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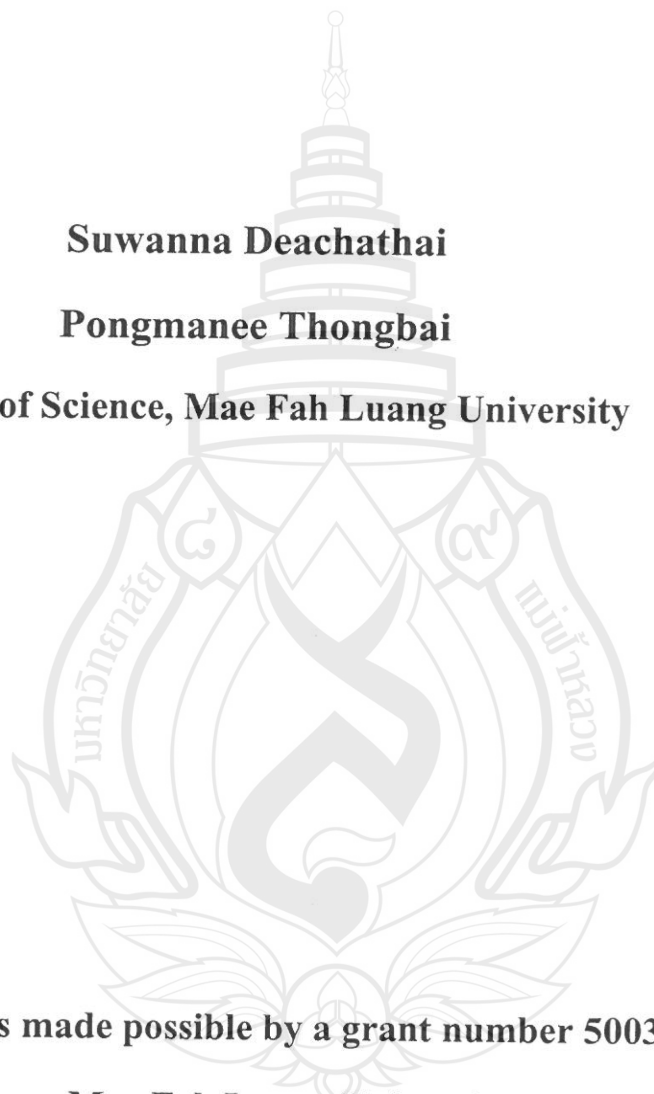
Investigation Techniques for Determination and Evaluation of Phorbolesters in *Jatropha curcus* Plant



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PREFACE

Phorbolesters, present in high level in the kernels, have been identified as the main toxic agent responsible for toxicity. They cause a large number of biological effects such as giddiness, vomiting diarrhea, inflammation and tumor promoter. The term phorbolester is used today to describe a naturally occurring family of compounds widely distributed in plant species of the family Euphorbiaceae and Thymelaecae. The oils from *J. curcas* contain phorbolester at least four different.

Phorbolesters (phorbol-12-myristate 13-acetate) have been identified as the major toxic principle in *Jatropha*. Phorbolesters are bioactive diterpene derivatives that have a multitude of effects in cells. Thus, to determine the phorbolesters content in leaves, stems, seeds and latex of *J. curcas* were collected in site Mae Fah Luang University form Chiangrai, Thailand, with the seeds contain oil that could be used for biodiesel production. There are many toxic substances most found importantly, phorbolesters. It is diterpere derivatives known as a tumor promoting agent with very little information about it content and determined phorbolesters in *J. curcas* was quantified used a HPLC method.

ABSTRACT

In this work, determination of phorbolesters in some parts that were stems, leaves and latex in *Jatropha curcas* grown in Chiang Rai, Thailand; Pak Chong 42 and Korat varieties, was performed by using reverse-phase high performance liquid chromatography (RP-HPLC). The conditions for the separation of phorbolesters by RP-HPLC were optimized. Acetonitrile (100 %) was used as the mobile phase. The column temperature at 30 °C was controlled. The absorbance of phorbolester was detected at 232 nm. The flow rate of mobile phase was 1.0 ml/min. The standard phorbolester peak appeared at the retention time of ~11 min. Method validation showed that the relative standard deviations (RSD) were in the rage of 0.38 to 3.57 %. The accuracy was tested by determination of recovery. Recoveries for phorbolester determination were found to be ranging from 32.23 to 107.99 %. The concentration of phorbolesters in the stem extracts from Pak Chong 32 and Korat samples were 21.75 and 24.63 ppm, respectively, whereas the phorbolesters concentrations in the leaf extracts were 26.81 and 31.10 ppm, respectively.

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Suwanna Deachathai

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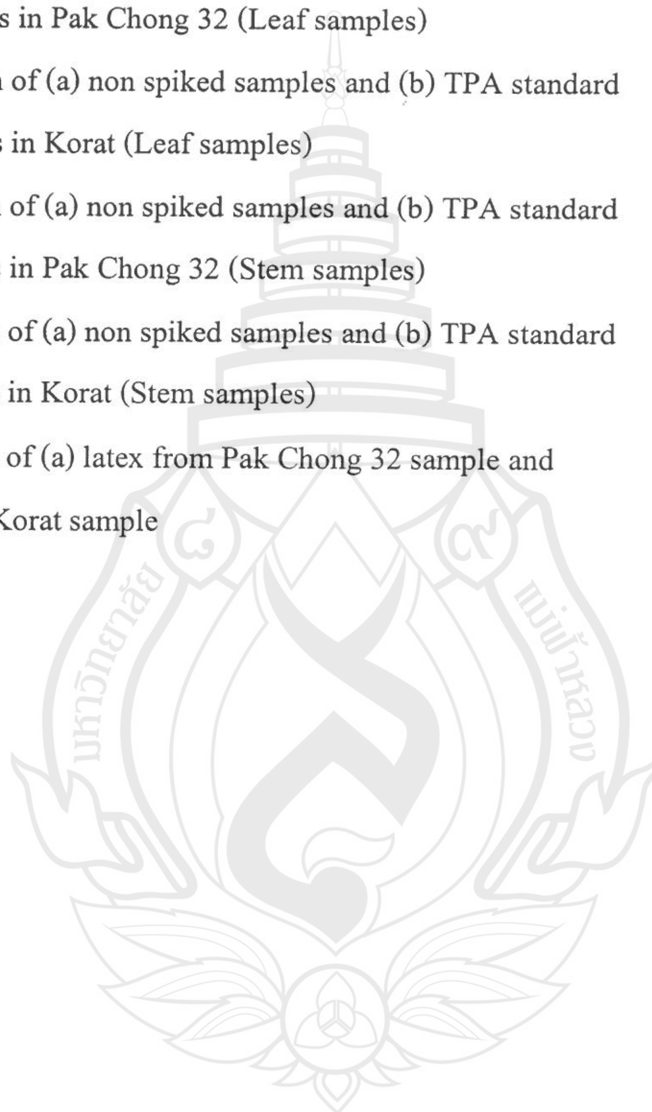


ABBREVIATIONS AND SYMBOLS

m	meter
g	gram
cm	centimeter
nm	nanometer
mm	millimeter
mL	milliliter
mg	milligram
μm	micrometer
μL	microliter
ppm	part per million
mg/g	milligrams per gram
μg/g	micrograms per gram
mL/min	milliliter per minute
°C	degree Celsius
C18	reverse-phase C18 column
DHPB	Phorbolesters 12-deoxy-16-hydroxyphorbol-4′-[12′,14′-butadienyl]-6′-[16′,18′,20′,5-nonatrienyl]-bicyclo[3.1.0]hexane-(13-*O*)-2′-[carboxylate]-(16-*O*)-3′- [8′-butenoic-10′]ate
HPLC	High Performance Liquid Chromatography
TPA	Phrobol-12-myristate-13-acetate

CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problem

Recently, the different concentration levels of phorbolesters have been found in *J. curcas* seeds cultivated in different sites. Previously reported that the seeds of *J. curcas* collected from various sites in Mexico contained different concentration levels of phorbolesters; the seeds from one site contained phorbolesters whereas the seeds from the other sites did not contain phorbolesters. Therefore, we wanted to determine phorbolesters in seeds of *J. curcas* grown in Chiang Rai, Thailand. Apart from the seeds of *J. curcas*, the other parts such as stems, leaves and latex have been used for many purposes such as in environment, medicine, cosmetic, food and animal feed, insecticide, pesticide, fungicide and fuel.

