Chapter 3

Derris Plant Species Identification and Rotenone Root Quantity as Determined by Isozyme Pattern Method and the Plant Morphological Characteristics

3.1 Introduction

Within the genus *Derris* about 25 species were documented with their distribution over the tropical regions of Asia and East Africa. All of these plant species are woody perennials, mostly climbers (Toxopeus, 1952). Later, Hooker(1961) reported about 40 species were belonged to the genus *Derris*. The two species include *elliptica* and *malaccensis* occur in South Eastern Asia and in the Malayan and both processed the insecticidal properties. To distinguish them with conventional identification scheme is quite a difficult task since both species have more similarity in morphological appearance. *D. elliptica* is local plant which is usually existed in natural habitats near the river side in the north of Thailand, while *D. malaccensis* was originally cultivated in Chontaburi. The latter plant species is currently established and distributed in the northern region of Thailand, especially in Lampang.

Isozyme pattern of plant by Electrophoresis method is employed for plant species identification: This is a separation method of chemical substance component of plant by transfer them in suitable media with electric field between anode and cathodes; the smaller chemical molecule could move faster than larger molecule, after staining, it strikethrough will provide different band pattern appearances on media for different plant varieties (Kawpet, 2004).

Pasteur *et al.* (1998) reported that polyacrylamide gel electrophoresis (PAGB) were method for separation protein and mixed protein solution by molecular sieving effect with polyacrylamide gel. It was the product of polymerization reaction between N,N-methylene bisacrylamide (BIS) and ammonium persulphat or riboflavin one of them and mixed catalyst with N,N,N,N-tetramethyl ethylene diamine (TEMED). In the case of riboflavin activate by light. Wendel and Weeden (1998) report that size of porous in acrylamide gel of importance effect to movement of protein molecule depended on ratio of acrylamide and BIS show in % T and % C. The mean of % T was concentration of total monomer (acrylamide + BIS) g/100 ml. (w/v) and %C was % BIS weight in total weight of monomer increase size of porous by decrease % T, 3-5% T was suitable for sieving weight molecule more than 100,000, 5-12% T for sieving weight molecule 20,000-150,000, 10-15% T for sieving 10,000-80,000 of molecule weight and more than 15% T for sieving lower than 15,000 of molecule weight. Hussain *et al.* (1988, 1989) found that a method was developed for identification of

cultivars of the legume, *Strylosanthes capitata* Vog., and *Pueraria phaseoloides* using electrophoretic patterns of seed proteins and forage in polyacrylamide gels as the genotypic markers.

The current objectives of these experiments are performing feasibility test on manipulation of isozyme pattern method for derris plant species identification, determination of their rotenone root quantity; in addition, the morphological characteristics of both *D. elliptica* and *D. malaccensis* are also investigated.

3.2 Material and method

3.2.1 Analysis isozyme pattern by polyacrylamide gel electropholysis method.

In analyzing the isozyme patterns of both *D. elliptica* and *D. malaccens*is, the polyacrylamide gel electrophoresis method is employed in this study. The electrophoresis which is a typically method in studying of genetics is an electrochemical process of separating large molecules from a mixture of similar molecules by using the electric current. This method encourages in separating and identifying the proteins and enzymes.

Steps in preparation of the samples for this analyzation were plantings the two selected species of derris plants in 80 cm. diameter cement containers under open-air. Selection of the young leave located in the third place from the tip of the plant and the old leave located next from the third position of the young leave were collected from the two years old derris plants. Then the leaves were washed thoroughly with distilled water and sliced into small pieces. Only one gram of sliced leaves was grinding collectively with liquid nitrogen with 0.05 gm PVPP and 3 ml extraction buffer (0.2 tris-HCl, pH 8.0). Each sample was centrifuged by 14,000 rpm at 2^0C for 20 minutes. Only clear supernatant was collected to analyze for isozyme pattern by using the polyacrylamide gel electrophoresis method (modified from Chuanpit, 1994). The three-color systems of marker enzyme applied were peroxidase (POX), esterase (EST), and acid phosphatase (ACP). The existed patterns were recorded and visualized by photographing and Zymogram diagram drawings (modified from Siripanit, 1988; and Aroonrungsikul, 1995).

