam* stomata at 5 hrs; I. digested *C. odollam* stomata at 48 hrs.

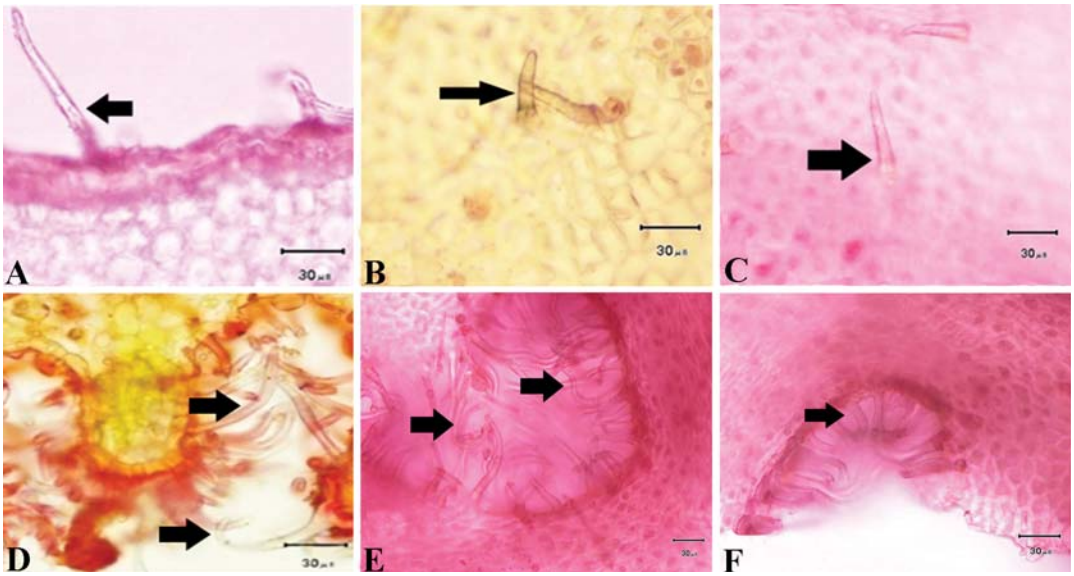

Figure 8. *N. oleander* trichomes. A. undigested sharp-end trichomes (arrow); B. digested sharp-end trichomes after 5 hrs (arrow); C. digested sharp-end trichomes after 48 hrs (arrow); D. undigested blunt-end trichomes (arrows); E. digested blunt-end trichomes after 5 (arrows); F. digested blunt-end trichomes after 48 hrs (arrow).

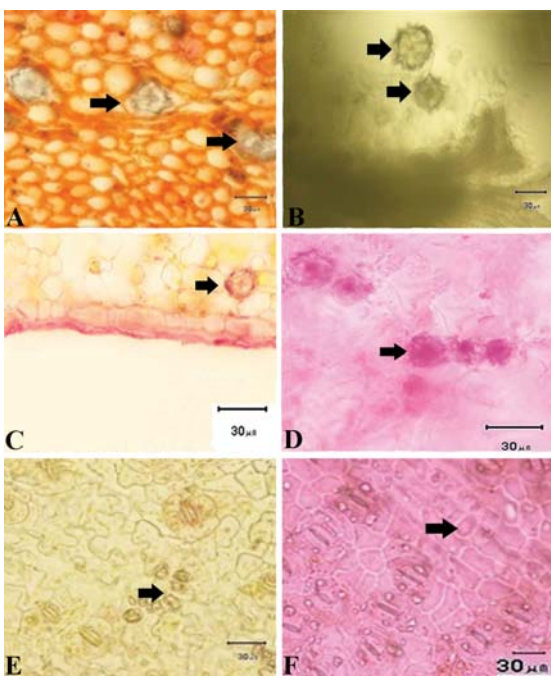

Figure 9. A. undigested rosette crystals of *N. oleander* (arrows); B. digested rosette crystals of *N. oleander* after 48 hrs (arrow); C. undigested rosette crystals of *T. peruviana* (arrows); D. digested rosette crystals of *T. peruviana* after 5 hrs (arrow); E. undigested prismatic crystals of *C. odollam* (arrow); F. digested prismatic crystals of *C. odollam* after 48 hrs (arrow).

