BOTANICAL AND CHEMICAL IDENTIFICATION OF PLANT CONTAINING CARDIAC GLYCOSIDES IN THAILAND

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Abstract: A protocol for forensic identification of 5 species of Asclediaceae which contain cardiac glycosides, namely *Calotropis gigantean* (L.) R. Br. ex Ait., *Cerbera odollum* Gaertn., *Nerium oleander* L., *Strophanthus gratus* Franch., and *Thevetia peruviana* (Pers.) K. Schum, was proposed based on botanical and chemical examination. Plant materials were collected and subjected to morphological, microscopic, and thin layer chromatography (TLC) examinations. Morphological comparison suggested that the shape of leaf was distinctive as each plant species has a unique leaf shape. Comparison of microscopic characteristic of powdered plant parts revealed the composition of different cell characteristics. Moreover, unique TLC fingerprints were generated when plant extracts were separatedin a EtoAC:MeOH:CH_2Cl_2 (81:11:8) solvent system. Results supported that these 5 Thai plant species can be accurately differentiated by botanical and chemical characters.

Introduction: Plants containing cardiac glycosides are classified as poisonous plants and have a long history in fatal poisoning. Cardiac glycosides have direct effect on Na^+/K^+ATPase pump of cardiac cells, resulting in the increase of intracellular Na^+ concentration which consequently affects the Na^+/Ca^{2+} exchange channels. Therefore, resulting in an increase in intracellular Ca^{2+} that leads to increased force of cardiac muscle contraction (positive inotropic effect)[1]. Consumption of plants containing cardiac glycosides may be either accidental or by intention. During 1984 – 2001, there were 14 cases of children consuming fruits and seeds of *Thevetia peruviana*[2] reported from a hospital in Columbia, Sri Lanka. These children were brought to the hospital with symptoms i.e., vomiting, abdominal pain, bradycardia, and loose motion. There are also reports of death caused from *Nerium oleander* poisoning. In these cases, *Nerium oleander* leaves were used to make tea as they were thought to be eucalyptus leaves,[3] another case report was on a male with diabetes consuming herbal medicine containing *N. oleander*[4]. Blood of the deceases were analyzed by LC-MS/MS for oleandrin and related compounds, and matched with those from *N. oleander* leaves. *T. peruviana* and *Cerbera odollam* seeds were also reported to be used for suicide and homicide incidents. According to an 11-month retrospective study in 1995, 79 people were admitted in a hospital located in northern Sri Lanka suspected to attempt suicide by eating seeds of *T. peruviana*, of which 6% did not survive when reached hospital[5]. During 1989 – 1999, there were 537 casualties from consumption of *C. odollam*. These incidents consisted of both suicide and homicide cases[6]. To identify the plant species in these poisoning cases, details of the ingested plant such as adescription of the plant, plant part and ingesting time, are essential information. However, in severe cases, immediate supportive treatment is given prior to identification of the plant. Most Thai plants containing cardiac glycosides belong to the family Apocynoceae, i.e., *Nerium oleander* L., *Cerbera odollam*

Gaertn., *Thevetia peruviana* (Pers.) K. Schum., and *Strophanthus gratus* Franch. There are also plants which belong to other families such as *Calotropis gigantea* (L.) R. Br. ex Ait. which belongs to the family Asclepiadacea. In forensic casework, the sample may be received in powdered form or as fragments which makes identification of the plant by morphology alone. Therefore, this study also aimed to investigate microscopic characteristics which can aid plant identification and thin layer chromatographic conditions to differentiate Thai plants containing cardiac glycosides in order to develop a simple identification protocol which is rapid, inexpensive and accurate. This would not only benefit the need for accurate treatment, but also providing scientific evidence to help determining the cause of death in forensic cases.

