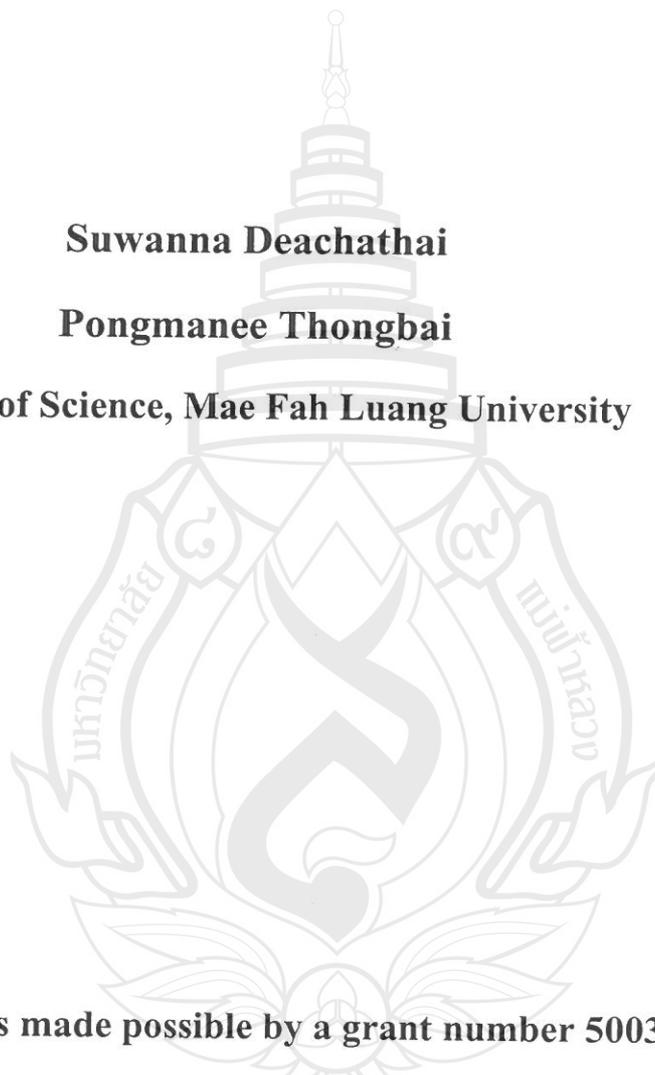


**Investigation Techniques for Determination and
Evaluation of Phorbolesters in *Jatropha curcus* Plant**

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PREFACE

Phorbol esters, 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 phorbol ester is used today to describe a naturally occurring family of compounds widely distributed in plant species of the family Euphorbiaceae and Thymelaeaceae. The oils from *J. curcas* contain phorbol ester at least four different.

Phorbol esters (phorbol-12-myristate 13-acetate) have been identified as the major toxic principle in *Jatropha*. Phorbol esters are bioactive diterpene derivatives that have a multitude of effects in cells. Thus, to determine the phorbol esters 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, phorbol esters. It is diterpene derivatives known as a tumor promoting agent with very little information about it content and determined phorbol esters in *J. curcas* was quantified used a HPLC method.

ABSTRACT

In this work, determination of phorbolsters 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 phorbolsters by RP-HPLC were optimized. Acetonitrile (100 %) was used as the mobile phase. The column temperature at 30 °C was controlled. The absorbance of phorbolster was detected at 232 nm. The flow rate of mobile phase was 1.0 ml/min. The standard phorbolster peak appeared at the retention time of ~11 min. Method validation showed that the relative standard deviations (RSD) were in the range of 0.38 to 3.57 %. The accuracy was tested by determination of recovery. Recoveries for phorbolster determination were found to be ranging from 32.23 to 107.99 %. The concentration of phorbolsters in the stem extracts from Pak Chong 32 and Korat samples were 21.75 and 24.63 ppm, respectively, whereas the phorbolsters concentrations in the leaf extracts were 26.81 and 31.10 ppm, respectively.