3.2.2 Determination of *D. elliptica* and *D. malaccensis* rotenone root contents

Analyzation of the rotenone content in *D. elliptica* and *D.* malacensis are conducted by collecting four of each derris plant species at the age of two years existed in the same habitat.

The following process of rotenone content analysis in this study is determined. Different parts of the two derris plants, including, the young tip, the mature leaf, the

stem and various sizes of 0.4, 0.9, and 1.5 cm root diameters were individually dried and grinded. One gram of each powder from each of the mentioned plant part was extracted with 10 ml dioxane solvent. Then they were soaked under shaking condition for 30 minutes, and the content materials were filtered with 0.45 micron pore size filter. Analyzation for rotenone concentration was performed by HPLC (High performance liquid chromatography) method (modified from Pityon and Sangwanit, 1997). HPLC method is an analytical method used for the separation and identification of chemical components of substances. The following information explains about the HPLC method used for this study.

Method of analysis rotenone by HPLC (High Performance Liquid Chromatography)

1. Principle of the method

Sample is extract with dioxane and rotenone analyzation is obtained by reversed phase HPLC and UV detector at 280 mm.

2. Apparatus and reagents

2.1 HPLC with variable UV detector

2.2 HPLC column ODS-3 4.6 x 150 mm, Prontosil C18 H, 5-micron with guard column

2.3 Mobile phase, Phosphoric acid 1ml/water 100ml and degas before use by methanol HPLC grad

2.4 Pure rotenone standard

3. Procedures

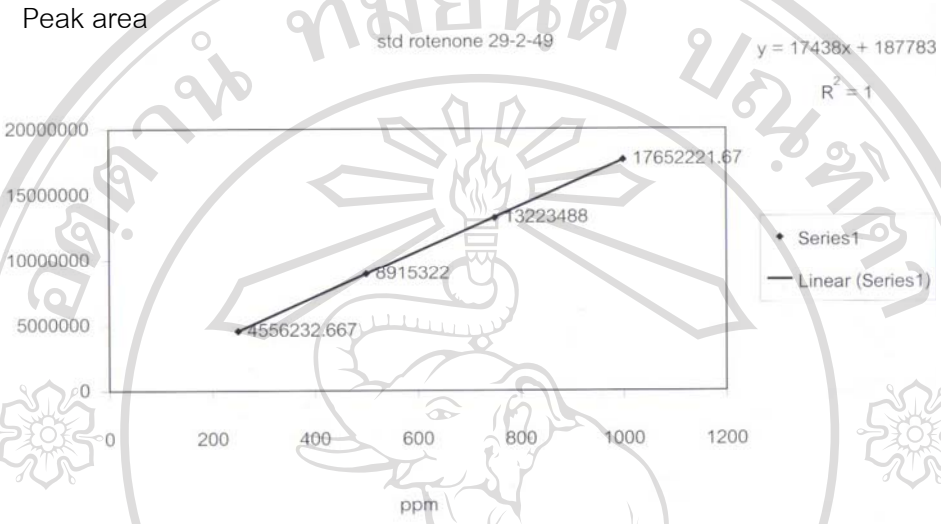
3.1 Standard preparations

Pure standard rotenone (99.5%) was accurately weighed at 0.1005 gm and it was filled up the volume with dioxane. The solution was left on shaker for 30 minute, 1,000 ppm stock solution was obtained. It was then dilute with dioxane were to prepare 750, 500 and 250 ppm solution (calculate with formula $N_1V_1 = N_2V_2$). Varied concentration of standard rotenone was subjected to HPLC and then linear regression from peak area and concentration was calculated as

$Y = 17438 (X) + 187783$

Y = Peak area from HPLC of each concentration

X = concentration unknown (ppm.)



3.2 Calculation of the rotenone content percentage in derris root

One gram of derris root powder was soaked for 30 minute in 10 ml of dioxane after that it was filtered through with no.1 filter paper and 45 micrometer filter membrane before subjected to HPLC

Rotenone content in solution detect by HPLC = A ppm.

= A mg/l

Solvent (dioxane) = 10 ml

In 10 ml. with rotenone content = $\frac{10\ A}{1,000}$ mg

Percent rotenone content in derris root was = $\frac{10\ A \times 100}{1,000 \times 10}$

= $\frac{A}{10}$

3.2.3 Morphological study on *D. elliptica* and *D. malaccensis.*

Morphological investigation on *D. elliptica* and *D. malaccensis* were performed on collecting the definite characteristics data on leaves, stems, and flowers, for ten times from the two-year old derris plants existed in the same habitat in order to obtain significant differential characteristic for identification of these two varieties.