1.2 Objectives

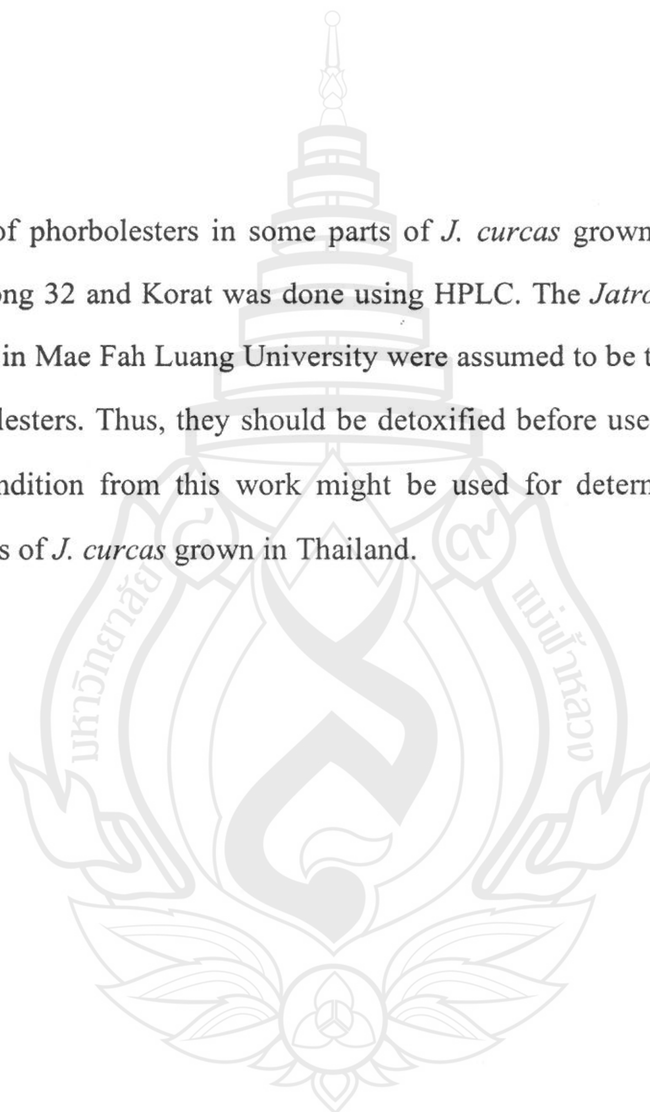
This project aimed to investigate the phorbolesters content in some parts of *J. curcas* using HPLC method. The HPLC conditions were optimized for the determination of trace amount of phorbolesters. Phorbolesters is extracted from the stems and leaves using methanol as the solvent.

1.3 Scope of study

To determine the phorbolesters content in leaves, stems and latex of *J. curcas* grown Chiang Rai, Thailand, the samples were collected from a site of Mae Fah Luang University. Phorbolesters in *J. curcas* have been quantified by a HPLC method.

1.4 Benefit

Determination of phorbolesters in some parts of *J. curcas* grown in Chiang Rai, Thailand; Pak Chong 32 and Korat was done using HPLC. The *Jatropha* leaves and stems from the site in Mae Fah Luang University were assumed to be toxic due to the presence of phorbolesters. Thus, they should be detoxified before used for many purposes. The best condition from this work might be used for determination of phorbolesters in all parts of *J. curcas* grown in Thailand.



CHAPTER 2

LITERATURE REVIEWS

Jatropha curcas L. belongs to the Euphorbiaceae family. It is a shrub of 3-8 m high, originates from Central America but is presently cultivated in Central and South America, West and South Africa, India and South-East Asia (Gübitz *et al.*, 1998). The oil from kernels of *J. curcas* can serve as fuel for diesel engines, indicating its potential as a renewable energy source (Makkar *et al.*, 1998). In the tropics, *J. curcas* is traditionally used for medicines and as hedges (Jones and Miller, 1992). Its use as green manure to rice grown on loamy acid soil was reported (Gübytz *et al.*, 1998). The seed weighs about 0.75 g, contains 30-32% protein and 60-66% lipid indicating good nutritional value. However, the seed and oil were found to be toxic to mice, rats, calves, sheep and goats, humans and chickens. Hence, its use as a food or feed source is presently limited (Makkar *et al.*, 1998).

Toxicity of *J. curcas* seeds can be caused by several components, including saponins, lectins (curcin), phytates, protease inhibitors, curcalonic acid, and phorbolesters. Phorbolesters are the tetracyclic diterpenoids generally known for their tumor promoting activity. However, *Jatropha* oil and phorbolesters exhibit insecticidal and molluscicidal activities over a wide range of organisms, suggesting their potential use in agiculture as biorational pesticides and as mollusc control agents (Gübytz *et al.*, 1998).

Phorbolesters are present in high levels in kernels. The level of phorbolesters ranges from 0.87 to 3.32 mg/g kernel (Makkar *et al.*, 1998). Phorbolesters have been found to be responsible for skin-irritant effects, beside tumor promotion, since they stimulate protein kinase C (PKC). Insecticidal activities of oil containing phorbolesters or of concentrated phorbolesters fractions have been recorded by Wink

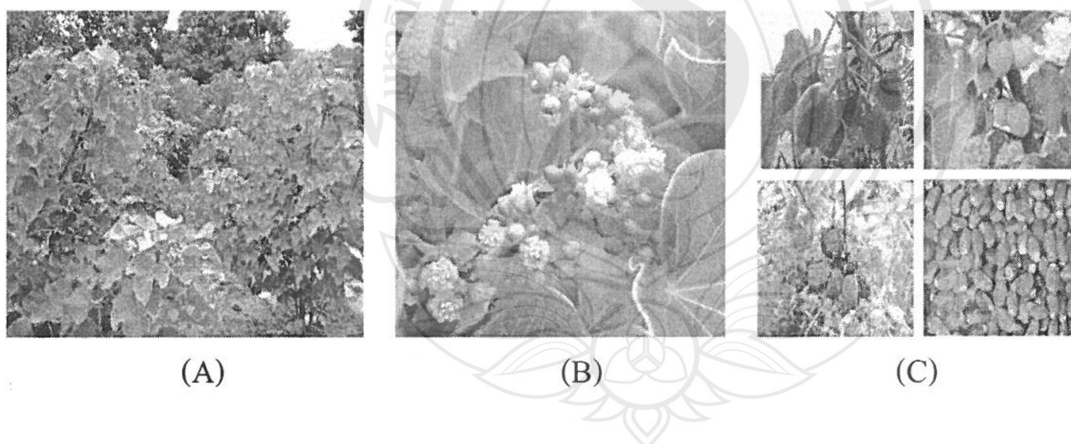
et al., 1997. The effect of 0.1% and 1% oil on the survival of some insects indicated that topical applications of phorbolesters containing oil have insecticidal properties over a wide range of insects. It is not a very strong activity, but it should be recalled that extracts and formulations had not been optimized for these trials (Wink *et al.*, 1997). Phorbolesters act as tumor promoters in mice which have been treated with a carcinogen beforehand but not in untreated animals. Therefore, phorbolesters are called co-carcinogens, although they are no carcinogens themselves (also the hormones present in the pill are co-carcinogens according to this definition) (Wink *et al.*, 1997).

Recently, the different concentration levels of phorbolesters have been found in *J. curcas* seeds cultivated in different sites. Makkar *et al.*, (1998), reported that the seeds of *J. curcas* collected from various sites in Mexico contained different concentration levels of phorbolesters; the seeds from one site contained phorbolesters whereas the seeds from the other sites did not contain phorbolesters. Therefore, we wanted to determine phorbolesters in seeds of *J. curcas* grown in Chiang Rai, Thailand. Apart from the seeds of *J. curcas*, the other parts such as stems, leaves and latex have been used for many purposes such as in environment, medicine, cosmetic, food and animal feed, insecticide, pesticide, fungicide and fuel (Chumphonwong and Sripisut, 2007). However, there have not been reports on the toxicity or the amount of phorbolesters in those parts of *J. curcas* plant. Thus we aimed to determine and compare the concentration of phorbolesters in *J. curcas* stems, leaves and latex with that in its seeds.

There have been several works that reported the determination of the phorbolesters in *J. curcas* using high performance liquid chromatography (HPLC). Wink *et al.*, (1997) separated phorbolesters from *J. curcas* oil by using HPLC because it is suitable for analyze such mixture compounds like phorbolesters. Wilhelm and Martin (2000) studied detoxification of seed oil from *J. curcas* and determined the

phorbolesters in *J. curcas* using HPLC method. Herrera *et al*., (2005) extracted and investigated the quantity of phorbolesters by using HPLC method. In this project, the HPLC method was used for the determination of the phorbolesters in *J. curcas*, because it has shown a good performance for the separation of the mixture compounds of phorbolesters from *J. curcas*. The optimization of the HPLC conditions for determination of phorbolester in extract samples was done.