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Suwanna Deachathai



TABLE OF CONTENTS

	Page
ABSTRACT	(1)
ACKNOWLEDGEMENT	(2)
TABLE OF CONTENTS	(3)
LIST OF TABLES	(4)
LIST OF ILLUSTRATIONS	(5)
ABBREVIATIONS AND SYMBOLS	(7)
CHAPTER 1 INTRODUCTION	1
1.1 Statement and significance of the problem	1
1.2 Objectives	1
1.3 Scope of study	2
1.4 Benefit	2
CHAPTER 2 LITERATURE REVIEWS	3
CHAPTER 3 METHODOLOGY	13
3.1 Plant material	13
3.2 Chemicals	14
3.3 Method	14
CHAPTER 4 RESULTS AND DICUSSION	16
4.1 Optimization of HPLC condition	16
4.2 Determination of phorbolesters	20
CHAPTER 5 CONCLUSION	26
REFERENCES	27
BIOGRAPHY	29

LIST OF TABLES

Table		Page
1	Useful properties of <i>J. curcas</i> extraction	7
2	Information of the samples	13
3	Summaries the condition varied in this work	16
4	Phorbolsters concentration (equivalent to TPA standard) in the samples	21
5	Recovery of extraction procedure	22

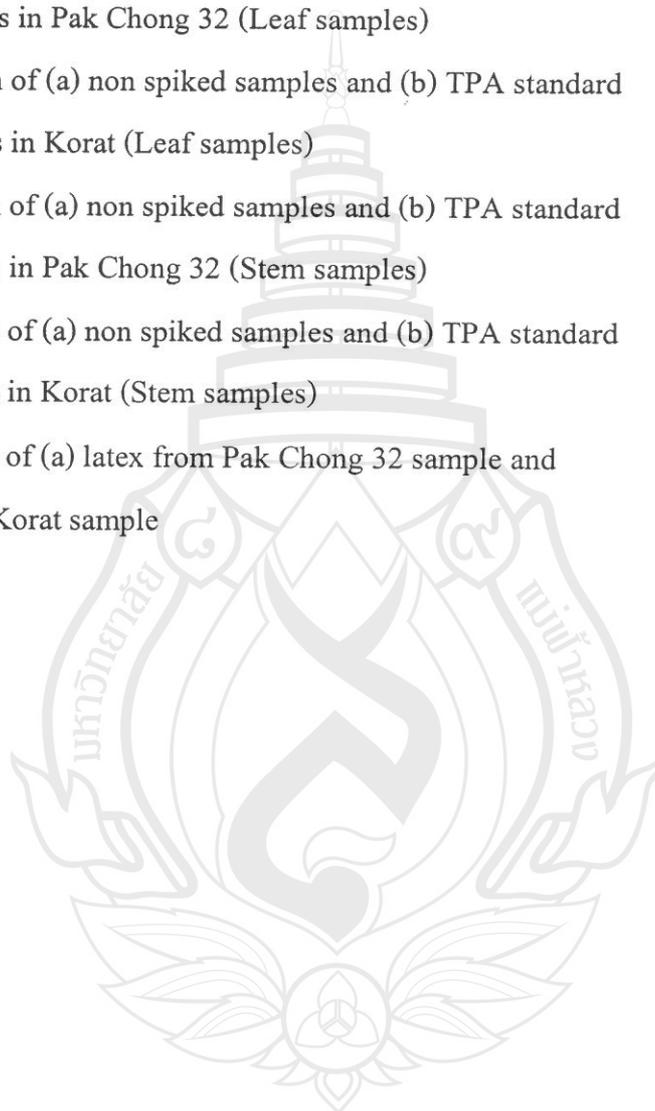


LIST OF ILLUSTRATIONS

Figure	Page
1 Morphological characteristics of <i>J. curcas</i> : (A) branches, (B) flowers and (C) seeds	5
2 Characteristics of <i>J. curcas</i> : 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; Characteristics of <i>J. curcas</i>	6
3 Tigliane	11
4 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 (DHPB)	11
5 Diterpenoid (12-Deoxy-16-hydroxyphorbol)	11
6 Chromatogram of standard solution containing 500 ppm of TPA, mobile phase: gradient acetonitrile: 85% <i>o</i> -phosphoric acid (80:20 v/v)	17
7 Chromatogram of standard solution containing 500 ppm of TPA, mobile phase: acetonitrile:water (80:20 v/v)	17
8 Chromatogram of standard solution containing 500 ppm of TPA, mobile phase: acetonitrile: 85% <i>o</i> -phosphoric acid (80:20 v/v)	18
9 Chromatogram of standard solution containing 500 ppm of TPA, mobile phase: 100% acetonitrile	18
10 Chromatogram of standard solution containing 500 ppm of TPA, mobile phase: 100% acetonitrile	19
11 Standard curve of TPA	19

LIST OF ILLUSTRATIONS (continued)