3.3 Result and discussion

3.3.1. Analysis isozyme pattern by polyacrylamide gel electropholysis method.

The results revealed the difference pattern appearances for the basic enzymes of young and mature derris leaves analyzed by peroxidase (POX), esterase (EST), and acid phosphatase (ACP) method was detected. With the POX isozyme pattern, the mature leaf of derris cultivar variety (*D. malaccensis*) appeared only one band with the Rf value of 0.60-0.66. On the other hand, two bands were noticed in mature leaf of the derris local variety (*D. elliptic*a) with the Rf value of 0.27, and 0.60-0.66 (see table 3.1). In addition, three of similarity bands with the Rf value of 0.19, 0.36-0.4, and 0.63 for both local and cultivar variety of the young derris leaves were obtained.

The EST isozyme pattern of the mature derris leaves of both varieties demonstrated in two bands with the definite Rf values of 0.36, and 0.80. Besides, the young leaf of derris cultivar variety exhibited only two bands with the Rf values of 0.19, and 0.58, while the young leaf of derris local variety appeared three bands with the Rf value of 0.29, 0.40, and 0.58, respectively. In conclusion, the EST isozyme pattern is convincingly an obvious method in differentiation of the young derris leaves of the two varieties.

When employing the ACP isozyme pattern, both the mature and the young leaves of cultivar variety showed only one band with the exact Rf value of 0.28, while the mature and the young leaves of local variety exhibited three bands with the Rf values of 0.15, 0.23, and 0.5 for the mature leaves, and the Rf values of 0.23, 0.38, and 0.47 for the young leaves, respectively. Hence, the ACP isozyme pattern is seemed to be the best method in differentiation of both the young and the mature derris leaves for both the cultivar and the local varieties.

Table 3.1 Rf values of young and mature leaves of derris cultivar and local varieties as determined by POX, EST, and ACP isozyme patterns

Leaf	Mature leaf					Young leaf					
	Cultivar		Local			Cultivar			Local		
	B.1	B.2	B.1	B.2	B.3	B.1	B.2	B.3	B.1	B.2	B.3
POX											
1	0.60		0.27	0.66		0.19	0.40	0.63	0.19	0.40	0.63
2	0.66		0.27	0.66		0.19	0.36	0.63	0.19	0.38	0.63
3	0.66		0.27	0.66		0.19	0.40	0.63	0.19	0.38	0.63
4	0.66		0.27	0.66		0.19	0.40	0.63	0.19	0.38	0.63
5	0.66		0.27	0.66		0.19	0.40	0.63	0.19	0.38	0.63
EST											
1	0.36	0.80	0.36	0.80		0.19	0.58		0.29	0.40	0.58
2	0.36	0.80	0.36	0.80		0.19	0.58		0.29	0.40	0.58
3	0.36	0.80	0.36	0.80		0.19	0.58		0.29	0.40	0.58
4	0.36	0.80	0.36	0.80		0.19	0.58		0.29	0.40	0.58
5	0.36	0.80	0.36	0.80		0.19	0.58		0.29	0.40	0.58
ACP											
1	0.28		0.15	0.23	0.5	0.28			0.23	0.38	0.47
2	0.28		0.15	0.23	0.5	0.28			0.23	0.38	0.47
3	0.28		0.15	0.23	0.5	0.28			0.23	0.38	0.47
4	0.28		0.15	0.23	0.5	0.28			0.23	0.38	0.47
5	0.28		0.15	0.23	0.5	0.28			0.23	0.38	0.47