Methodology: Materials of 5 plants containing cardiac glycosides in Thailand (*Calotropis-gigantea*, *Cerbera odollam*, *Nerium oleander*, *Strophanthus gratus*, and *Thevetia peruviana*.) were collected from Siri Ruckachat and King Rama 9 botanical gardens. Firstly, species of each plant were confirmeded by their morphological characteristics before separating plant parts which can be accidentally consumed by human to dry and grind to powder. For chemical examintion, 5 g of powdered plant was extracted by refluxing with 40 ml of 70% ethanol at 45-60^{o}C for 15 minutes, followed by addition 25 ml of 10% lead acetate solution, then incubated at 45-60^{o}C for 15 minutes. The clear filtrate was collected and further extracted with 30 ml of dichloromethane in a separatory funnel. Re-extraction was carried out twice using 20 ml of dichloromethane. Anhydrous sodium sulphate was then added for dehydration. The dichloromethane extract was filtered and concentrated to 10 ml. Aliquots of 1 ml were subjected to cardiac glycoside screening by 3 color tests; Kedde, Liberman Burchard (acetic anhydride-sulfuric acid), and Keller-Kiliani (ferric chloride-acetic acid-sulfuric acid). The remaining 7 ml was evaporated to dryness. Then, reconstituted in 1 ml of dichloromethane : methanol (50 : 50) solution, and subjected to TLC examination. Separation was carried out using silica gel $60F_{254}$ plate (20 x 20 cm) and ethyl acetate : methanol : water (81:11:8) as the developing solvent. TLC plates were sprayed with Kedde and sulfuric reagent. For Microscopic examination, 10% sodium hypochlorite was used to clear pigments in plant tissue, and stained with Aniline sulfate solution.

Results, Discussion and Conclusion: Macroscopic examination showed that the 5 plant species can be differentiated by morphological characteristic of leaves, as demonstrated in figure 1.

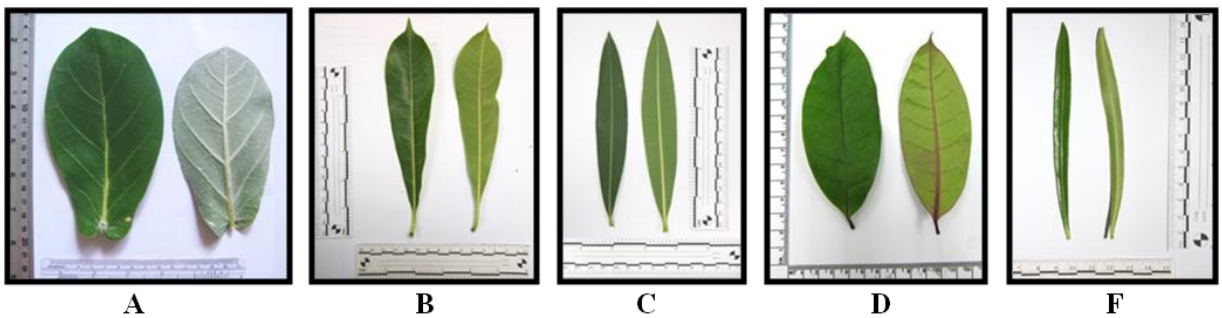
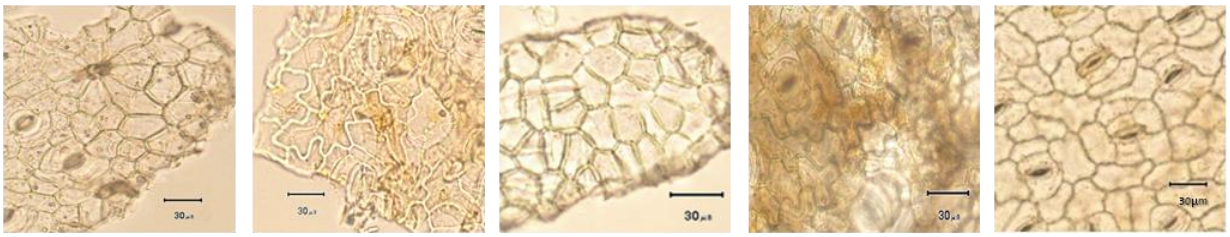


Figure 1. Comparison of leaf shape of the 5 plants containing cardiac glycosides in Thailand; A) *Calotropis gigantea* : obovate; B) *Cerbera odollum* : oblanceolate; C) *Nerium oleander* : narrowly elliptic; D) *Strophanthus gratus* : elliptic; and E) *Thevetia peruviana* : narrowly oblong shaped.