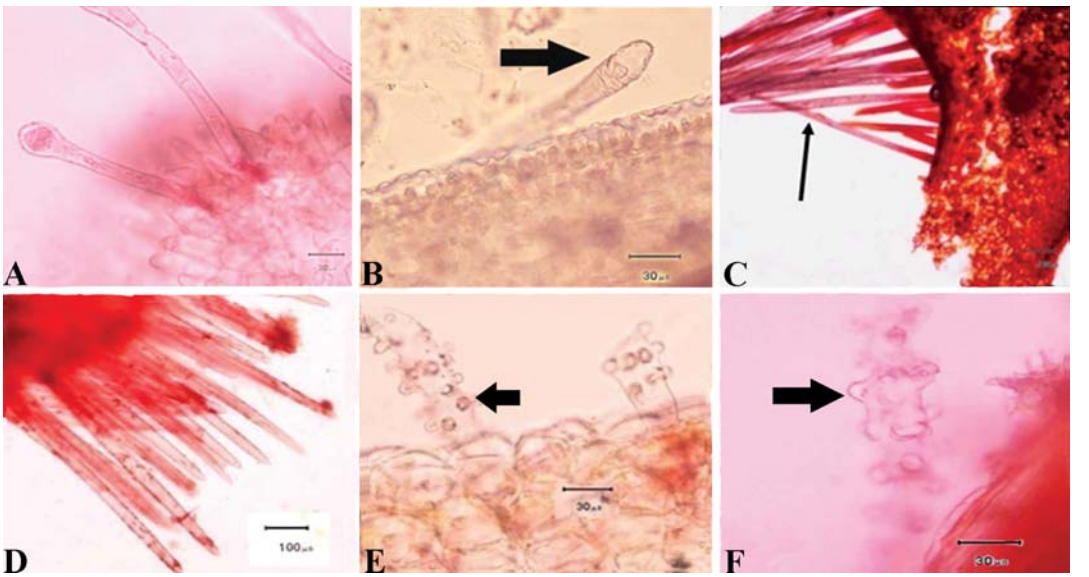

FIGURE 10. Trichomes found in flowers: A. undigested trichome of *N. oleander*; B. digested trichomes of *N. oleander* after 48 hrs*;* C. undigested trichomes of *T. peruviana*; D. digested trichomes of *T. peruviana* after 48 hrs; E. undigested trichomes of *C. odollam*; F. digested trichome of *C. odollam* after 48 hrs (arrow).