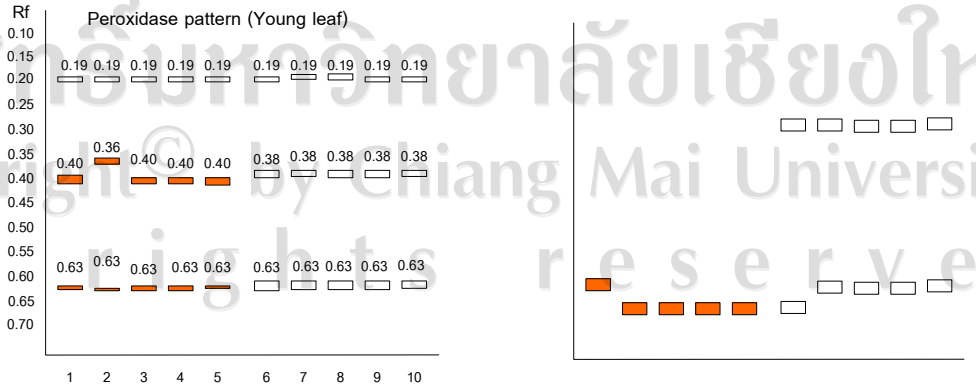


Figure 3.1 Peroxidase isozyme cymogram patern of mature leaf (right) and young leaf (left) column 1-5 were of cultivar variety, column 6-10 were of local variety

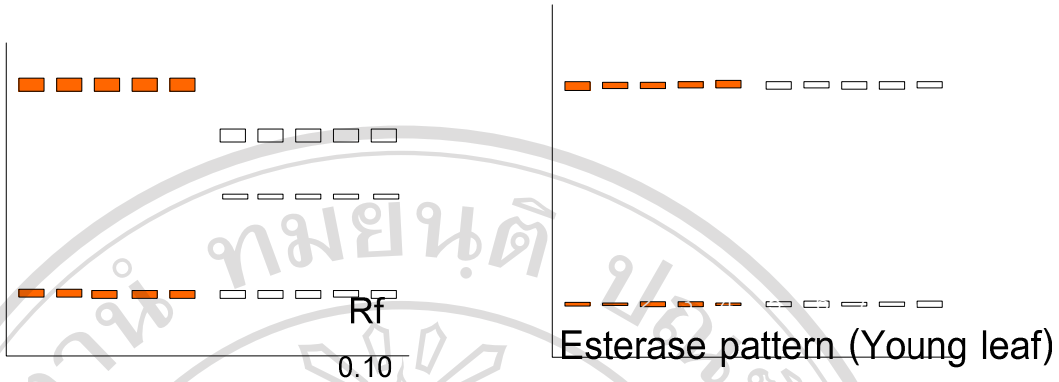


Figure 3.2 Esterase isozyme zymogram patern of mature leaf (right) and young leaf (left) column 1-5 were of cultivar variety, column 6-10 were of local variety

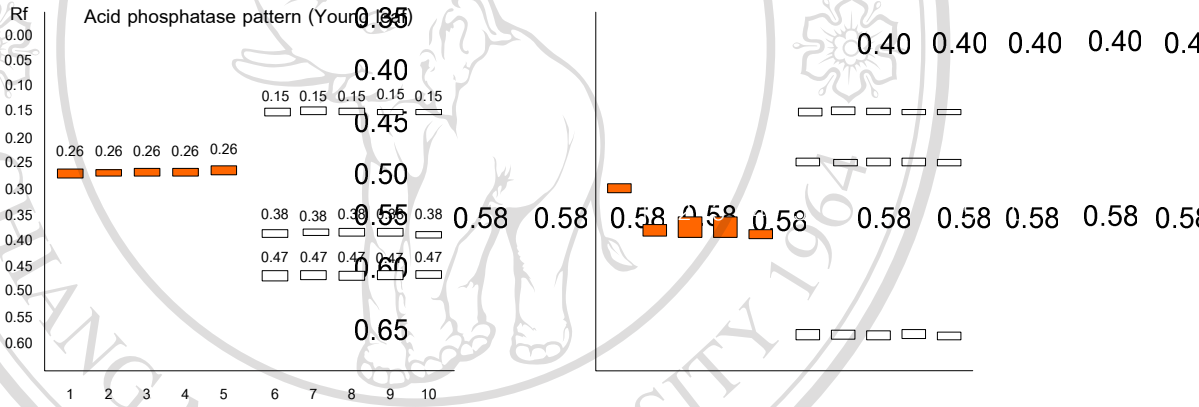


Figure 3.3 Acid phosphatase isozyme zymogramand patern of mature leaf (right) and young leaf (left) column 1-5 were of cultivar variety, column 6-10 were of local variety