Microscopic examination of powdered leaf part showed specific characters which were useful for the differentiation of the 5 plants containing cardiac glycosides. Different combinations of cell types were found in each species. In addition, two forms of calcium oxalate crystals, i.e., rosette aggregate (druse) and prism forms were found in leaves of *Nerium oleander* L., *Strophanthus gratus* Franch., and *Thevetia peruviana* (Pers.) K. Schum. Details are shown in table 1, and figure 2 – 5.

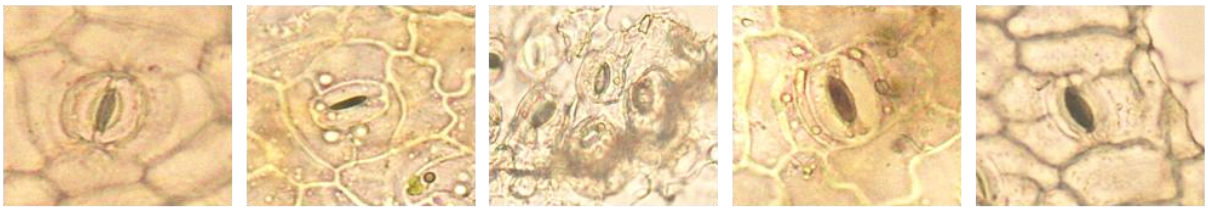
Table 1. Comparison of microscopic characteristic comparison of leaf part.

Plant	Margin of epidermal cell wall	Stomata type	Trichome	Calcium oxalate
Calotropis gigantia.	entire	tetracytic	multicellular-uniseriate	not found
Cerbera odollum.	wavy	anomocytic	not found	not found
Nerium oleander.	entire	sunken-stomata	unicellular twisted and hook	rosett aggregate and prism
Strophanthus gratus.	wavy	anomocytic	not found	rosett aggregate and prism
Thevetia peruviana.	slightly wavy	anomocytic	not found	rosett aggregate



A B C D E

Figure 2. Epidermis in surface view of leaf of *Calotropis gigantia* (A), *Cerbera odollam* (B), *Nerium oleander* (C), *Strophanthus gratus* (D), *Thevetia peruviana* (E).

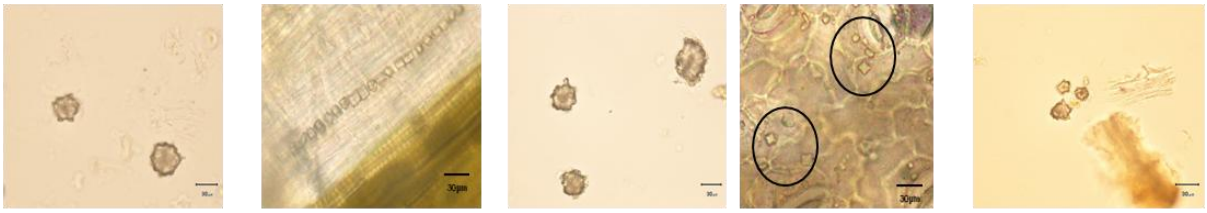


A B C D E

Figure 3. Stomata types : Tetracytic stomata found in *Calotropis gigantia* (A), Anomocytic stomata found in *Cerbera odollam* (B), sunken stomata found in *Nerium oleander* (C), Anomocytic stomata found in *Strophanthus gratus* (D), Anomocytic stomata found in *Thevetia peruviana* (E).

A B C D

Figure 4. Trichomes : Multicellular uniseriate trichome (A and B) found in *Calotropis gigantia*, Unicellular twisted trichome and hook trichome (C and D) found in *Nerium oleander*.