Jatropha curcas, called physic nut, was classified into Kingdom of Plantae, Division Magnophyta, Class Magnoliopsida, Order Malpighiales, Family Euphorbiaceae, Subfamily Crotonoideae, Genus *Jatropha* and Species *J. curcas*. The plant grows in all regions as well as in wasteland and ravine land (Azam *et al*., 2005). The morphological characteristics of *J. curcas* are a large shrub or small tree which can reach a height of 7 m, branches contain latex, black seeds contain oil, and yellow flowers are unisexual (**Figure 1** and **2**)



(A) (B) (C)

Figure 1 Morphological characteristics of *J. curcas*: (A) branches, (B) flowers and (C) seeds

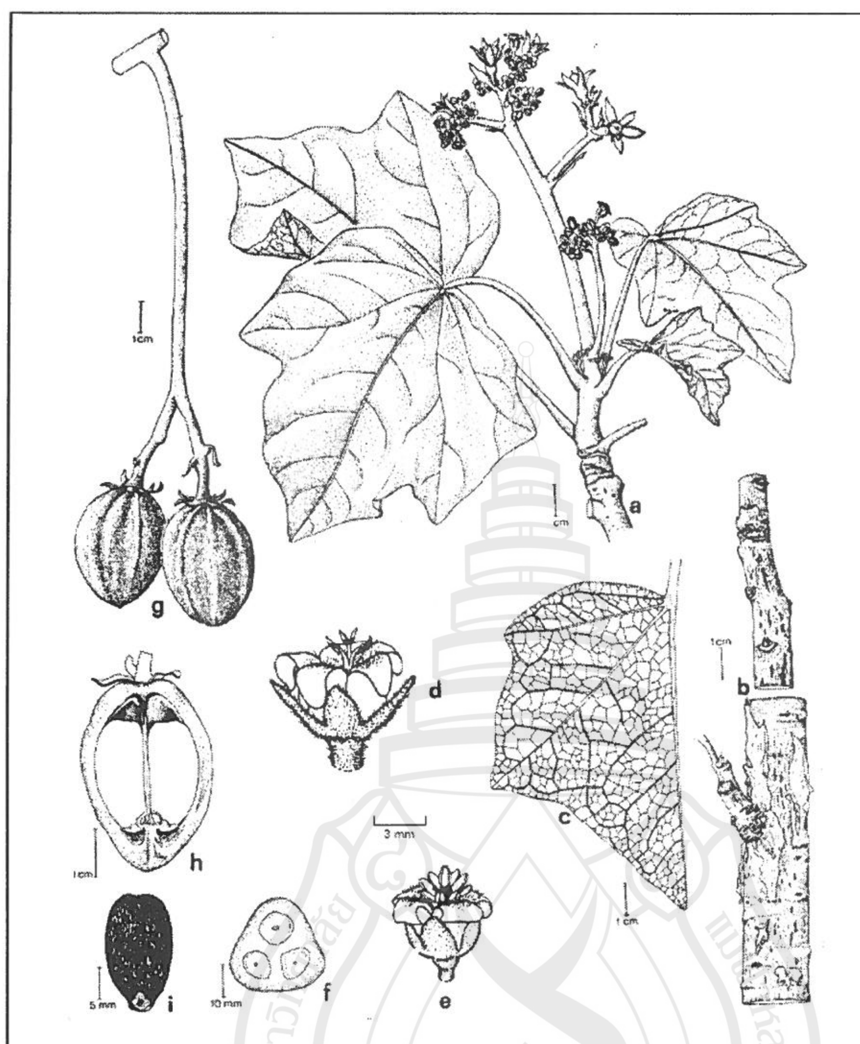


Figure 2 Characteristics of *J. curcas*: a: flowering branch, b: bark, c: leaf veinature, d: pistillate flower, e: staminate flower, f: cross-cut of immature fruit, g: fruits, h: longitudinal cut of fruits (International Plant Genetic Resources Institute, Heller, Joachim, 1996)

J. curcas is useful to various fields such as in environment, medicine, cosmetic, food and animal feed, insecticide, pesticide, fungicide and fuel as summarized in **Table 1**.

Table 1 Useful properties of *J. curcas* extraction

Field	Parts	Use	Reference
Environment	All parts	- Prevent and control ersion. - Improve land. - Fix atomospheric carbon store in wood and assist in the built of soil carbon.	Duke *et al.*, 1985
Cosmetics	Fruit	- Soaps	Duke *et al.*, 1985
Food	Leaves and stems	- Young leaves after steamed could be eaten	
Insecticide	seed oil	- Inhibit *Helicoverpa armigera* in cotton.	Chumphonwong and Sripisut, 2007
	Aqueous	- Inhibit *Aphis gossypii* in cotton.	
		- Inhibit *Pectionophra gossypiella* in cotton.	
	Seed oil	- Inhibit Empoasca biguttula in cotton (syn. Amrasca biguttula)	
		- Inhibit *Phthorimaea opercullela* in potato.	
		- Inhibit *Callosobruchus maculatus* in pulse.	

Table 1 (continued)

Field	Parts	Use	Reference
Fungicide	Shells	- Inhibit *Pytophthora pamivora* - Inhibit pathogen of durian - Inhibit *Colletorichum glorosprioides* - Inhibit sporangia - Zoospore growth of *p. palmivora* - Inhibit conidia production and growth of *C. gloeorioides*	Chareonsataporn *et al.*, 2005
Fuel	Seeds	Biodiesel	Chumphonwong and Sripisut, 2007
Medicine	Bark	Laxative, parasiticide and stomachache cures	Duke *et al.*, 1985
	Seeds	Skin disease cure, pain joint heath and laxative	

In addition traditionally, shrubs and trees serve many purposes. Le Houérou *et al.*, 1989 distinguished 13 groups according to how they are used:

1) Food and drink for humans
2) Browse for livestock and wildlife

3) Beekeeping and honey production
4) Source of energy – firewood and charcoal
5) Building and fencing material
6) Fiber for cloth, rope and handicrafts
7) Tools for agriculture and cottage industry
8) Handicraft, art and religious objects
9) Dye and tanning
10) Drugs, medicinal and veterinary uses
11) Shade and shelter for plants, animals and humans ('palaver' trees)
12) Protection against erosion, maintenance of soil fertility and productivity
13) Water storage

Phorbolesters

Phorbolesters, present in high level in the kernels of *J. curcas*, have been identified as the main toxic agent responsible for toxicity. They cause a large number of biological effects such as giddiness, vomiting, diarrhea, inflammation and tumor promoter. The term phorbolester is used today to describe a naturally occurring family of compounds widely distributed in plant species of the family Euphorbiaceae and Thymelaelaecae. Phorbolesters are bioactive diterpene derivatives that have a multitude of effects in cells (Goel *et al.*, 2007). The fundamental substance of phorbolester is tigliane (**Figure 3**). Tigliane is tetracyclic diterpene that react with hydroxylation and has hydroxyl (OH) group attach with tigliance at position. The product from this reaction is alcohol. When substance combines with acid, it will produce ester, called phorbolester 12-deoxy-16-hydroxy-phorbol (**Figure 4**). The oils from *J. curcas* contain phorbolester at least four different. The structure of the major compound is 12-deoxy-16-hydroxyphorbol-4′-[12,14′-butadienyl]-6′-[16′,18′,20′-

nonatrienyl]-bicyclo[3.1.0]hexane-(13-0)-2′[carboxylate]-(16-0)-3′-[8′-butenoic-10′]ate (DHPB) (Figure 5) (Chumphonwong and Sripisut, 2007).

Phorbolesters (phorbol-12-myristate-13-acetate) have been identified as the major toxic principle in *Jatropha* (Makkar *et al.*, 1997; Makkar and Becker, 1998). Major antinutrients present in *Jatropha* seed/seed meal are trypsin inhibitor, lectin and phytate. Diets containing *Jatropha* meal with 1.5 to 2 mg/g of phorbolesters have been found to cause suppression of feeding, lesions on the skin, weight loss and death in both fish and rats. Toxicity of *Jatropha* seeds has been studied extensively in different animal models like goats, sheep, mice, rats and fish when fed with phorbolester containing feeds (Goel *et al.*, 2007). Carp (*Cyprinus carpio* L.) were found to be highly susceptible to phorbolesters present in *Jatropha*. The threshold level at which phorbolesters caused adverse effects was 15 ppm (15 μg/g) in the diet whereby a level higher than of 31 μg/g of extract in the diet resulted in lower average metabolic rate, increase fecal mucus production and rejection of feed (Becker and Makkar, 1998). Makkar and Becker (1999) further explored the non-toxic variety of *J. curcas* as animal feed and reported that this variety after heat treatment resulted in a promising protein efficiency ratio and feed conversion ratio in rats. The heat treatment was required to inactivate lectins and trypsin inhibitor. In ruminants, microorganisms in the rumen are unable to break. Thus, to determine the phorbolesters content in leaves, stems and latex of *J. curcas* grown Chiang Rai, Thailand, the samples were collected from a site of Mae Fah Luang University. Phorbolesters in *J. curcas* have been quantified by a HPLC method (Makkar *et al.*, 1998).