POX isozyme pattern for young derris leaves of both local and cultivar varieties demonstrated in three bands with similar appearance, while two distinct bands exhibited in mature leaves of local variety as compare to only one band existed in the cultivar variety. Never the less, Kawpet (2003) demonstrated the POX isozyme pattern of Longan (varieties: 'Chompoo' and 'Biewkiew') did not deviated with plant ages. The EST isozyme pattern for young derris leaves of both varietal species showed brightness bands, although, the cultivar variety (*D. malaccensis*) provided only two bands, while the local variety (*D. elliptic*a) appeared in three bands. Besides, two similarity bands were recorded from mature leaves of both varietal species. Two selected species of derris plants were obviously distinguished by using the ACP

isozyme pattern. Since both mature and young leaves of the cultivar variety displayed only in one band whereas three bands occurred in local variety.

We may conclude that in determining the derris plants varietal species the POX isozyme pattern is clearly being one of the methods in identifying the derris mature leaves, while the EST isozyme pattern is better for the young leaf identification. Certainly, the ACP pattern is the best method for mature and young leaf identifications of both derris varietal species. Other corresponding studied also exhibited similarity result in application of isozyme patterns for identification of *Eulophia graminea* Lindley, and *Eulophia andamanensis* Reichenbach (Prarasri *et al.*, 2005).

3.3.2 Rotenone quantity in the both derris cultivar and local varieties

Table 3.2 Amount of Rotenone content (%) per 1 gram dry weight of mature leaf, shoot, stem and various root diameter of 2-year-old derris varieties grown in the same environment factor at LARTC (4 replications)

	Rotenone content (%)	
Plant part	Cultivar variety	Local variety
Mature leaf	0.006	0.003
Shoot	0.025	0.003
Stem	0.043	0.045
Root Ø 0.4 (cm.)	3.163	0.467
Root Ø 0.9 (cm.)	4.105	0.329
Root Ø 1.5 (cm.)	3.457	0.341

Rotenone contents from various parts of derris cultivar variety, including, mature leaf, young tip, stem, and various roots with 0.4, 0.9, and 1.5 cm in diameters were 0.006, 0.025, 0.043, 3.163, 4.105, and 3.457%, respectively. On the other hand, the rotenone quantities of derris local variety of the identical plant parts and root diameters were 0.003, 0.003, 0.045, 0.467, 0.329, and 0.341%, respectively. The result convincingly demonstrated the superior quantity of the rotenone content existed in the cultivar variety (*D. malaccensis*) in almost all plant parts with the highest content of 4.105% prevailed in 0.9 diameter root. Almost equal amount of rotenone content were recorded only in the stems of both cultivar and local varieties (Table 3.2). Accordingly, in pest management practice the mass cultivation of the cultivar variety for better rotenone quantity is highly recommended. The optimum derris root size range 0.8-1.0 cm in diameter should provide the highest level of rotenone content.

3.3.3 Morphological Characteristics of *D. elliptica* and *D. malaccensis*.

Studies on morphological characteristics of *D. elliptica* and *D. malaccensis* were conducted. Both varieties were woody climber with small rough greenish brown bark, compound leaf in odd-pinnate and spiral pattern with the sizes of 21-44.0 x 18.7-55.0, and 13.0-29.0 x 23.5-53.50 cm, respectively. Petiole long 4.5-12.0 and 8.5-14 cm had stipule number of leaflet 3-11 and 3-13 leafs with elliptic-lanceolate, shaped and size of flower cluster were 2.5-6.0 x 6.0-16.0 cm and 2.8-8.9 x 6.4-20.3 cm respectively, flower developed from bud between stem and petiole, peduncle were 0.6-1.7 and 2.5-2.8 cm, respectively with fine brown hair and petiole length 0.5 – 0.7 and 0.6-1.0 cm, complete flower and side view symmetry calyx cup shaped with not equal 4 lops and sharp terminal in brown color size 0.6-0.8 x 0.6-1.0 (cm) and 0.9-1.0 x 1.0-1.2 cm fetal component with not equal 5 corolla upper of flower was standard with broadly ovate 1.0-1.1 x 1.4-1.5 cm and 1.6-1.7 x 1.6-1.7 cm with softly pink color base of standard with blue trine in long channel with fine hair cover, size of oblong side wing shape were 0.4-0.5 x 1.3-1.4 cm and 0.6-0.7 x 1.6-1.7 cm with softly pink color, size of kell inner wing shape were 0.4-0.5 x 1.3-1.4 (cm) and 0.5-0.6 x 1.6-1.7 cm in white color diadelphous ten anthers were connect 9-anthers and one anther separate, filament of anther long 1.1-1.2 cm and 1.2-1.4 cm, 2 pollen sac and in longitude open size of anther was 0.8 0.9 x 0.9-1.0 and 0.8-0.9 x 1.0-1.2 mm superior one ovary flat and long with yellow green color, no gynophores, size of violet line on long middle ovary was 1.4-1.5 x 10.0-10.1, and 1.5-1.6 x 10.1-10.2 mm, and one locule with many ovule fix in yellow color long 6.0-7.0 and 7.0-9.0 mm cover with hair accept on terminal and one stigma with green in yellow (Humbert et Gagnepain, 1920; Hooker, 1961; Backer and Bakhuizen Van Den Brink, 1963).