A B C D E

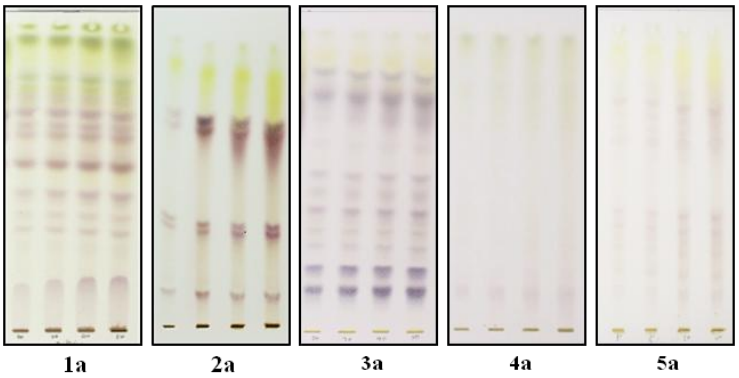
Figure 5. Calcium oxalate crystals : Rosett aggregate (A) and prism sheath (B) found in *Nerium oleander*, Rosett aggregate (C) and prism (D) found in *Strophanthus gratus*, Rosett aggregate (E) found in *Thevetia peruviana*.

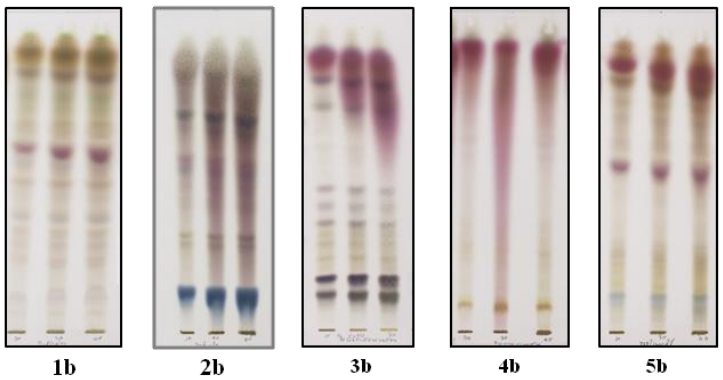
In chemical tests, all 5 plant extracts gave positive test results with all 3 reagents which are specific for 3 reactive sites for the chemical structure of cardiac glycosides, Kedde, Liberman, and Keller-Kiliani reagent, thus indicating the presence of cardiac glycosides. Example for extract of *Nerium oleander* leaves (Figure 6)

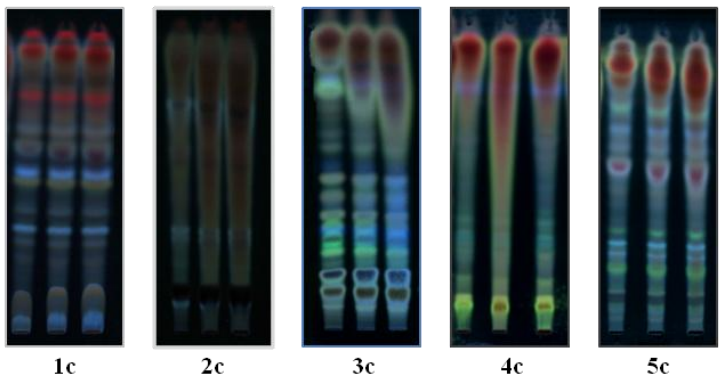
A B C

Figure 6. Color test of *Nerium oleander* leaf extract., Kedde reagent : a pinkish-violet colour is indicative of the presence of a lactone ring (A), Liebermann Burchard reagent : the color changes from reddish pink to blue and green indicating the presence of steroid (B), Keller - Kiliani reagent : a blue-green ring indicating the presence of deoxy sugar (C).

As shown in Figure 7, TLC fingerprints of 5 plant species containing cardiac glycosides had diatinctive band patterns when separated by TLC (Figure 6, 1 – 5) , and had different colors when sprayed with different spray reagents (Figure 6, a – c). Results therefore demonstrated that the 5 plant species containing cardiac glycosides found in Thailand can be distinguished based on their macroscopic and microscopic characteristics, and be identified by their TLC fingerprints.