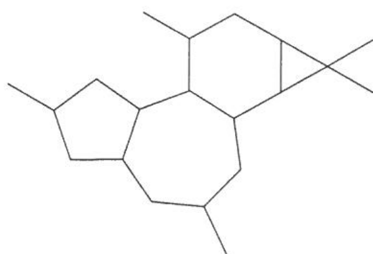


Figure 3 Tigliane

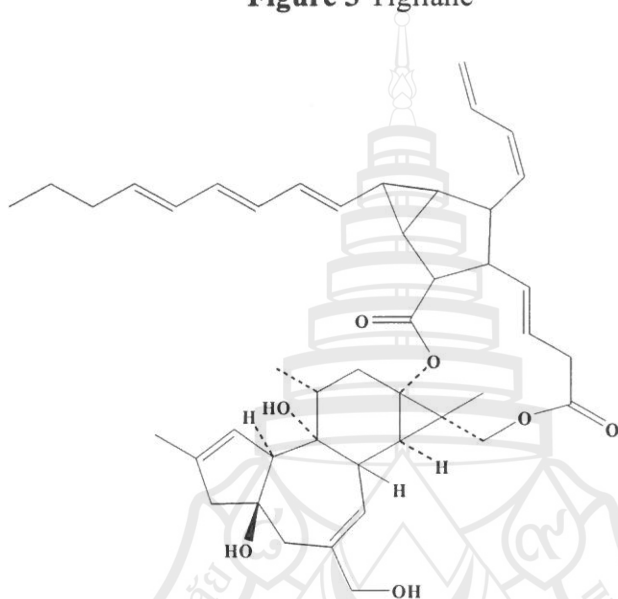


Figure 4 Phorbolesters (12-Deoxy-16-hydroxyphorbol-4′-[12′,14′-butadienyl]-6′-[16′,18′,20′nonatrienyl]-bicyclo[3.1.0]hexane-(13-*O*)-2′-[carboxylate]-(16-*O*)-3′-[8′-butenoic-10′]ate (DHPB).

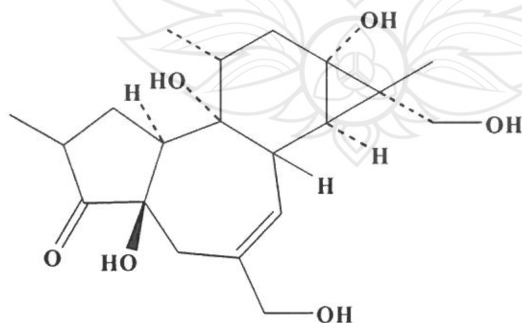


Figure 5 Diterpenoid (12-Deoxy-16-hydroxyphorbol)

High Performance Liquid Chromatography

Reverse-phase high performance liquid chromatography (RP-HPLC) provides high sensitivity for analyzing small amount of composition like phorbolesters. In principle, HPLC is used to separate components of mixture by using a variety of chemical interactions between the substance and the chromatography column at high pressure, so that different amount of time (Martinez and Herrer *et al.*, 2006). Compounds stick to reverse phase HPLC columns in high aqueous stationary phase and are eluted from RP-HPLC columns with high organic mobile phase. In RP-HPLC, compounds are separated based on their hydrophobic character. Phorbolesters extracted from defatted seed kernels in methanol or dichloromethane, aliquot of this extract is loaded on a HPLC reverse-phase C18 LiChrospher 100 (Merck, Darmstadt, Germany), end-capped 5μm column. The separation is done using a gradient elution with solvents comprising diluted *o*-phosphoric acid, acetonitrile, and tetrahydrofuran. The absorbance is recorded at 280 nm, by HPLC confirmed were found to be highly susceptible to phorbolesters in *Jatropha*. The threshold level in carp at which phorbolesters caused adverse effects was 15 ppm (15 μg/g) in the diet whereby a level higher than of 31 μg/g of extract in the diet resulted in lower average metabolic rate, increase fecal mucus production and rejection of feed (Becker and Makkar, 1998). Herrera *et al.*, (2005) extracted and investigated the quantity of phorbolesters by using HPLC method. In this project, the method was used for the determination of phorbolesters in *J. curcas* extracts, because HPLC can separate mixture compounds such as phorbolesters from *J. curcas*.

CHAPTER 3

METHODOLOGY

3.1 Plant material

Stems, leaves, and latex of *Jatropha curcas* L. samples, listed in **Table 2**, were collected from a site in Mae Fah Luang University, Chiang Rai.

Table 2 Information of the samples

Part	Variety	Sample name
Stem	Pak Chong 32	SPC
		SPCE *
	Korat	SKR
		SKRE *
Leaf	Pak Chong 32	LPC
		LPCE *
	Korat	LKR
		LKRE *
Latex	Pak Chong 32	APC
		APCE *
	Korat	AKR
		AKRE *

* Evaporated extracts

3.2 Chemicals

1. 100% Methanol, HPLC grade (Mallinckrodt Baker, S.A., USA)
2. 100% Tetrahydrofuran (MERCK, Germany)
3. 85% Orthophosphoric acid (MERCK, Germany)
4. 100% Acetonitrile, HPLC grade (Mallinckrodt Baker, S.A., USA)
5. Phorbol-12-myristate-13-acetate (Sigma, Prod. No. P 8139, USA)

3.3 Method

3.3.1 Preparation of extracts

Stems (500 g), leaves (300 g) and latex (3 mL) of *Jatropha curcas* were extracted with methanol (4 L) at room temperature (Makkar *et al.*, 1997). The methanolic extract was separated into two fractions. One fraction was filtered with cotton pads without preconcentration. The other fraction was filtered with cotton pads and preconcentrated by evaporation (40 °C) giving a honey-like viscous residue (500 mL). The extracts were filtered through a 0.45 μm syringe filter into amber glass shell vial 1 mL before injected (20 μL) into the HPLC system for analysis.

3.3.2 Standard solutions

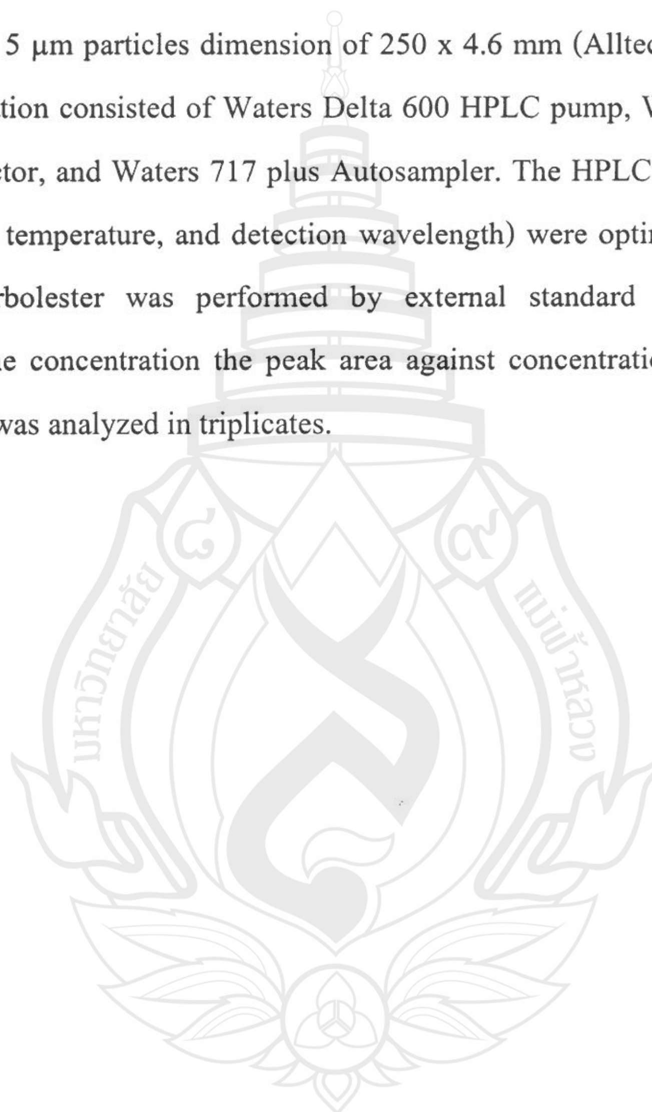
Phorbol-12-myristate-13-acetate (TPA) used as standard stock solution (5,000 ppm), was prepared by dissolving 5 mg of TPA and diluting with 100 % methanol in a 100 mL volumetric flask. The TPA standard stock solution was stored in a refrigerator at a temperature between 2-8 °C.

TPA working solutions (10, 20, 30, 40 and 50 ppm) were prepared by diluting the stock TPA standard with 100% methanol. Methanol 100% was used as a blank. The

standard solutions were filtered through 0.45 μm, PTFE syringe filter and injected solutions into HPLC. The quantity of TPA was determined from a standard curve.