Table 3.3. Morphological characters of derris local variety (*D. elliptica*) and derris cultivar variety (*D. malaccensis*)

Plant part	Local variety	Cultivar variety
Stem	Woody climber	Woody climber
Compound leaf (Size)(cm)	21-44.0 x 18.7 x 59.0	13.0-29.0 x 23.5 x 53.50
Arrange	Alternate	Alternate
Leaflet (Size)	2.5-6.0 x 6.0-16.0 (cm)	2.8-8.9 x 6.4-20.3 (cm)
- arrange	add-pinnate	add-pinnate
- number	3-11 leaves	5-13 leaves
- shape	elliptic-lanceolate	elliptic-lanceolate
Petiole (long)	5.5-12.0 (cm)	8.5-14.0 (cm)
Petiolule (long)	0.30-0.80 (cm)	0.50-0.70 (cm)
Flower cluster (type)	panicle	panicle
(Size)	4.5-5.5 x 16.0-21.3	6.0-7.5 x 14.5-22.0
(arrange)	flower-triate	flower-triate
Peduncle (long)	0.60-1.70 (cm)	2.5-2.8 (cm)
(color)	red	brown
Pedicel (long)	0.50-0.70 (cm)	0.6-1.0 (cm)
(color)	red	brown
Calyx (cup shaper)	0.6-0.8 x 0.6 x 0.9	0.9-1.0 x 1.0 x 1.2 cm
(no lop)	4 lops	4 lops
(color)	red	brown
Petal (no)	5	5
Standard (shape)	broadly-ovate	broadly-ovate
(Size)	1.0-1.1 x 1.4-1.5	1.6-1.7 x 1.6-1.7
Plant part	Local variety	Cultivar variety
Wing (shape)	oblong	oblong
(Size)	0.4-0.5 x 1.3 x 1.4 (cm)	0.6-0.7 x 1.6 x 1.7 (cm)
inner wing (shape)	keel	keel
(Size)	0.4-0.5 x 1.3-1.4	0.5-0.6 x 1.6-1.7 (cm)
Stamen (no)	10	10
(arrange)	diadelphous	diadelphous
(filament)	1.1-12 cm	1.6-1.7 cm
Anther (no. sac)	2	2
(Size)	0.8-0.9 x 0.9-1.0 cm	08.-0.9 x 1.0-1.2 cm
Ovary (no)	1	1
(type)	Superior	Superior
no. locule	1	1