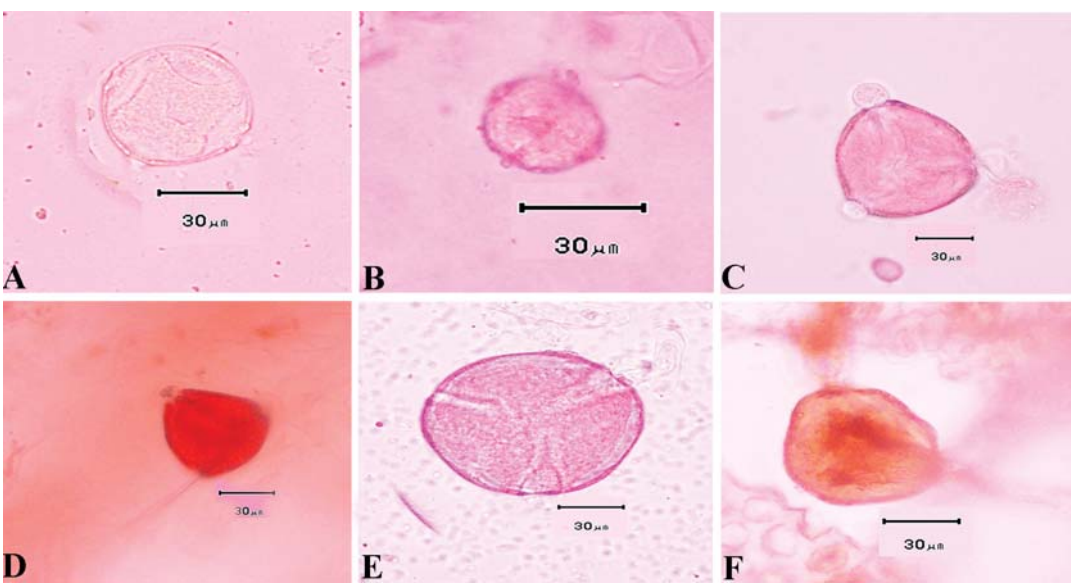

FIGURE 11. Pollen grain found in the flowers: A. undigested *N. oleander*'s pollen; B. digested *N. oleander*'s pollen after 48 hrs; C. undigested *T. peruviana*'s pollen; D. digested *T. peruviana* pollen after 48 hrs; E. undigested *C. odollam*'s pollen; F. digested *C. odollam* pollen after 48 hrs.

mesophyll, the 2-3 layers of hypodermis and the prismatic calcium oxalate crystals in *N. oleander*'s leaves (Table 5), the single-layer palisade mesophyll of *T. peruviana*'s leaves (Table 6), and the single-layer palisade mesophyll and prismatic calcium oxalate crystals of *C. odollam*'s leaves (Table 4), this might due to the grinding process (chewing). As the leaves were ground, the layers of the palisade mesophyll and hypodermis, then, were destroyed and became difficult to observe. For the other anatomical structures that were absent at some time points during the digestion, which are: the trichomes at 5 hrs of digestion and pollen grains of *T. peruviana* at 5 and 48 hrs, respectively (Table 6), and the trichomes of *C. odollam* in flowers at 5 and 24 hrs of digestion (Table 4), this might due to the darken colours of the samples that disguised the structures. Moreover, the samples were ground and mixed, the structures can be scattered all over the zippered bags which made them difficult to be observed. However, the presence of the unobserved features at other time points can support the identification. The prismatic crystals and the numbers of the layers of both palisade mesophyll and hypodermis are not the effective parameters and should not be used in the identification of the digested leaves. On the other hand, the three characteristics are, still, useful for identification of the undigested unknown plant leaves. For flowers, both the types of trichomes and the shape and the type of the pollen's apertures are the effective parameters for the identification of the undigested and digested material.

Previous studies also used the different types of stomatal complexes found in leaves, types of trichomes (both in leaves and flowers), and the shape and apertures' types of the pollen grains to identify the plant species which corresponded to the present study (Mauseth, 2008; Pongpijid *et al.*, 2011). However, the type or name of each structure had been identified differently. These might due to the different definitions or references used in the identifications. For the study of the identification of the digested plant materials in forensic cases, former studies mostly had been done with other plants (rather than the three species), and usually concerned with macro morphology or chemical test for the presence and type of cardiac glycoside Roll *et al.*, 2009; Pongpijid *et al.*, 2011)

Conclusions

The microscopic technique is one of the methods used to confirm or identify plants species. Moreover, it is cheap and rapid than the TLC method (Gaillallard *et al.*, 2004; Pongpijid *et al.*, 2011). The results of the study showed the specific structure can be used for plant identification. For *N. oleander*, the significant microscopic structures for the identification of both undigested and digested samples are the sunken stomata, the sharp-end non-glandular unicellular trichomes, the blunt-end non-glandular unicellular trichomes and the rosette-aggregate calcium oxalate crystals. For the flowers, the significant microscopic structures for the identification of both undigested and

digested samples are the smooth-surfaced glandular unicellular trichomes and the 4-porate, oblate-spheroidal shaped pollen grains. For *T. peruviana*, the significant microscopic structures for the identification of both undigested and digested samples are the anomocytic stomata, the absence of trichome, and the rosette-aggregate calcium oxalate crystals. For the flowers, the significant microscopic structures for the identification of both undigested and digested samples are the smooth-surfaced non-glandular unicellular trichomes and the 3-colporate, oblate or oblate-spheroidal shaped pollen grains. For *C. odollam*, the significant microscopic structures for the identification of both undigested and digested samples are the anisocytic stomata, the absence of trichome and the prismatic calcium oxalate crystals. For the flowers, the significant microscopic structures for the identification of both undigested and digested samples are the rough-surfaced non-glandular unicellular trichomes and the 4-colporate spheroidal shaped pollen grains. The advantages of these criteria can apply for determination of plant material found in gastric contents in forensic cases in order to verify the cause of death.

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