3.3.3 HPLC analysis

The filtered samples (20 μL) were analyzed by HPLC using a reverse phase C18 analytical column with 5 μm particles dimension of 250 x 4.6 mm (Alltech Prevail). The HPLC instrumentation consisted of Waters Delta 600 HPLC pump, Waters2996 photodiode Array detector, and Waters 717 plus Autosampler. The HPLC conditions (mobile phase, column temperature, and detection wavelength) were optimized. The determination of phorbolester was performed by external standard calibration obtained by plotting the concentration the peak area against concentration of TPA standard. Each sample was analyzed in triplicates.



CHAPTER 4

RESULTS AND DISCUSSION

In this work, phorbolesters in some parts (stems, leaves and latex) of *J. curcas* families Euphorbiaeae were studied by HPLC.

4.1 Optimization of HPLC condition

The HPLC conditions (mobile phase, flow rate, detection wavelength, column temperature) were optimized to obtain a good separation (**Table 3**). The HPLC chromatograms of standard solutions obtained by using those conditions are shown in (**Figure 6-10**).

Table 3 Summaries the condition varied in this work

Condition	Mobile Phase	Flow rate	Detector	Temperature	Injection
		mL/min	nm	°C	μL
1	Gradient CH_3CN/H_3PO_4 (80:20 v/v)	1.3	280	No	25
2	CH_3CN/H_2O (80:20 v/v)	1.0	280	No	20
3	CH_3CN/H_3PO_4 (80:20 v/v)	1.0	250	No	20
4	100% CH_3CN	1.0	254	30	20
5	100% CH_3CN	1.0	232	30	20

The parameters used for optimization in condition 1, 2, and 3 were selected from some literature (Makkar *et al.*, 1998). However, the separation patterns obtained by using those conditions (**Figure 6-8**) were not as good as those using 100% acetonitrile and column temperature at 30% (**Figure 9-10**). Therefore, in this work the parameters in condition 5 were used. A good separation can be achieved with 20 min. The retention time of standard peak was founds at 10.88 min.

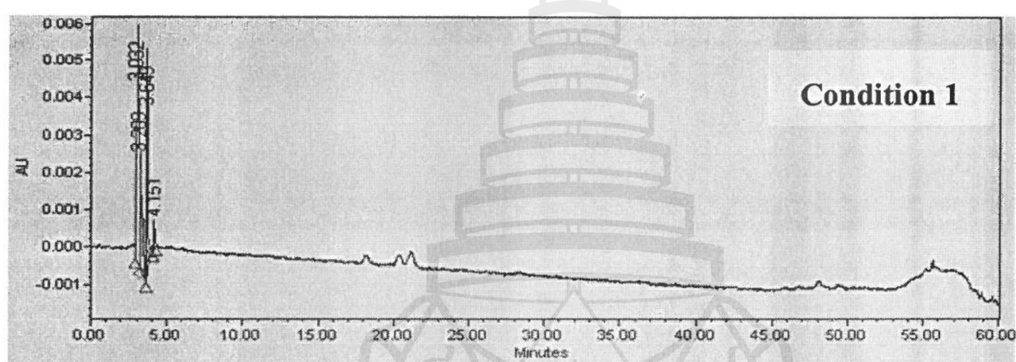


Figure 6 Chromatogram of standard solution containing 500 ppm of TPA, mobile phase: gradient acetonitrile:85% *o*-phosphoric acid (80:20 v/v)

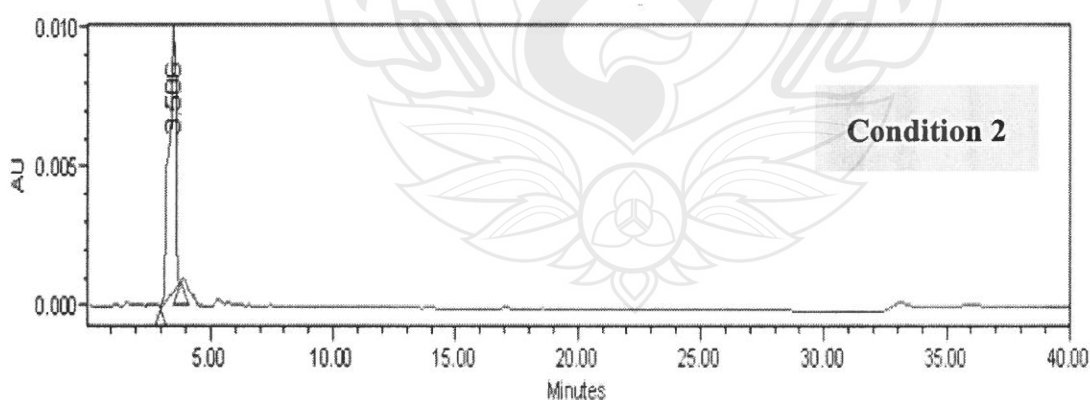


Figure 7 Chromatogram of standard solution containing 500 ppm of TPA, mobile phase: acetonitrile:water (80:20 v/v)

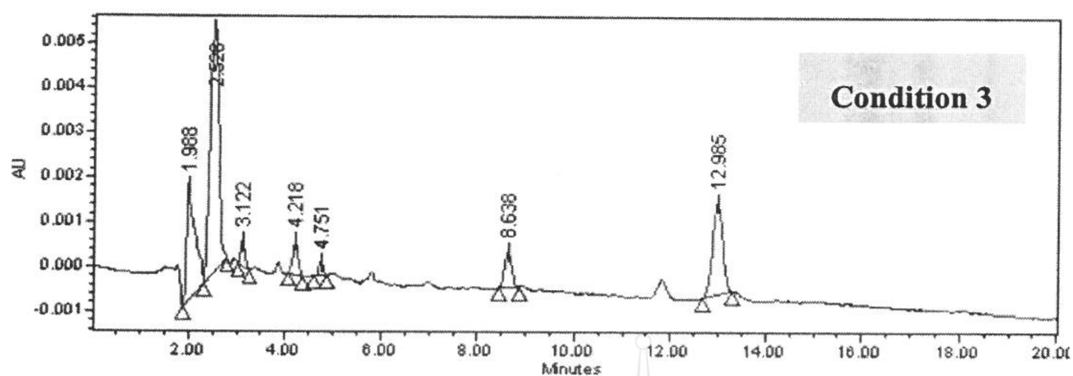


Figure 8 Chromatogram of standard solution containing 500 ppm of TPA, mobile phase: acetonitrile:85% *o*-phosphoric acid (80:20 v/v)

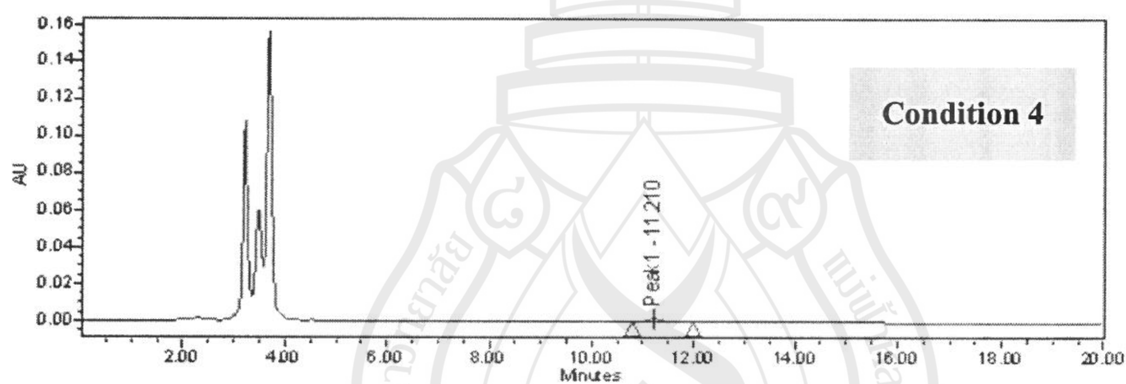


Figure 9 Chromatogram of standard solution containing 500 ppm of TPA, mobile phase:100% acetonitrile

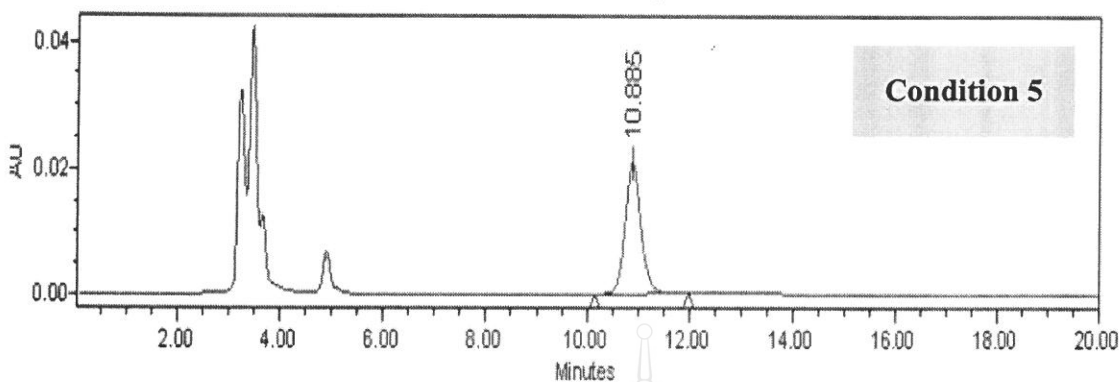


Figure 10 Chromatogram of standard solution containing 500 ppm of TPA, mobile phase: 100% acetonitrile.