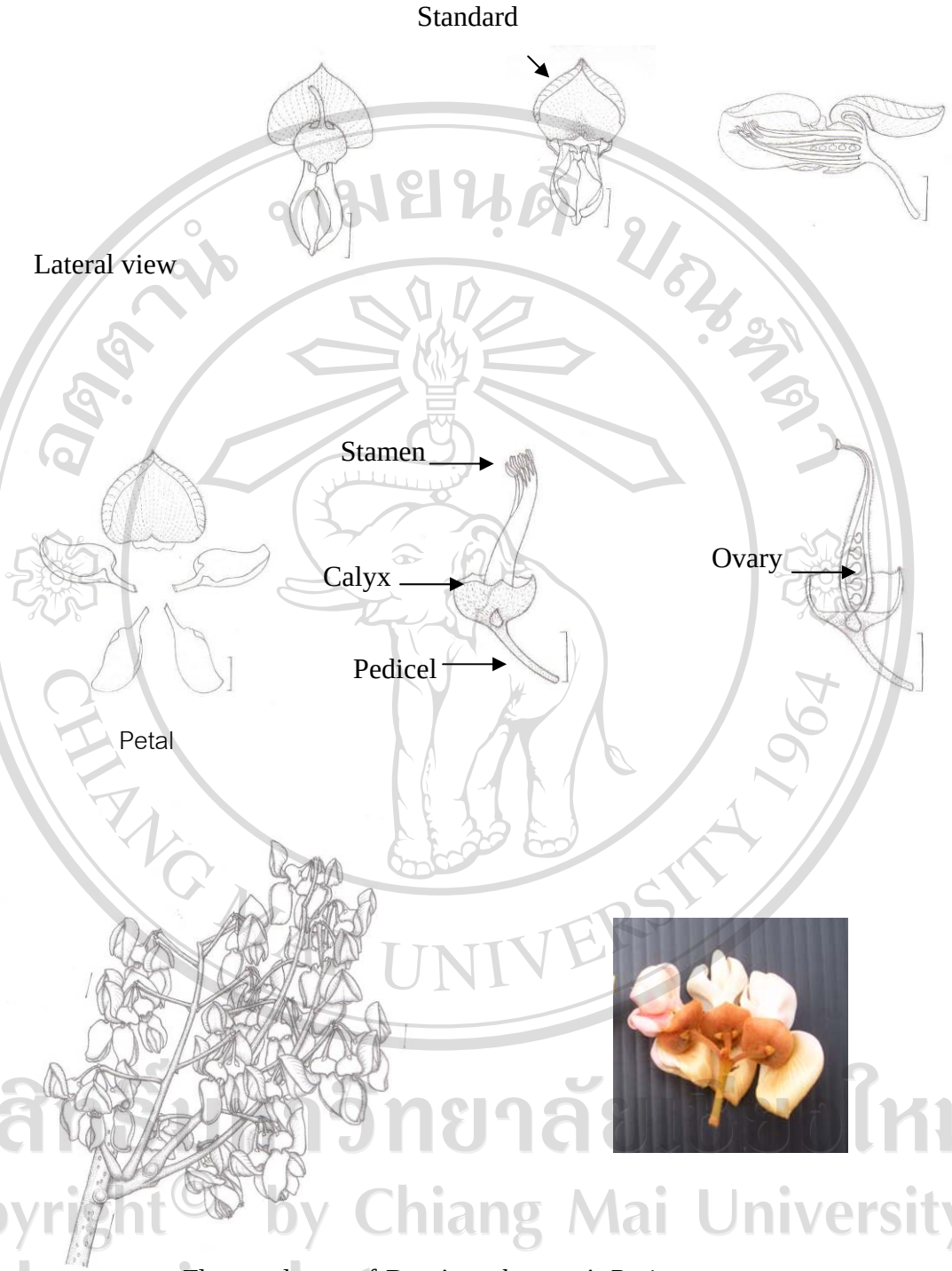


Figure 3.4 Morphological Characteristics of *D. malacensis* Flower (1cm)