Chromatogram	Plant	Volume (µl)	Detection
1a	*Calotropis gigantia*	20 – 50	Kedde- UV vis.
1b	"	20 – 40	H_2SO_4-UV vis.
1c	"	20 – 40	H_2SO_4-UV 366nm
2a	*Cebera odollam*	20, 40, 60,80	Kedde- UV vis.
2b	"	20, 40, 60	H_2SO_4-UV vis.
2c	"	20, 40, 60	H_2SO_4-UV 366nm
3a	*Nerium oleander*	20 – 50	Kedde- UV vis.
3b	"	10 – 30	H_2SO_4-UV vis.
3c	"	10 – 30	H_2SO_4-UV 366nm
4a	*Strophanthus gratus*	20 – 50	Kedde- UV vis.
4b	"	20 – 40	H_2SO_4-UV vis.
4c	"	20 – 40	H_2SO_4-UV 366nm
5a	*Thevetia peruvena*	30 – 60	Kedde- UV vis.
5b	"	30 – 50	H_2SO_4-UV vis.
5c	"	30 – 50	H_2SO_4-UV 366nm

Figure 7. TLC fingerprint of leaves extract 50 mg/ml in CH_2Cl_2 :CH_3OH (50:50).

Because materials originated from plants in forensic casework may be present in various forms, such as whole plant, plant parts, small pieces, powdered materials, herbal extracts, etc., a protocol for forensic identification of plants containing cardiac glycosides is proposed in figure 8.

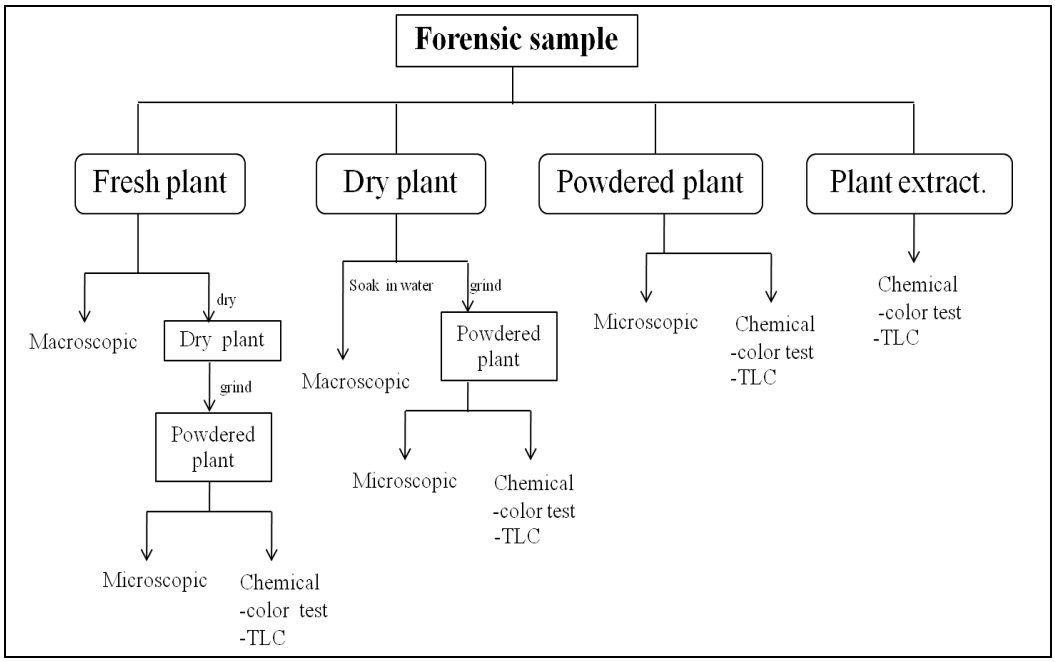


Figure 8. Protocol for identification plants containing cardiac glycosides

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Keywords: cardiac glycoside, plant identification, poisonous plants, TLC fingerprint