Using the optimum condition, good linearity of calibration was obtained over the concentration range of 10, 20, 30 and 50 ppm (R^2 = 0.998) were resulted in (**Figure 11**).

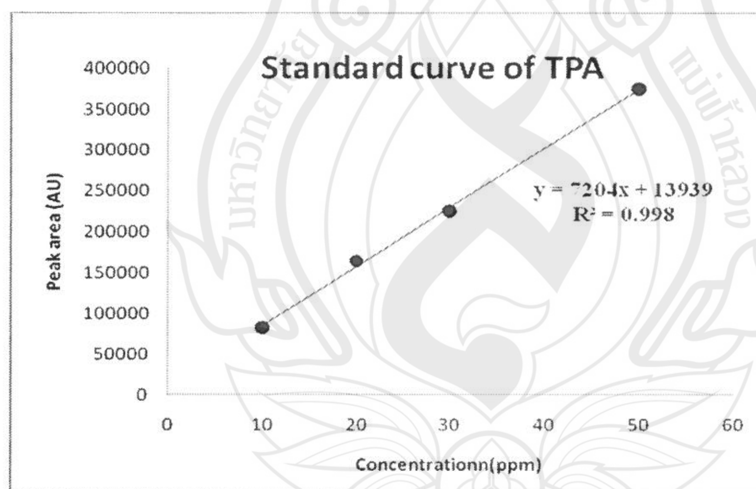


Figure 11 Standard curve of TPA

4.2 Determination of phorbolesters

The HPLC method using the optimum condition was applied to determine phorbolesters in stems, leaves and latex of *J. curcas*.

4.2.1 Leaf samples

The chromatograms of the extracts from leaf samples were shown in **Figure 12**. There were two peaks at the retention time (10-12 min) close to the retention time of TPA standard (10-11 min) (**Figure 10-11**). To confirm the TPA peak, known amount of TPA standard was spiked into samples. It was found that the peak at retention time of 11.60 min was increased. Thus, it was assumed that this peak represented the presence of phorbolesters. However, the peak nearby might be other forms or isomers of phorbolesters. This is needed to be investigated further.

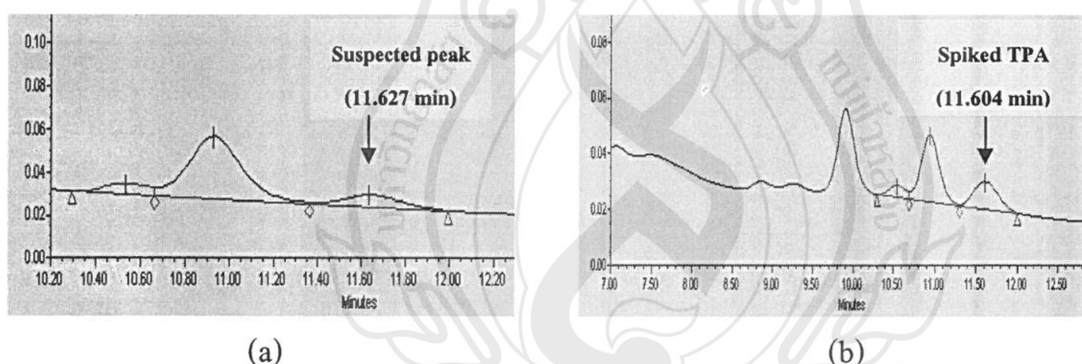
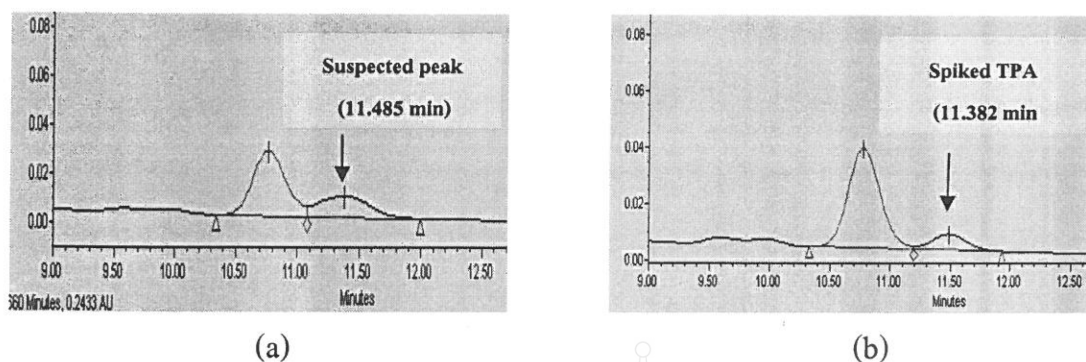


Figure 12 Chromatogram of (a) non spiked samples and (b) TPA standard spiked samples in Pak Chong 32 (Leaf samples)



(a) (b)

Figure 13 Chromatogram of (a) non spiked samples and (b) TPA standard spiked samples in Korat (Leaf samples)

The precision of the analysis was determined by measuring individually prepared three series of spiked standard at 20 ppm of TPA. The quantity of phorbolesters was calculated by using the obtained calibration curves, as shown in **Table 4**.

Table 4 Phorbolesters concentration (equivalent to TPA standard) in the samples

Part	Sample	Concentration of phorbolesters (ppm)	%RSD
Leaf	LPC	26.81	1.88
	LPCE	48.29	3.48
	LKR	31.10	0.90
	LKRE	45.40	1.84
Stem	SPC	21.75	1.88
	SPCE	18.40	0.38
	SKR	24.63	0.38
	SKRE	19.66	3.57

From **Table 4**, the concentrations of phorbolesters in the unevaporated leaf extracts for Pak Chong 32 (LPC) and Korat (LKR) were less than the evaporated ones (LPCE and LKRE). The solvent lost during evaporization might lead to more concentrated extracts. In addition, more volume of extract used for the LPCE and LKRE samples might dissolve more phorbolesters into the extracts. Thus, concentrations of phorbolesters in evaporized extracts were higher than that in unevaporized extracts.

Recovery of the method used was also determined (**Table 5**). The recoveries were found in the range of 68-108%, except the LPCE and LKRE that may be affected from evaporization process.

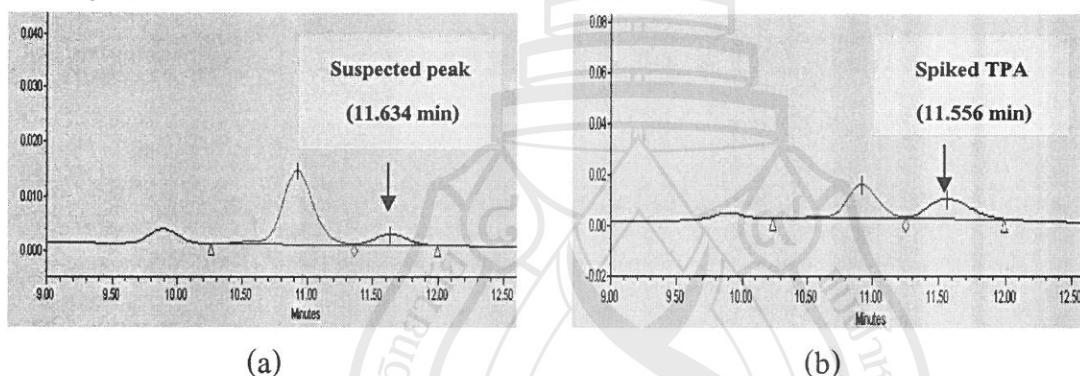
Table 5 Recovery of extraction procedure

Part	Sample	Concentration of phorbolesters (ppm)	Spiked standard (ppm)	Recovered concentration	%Recovery
Leaf	LPC	26.81	20	6.81	68.37
	LPCE	48.29	20	28.29	*
	LKR	31.10	20	11.10	79.64
	LKRE	45.4	20	25.40	32.23
Stem	SPC	21.75	20	1.75	81.84
	SPCE	18.40	20	-1.60	92.00
	SKR	24.63	20	4.63	102.76
	SKRE	19.66	20	-0.34	107.99