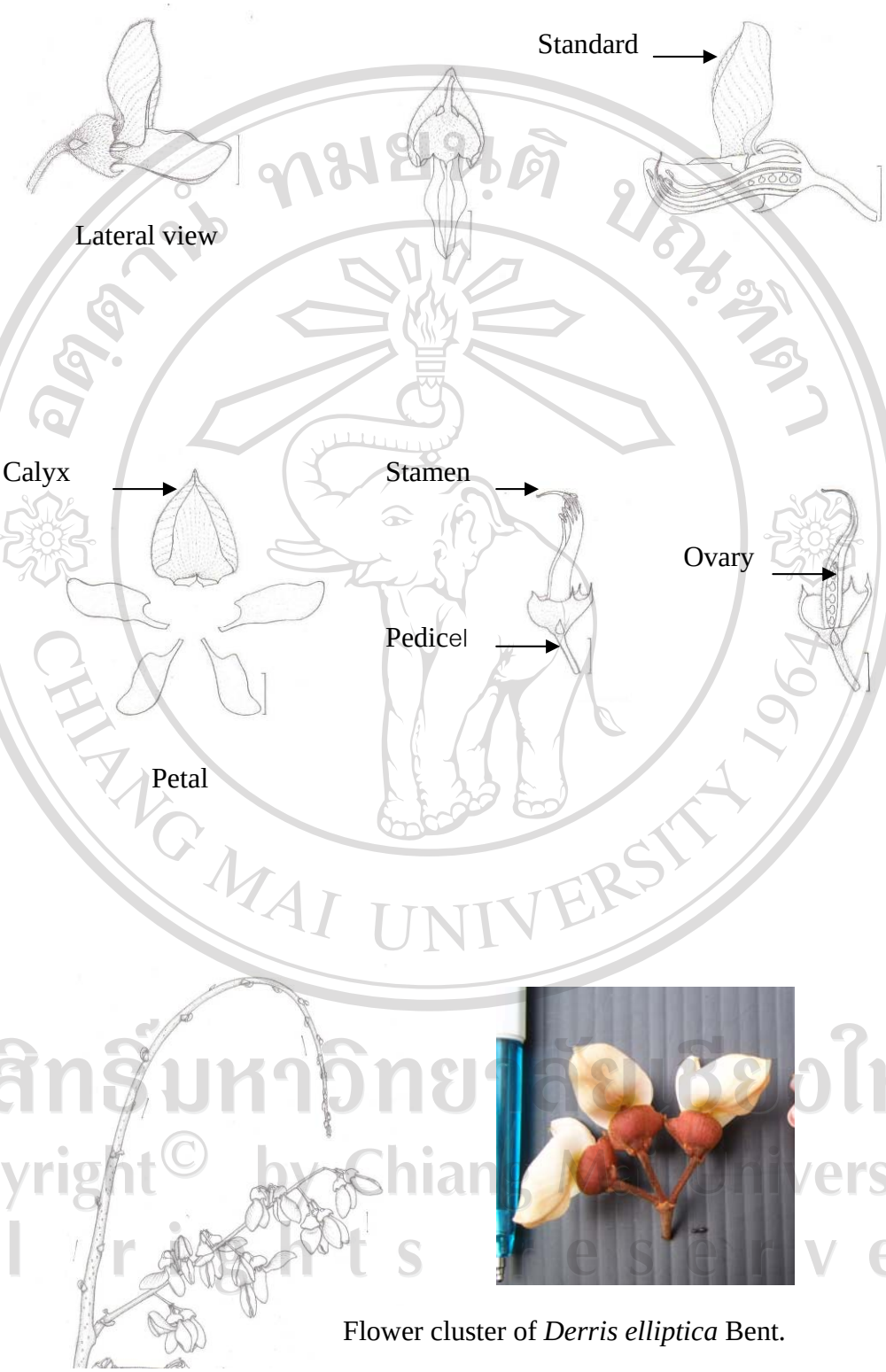


Figure 3.5 Morphological Characteristics of *D. elliptica* Flower (1cm)

Figure 3.6 Flower cluster of *D. elliptica* on stem

Figure 3.7 Flower cluster of *D. malaccensis* (Right) and *D. elliptica*(Left)

Figure 3.8 Compound leave of *D. malaccensis* (Left) and *D. elliptica* (Right)

Figure 3.9 Young leave of *D. malaccensis* (Right) and *D. elliptica*(Left)

Figure 3.10 Higher rotenone content in solutions of *Derris* cultivar (Right) and local varieties (Left)

Figure 3.11 More than 12 months old of cultivar derris root

Figure 3.12 HPLC machine (Shimadzu LC 14AD)

3.4 Conclusion

The identification of the two varieties of derris, *Derris malaccensis* Prain (cultivated variety) and *Derris elliptica Benth* (native variety) was investigated by isozyme patterns of acid phosphatase (ACP), esterase (EST), and peroxidase (POX) using both mature and young leaf of derris. The ACP pattern of mature and young leaf was clearly identified whereas identification could be made on young leaf only when using EST pattern. Only mature leaf could be identified when applying POX pattern. Also there are morphological differences between *D. elliptica* and *D. malaccensis*, especially, at young shoot color and flower cluster type. The distincquishable morphological characteristics of both varieties were also observed.