* Error recovery experiment

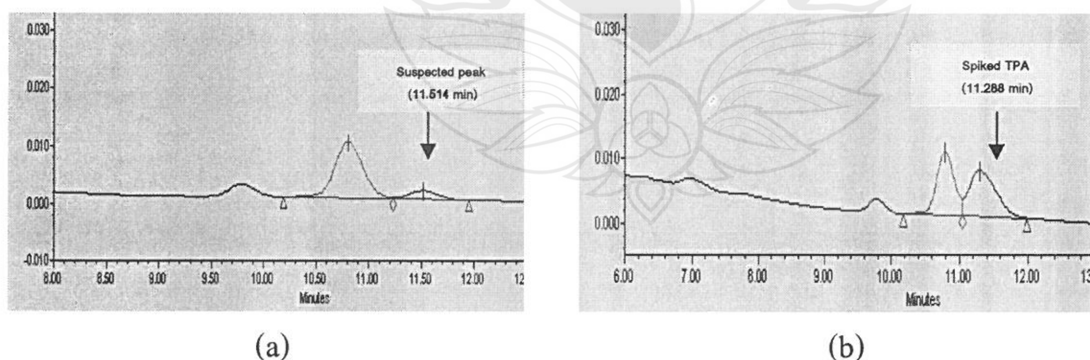
4.2.2 Stem samples

The chromatograms of stem extracts of Pak Chong 32 and Korat samples (**Figure 13**) showed two peaks at retention time close to the retention time of the pure standard (between 10-12 min). To confirm that the peak present at 11.63 min was phorbolesters, known amount of TPA standard was spiked into the samples. The peak at 11.55 min was increased (**Figure 13(b)** and **14(b)**). Thus the phorbolesters concentration was determined by using the peak area of this peak. The concentrations of phorbolesters in stem samples were 21.75 ppm in Pak Chong 32 and 24.63 ppm in Korat samples (**Table 4**). In addition, % RSD of 0.38-3.57 % showed good precision of analysis.



(a) (b)

Figure 14 Chromatogram of (a) non spiked samples and (b) TPA standard spiked samples in Pak Chong 32 (Stem samples)



(a) (b)

Figure 15 Chromatogram of (a) non spiked samples and (b) TPA standard spiked samples in Korat (Stem samples)

However, from **Table 4**, the phorbolester concentration in unevaporized SPC and SKR extracts were a little higher than that in evaporized SPCE and SKRE extracts. This is different from the results obtained from the leaf samples. Although more volume of extracts was used for the SPCE and SKRE samples, the dissolution of phorbolesters may be limited by its concentration level. This might lead to the insignificant difference of phorbolester concentration in the evaporized and unevaporized stem extracts. The little difference of concentration was assumed to be from the evaporation process.

The phorbolester concentrations in leaf and stem samples (**Table 4**) were higher than those in seed reported in literature. The seed oil contains 13.85 ppm of phorbolester (Becker and Makkar, 1998). Indeed, in further work we should compare the results between the seed of Pak Chong 32 and Korat samples.

Nevertheless, the concentrations of phorbolesters in the stem and leaf samples were higher than 20 ppm. It indicated that the *Jatropha* stems and leaves from the sampling site were assumed to be toxic and should not be used as animal feed. Many literatures studied the toxicity of phorbolesters in animals, e.g. rats, goats, mice, and fish. Different threshold levels were found for different animals (Goel *et al.*, 2007).

4.2.3 Latex samples

The chromatogram of latex of Pak Chong 32 and Korat samples (**Figure 16**) did not show any peak of phorbolesters in 20 min. It may be because the sample preparation method was not suitable to separate phorbolesters in latex samples. The samples preparation method for analysis of phorbolesters in latex samples should be optimized in further work.

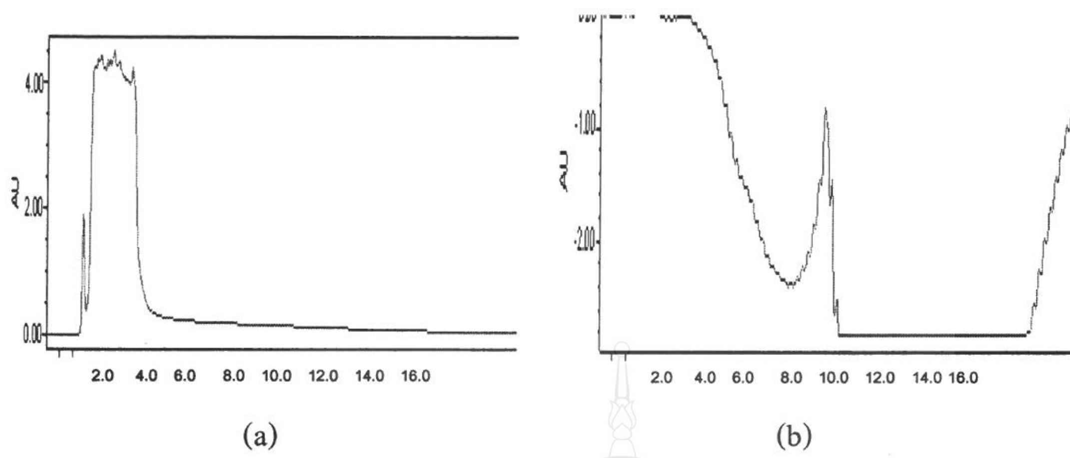
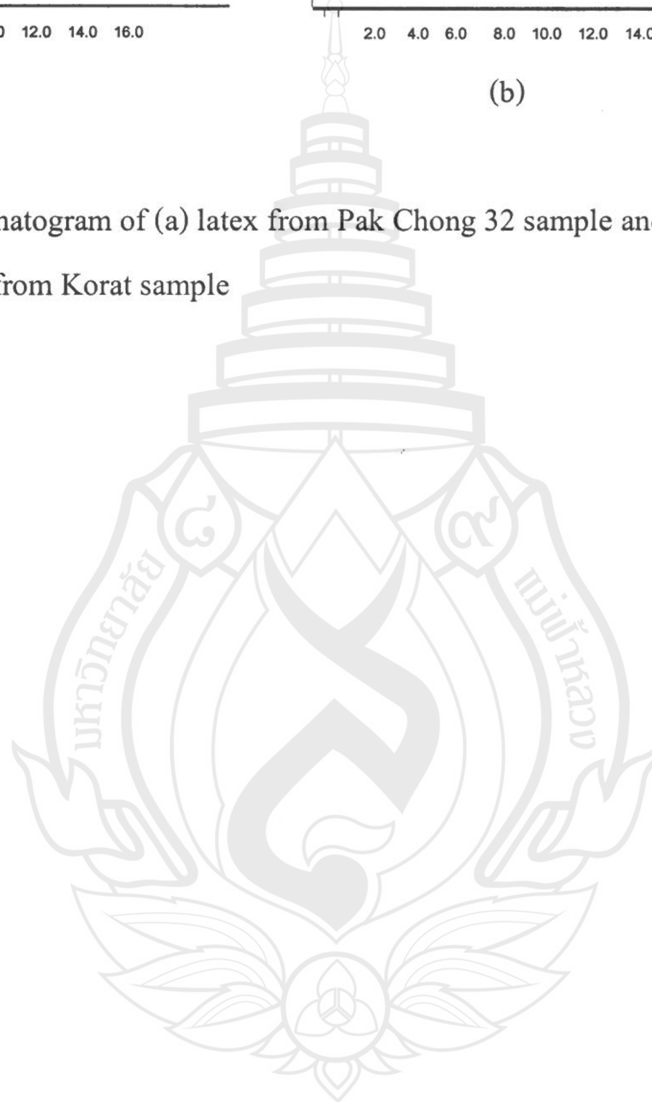


Figure 16 Chromatogram of (a) latex from Pak Chong 32 sample and (b) latex from Korat sample



CHAPTER 5

CONCLUSION

In this experiment, determination of phorbolesters in some parts such as the stems, leaves and latex of *J. curcas* grown in Chiang Rai, Thailand; Pak Chong 32 and Korat was done using HPLC. In order to properly analyze the phorbolesters, optimization of HPLC condition was performed. It was found that 100% acetonitrile was the best mobile phase solvent system. The column was controlled at 30 °C. The used flow rate was 1 mL/min, and the detect wavelength was 232 nm. Method validation showed that the relative standard deviations (RSD) were in the rage of 0.38 % to 3.57 %. The accuracy was tested by determination of recovery. Recoveries for phorbolester determination in *Jatropha* were found to be ranging from 32.23 to 107.99 %.

The concentration of phorbolesters in the stem samples from Pak Chong 32 and Korat were found at 21.75 and 24.63 ppm, respectively. The phorbolesters concentration in the leaf samples were found at 26.81 and 31.10 ppm in Pak Chong 32 and Korat, respectively. The results suggested that phorbolesters concentration in the leaf samples of both varieties (Pak Chong 32 and Korat) were more than those in stem samples.

The *Jatropha* leaves and stems from the site in Mae Fah Luang University were assumed to be toxic due to the presence of phorbolesters. Thus, they should be detoxified before used as animal feed.

REFERENCES

Adolf, W.; Opferkuch, H.J. and Hecker, E., (1984). Irritant phorbol derivates from four *Jatropha* species. *Phytochemistry* 23 (1), 129–132.

Chumphonwong, N. and Sripisut, T. (2007). Charaterization of some chemical compositions of *Jatropha curcas* seed (in Thai).

Goel, G.; Makkar, H.P.S.; Francis, G. and Becker, K. (2007). Phorbol esters: structure, occurrence and biological activity. *Int. J. Toxicol.* 26, 279–288.

Gübitz, G.M.; Mittelbach, M. and Trabi, M. (1998). Exploitation of the tropical oil seed plant *Jatropha curcas* L. *Biores. Technol.* 67, 73–82.

Haas, W. and Mittelbach, M. (2000). Detoxification experiments with the seed oil from *Jatropha curcas* L. *Industrial crops and products*. 12: 111-118.

Hass, W.; Sterk, H. and Mittelbuch, M. (2002). Novel 12-Deoxy-16- hydroxy phorbol diesters isolated from the seed oil *Jatropha curcas J. Nat. Prod.* 65, 1434-1440.

Hirota, M.; Suttajit, M.; Suguri, H.; Yasuyuki, E.; Shudo, K.; Wongchai, V.; Hecker, E. and Fujiki, H. (1988). A new tumorpromoter from the seed oil of *Jatropha curcas* L., anintramolecular diester of 12-deoxy-16-hydroxyphorbol.*Cancer Res*. 48, 5800–5804.

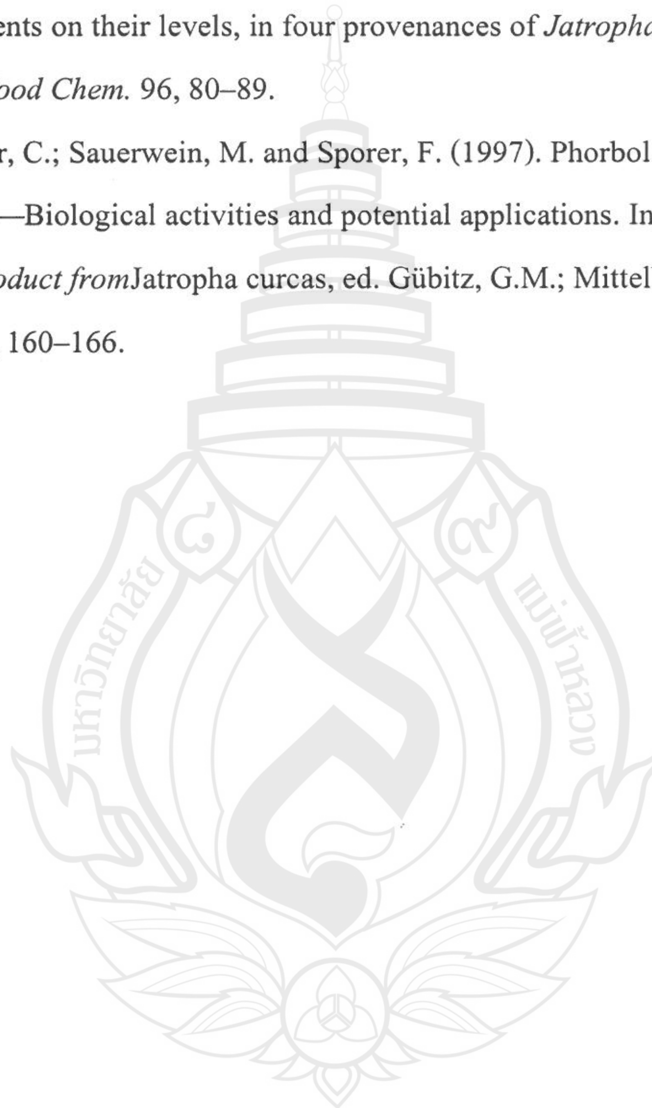
Kingsbury, J.M. (1964). Poisonous Plants of the United States and Canada, Prentice-Hall, Inc., Englewood Cliffs, NJ. 626 p.

Makkar, H.P.S. and Becker, K. (1997). Potential of *J. curcas* seed meal as a protein supplement to livestock feed, constraints to its utilisation and possible strategies to overcome constraints. In: Gübitz, G.M.; Mittelbach, M.; Trabi, M. (Eds.), Biofuels and Industrial Products from *Jatrophacurcas*. Dbv, Graz, pp. 190–205.

Makkar, H.P.S.; Becker, K.; Sporer, F. and Wink, M. (1997). Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. *J. Agric. Food Chem.* 45: 3152 - 3157.

Martínez-Herrera, J.; Siddhuraju, P.; Francis, G.; Dàvila-Ortíz, G. and Becker, K. (2006). Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of *Jatropha curcas* L. from Mexico. *Food Chem.* 96, 80–89.

Wink, M.; Koschmieder, C.; Sauerwein, M. and Sporer, F. (1997). Phorbol esters of *Jatropha curcas*—Biological activities and potential applications. In *Biofuel and industria product from*Jatropha curcas, ed. Gübitz, G.M.; Mittelbach, M.; Trabi, M. (Eds.), 160–166.



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Education background

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2006	Ph.D.	Organic Chemistry	Prince of Songkla University
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1999	B.Sc.	Chemistry	Prince of Songkla University

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1995-1999 Bachelor of Science in Chemistry. Prince of Songkla University.

1999-2001 Master of Science in Organic Chemistry. Prince of Songkla University.
Research Supervisor: Asst. Prof. Dr. Wilawan Mahabusarakam.
Thesis Title: Chemical Constituents from *Derris scandens* and Antioxidation Properties.

2002-2005 Ph.D. Student in Organic Chemistry. Prince of Songkla University.
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Thesis Title: Chemical Constituents from the flowers, fruits and seeds of *Garcinia dulcis* and Antioxidation Properties.

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Research Supervisor: Prof. Dr. Chong-Ren YANG & Assoc. Prof. Dr. Ying-Jun Zhang.

Research Title: Isolation of High Polarity Compounds from *Garcinia dulcis*.

Publications

1. W. Mahabusarakam; **S. Deachathai**; S. Phongpaichit; C. Jansakul and W.C. Taylor. 2004. "A benzil and isoflavone derivatives from *Derris scandens* Benth." *Phytochemistry*, 65, 1185-1191.
2. **S. Deachathai**; W. Mahabusarakam; S. Phongpaichit and W.C. Taylor. 2005. "Phenolic Compounds from the Fruit of *Garcinia dulcis*" *Phytochemistry*, 66, 2368-2375.
3. **S. Deachathai**; W. Mahabusarakam; S. Phongpaichit; W.C. Taylor; Y.-J. Zhang and C.-R. Yang. 2006. "Phenolic Compounds from the Flowers of *Garcinia dulcis*" *Phytochemistry*, 67, 464-469.
4. **S. Deachathai**; S. Phongpaichit and W. Mahabusarakam. 2008. "Phenolic Compounds from the Seeds of *Garcinia dulcis*" *Natural Product Research*, 22(15), 1327-1332.

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1994	Ph.D.	Plant Sciences	Adelaide University
1986	M.Sc.	Soil Science	Kasetsart University
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Publications

1. Bange, M.P.; Milroy, S.P. and **Thongbai, P**. 2004. Growth and yield of cotton in response to waterlogging. *Field Crop Res.* 88:129-142.
2. **Thongbai, P**. and Goodman, B.A. 2000. Oxidative free radicals generation and post-anoxic injury of rices (Oryza Sativa L.) in an iron-toxic soil. *J. Plant Nutrition*. 23: 1887-1900.
3. Kilcoyne, S.H.; Bentley, P.M.; **Thongbai, P**; Gordon, D.C. and Goodman, B.A. 2000. The application of Fe-57 Mössbauer Spectroscopy in the investigation of iron uptake and translocation in plants. *Nuclear Instruments & Methods in Physics Research Section B-Beam Interaction with Material & Atom.* 160:157-166.
4. Puckridge, D.W.; Kongchum, M.; **Thongbai, P.**; Sattarasart, A. and Sinoupakarn, S. 1994. Nitrogen uptake and yield of deepwater rice in the Central Plain of Thailand. *Field Crop Res.* 37:193-204.

5. **Thongbai, P.**; Hannam, R.J.; Graham, R.D. and Webb, M.J. 1993. Interaction between zinc nutritional status of cereals and *Rhizoctonia* root rot severity. I. Field observations. *Plant and Soil.* 153: 207-214.

6. **Thongbai, P.**; Graham, R.D.; Neate, S.M. and Webb, M.J. 1993. Interaction between zinc nutritional status of cereals and *Rhizoctonia* root rot severity. II. Effect of Zn on disease severity of wheat under controlled conditions. *Plant and Soil.* 153: 215-222.

7. Kupkanchanakul, T.; Puckridge, D.W.; Petchrit, K.; Kupkanchanakul, K. and **Thongbai, P.** 1986. Production from deepwater rice and early maturing rice intercrops with different row combinations. *Field Crop Res.* 14: 53-62.