Figure	Page
12 Chromatogram of (a) non spiked samples and (b) TPA standard spiked Samples in Pak Chong 32 (Leaf samples)	20
13 Chromatogram of (a) non spiked samples and (b) TPA standard spiked samples in Korat (Leaf samples)	21
14 Chromatogram of (a) non spiked samples and (b) TPA standard spiked samples in Pak Chong 32 (Stem samples)	23
15 Chromatogram of (a) non spiked samples and (b) TPA standard spiked samples in Korat (Stem samples)	23
16 Chromatogram of (a) latex from Pak Chong 32 sample and (b) latex from Korat sample	25



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
$\mu\text{g/g}$	micrograms per gram
mL/min	milliliter per minute
$^{\circ}\text{C}$	degree Celsius
C18	reverse-phase C18 column
DHPB	Phorbol-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	Phorbol-12-myristate-13-acetate

CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problem

Recently, the different concentration levels of phorbolsters 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 phorbolsters; the seeds from one site contained phorbolsters whereas the seeds from the other sites did not contain phorbolsters. Therefore, we wanted to determine phorbolsters 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

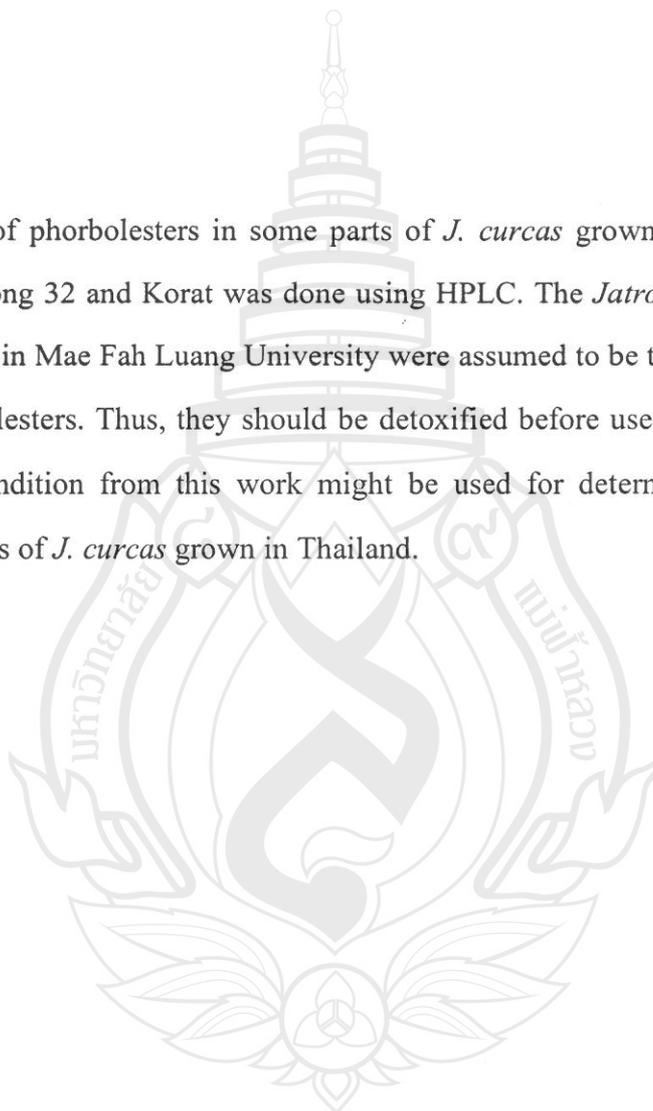
This project aimed to investigate the phorbolsters content in some parts of *J. curcas* using HPLC method. The HPLC conditions were optimized for the determination of trace amount of phorbolsters. Phorbolsters is extracted from the stems and leaves using methanol as the solvent.

1.3 Scope of study

To determine the phorbolsters 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. Phorbolsters in *J. curcas* have been quantified by a HPLC method.

1.4 Benefit

Determination of phorbolsters 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 phorbolsters. Thus, they should be detoxified before used for many purposes. The best condition from this work might be used for determination of phorbolsters 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 phorbolsters. Phorbolsters are the tetracyclic diterpenoids generally known for their tumor promoting activity. However, *Jatropha* oil and phorbolsters exhibit insecticidal and molluscicidal activities over a wide range of organisms, suggesting their potential use in agriculture as biorational pesticides and as mollusc control agents (Gübytz *et al.*, 1998).

Phorbolsters are present in high levels in kernels. The level of phorbolsters ranges from 0.87 to 3.32 mg/g kernel (Makkar *et al.*, 1998). Phorbolsters 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 phorbolsters or of concentrated phorbolsters 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 phorbol esters 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). Phorbol esters act as tumor promoters in mice which have been treated with a carcinogen beforehand but not in untreated animals. Therefore, phorbol esters are called co-carcinogens, although they are not 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 phorbol esters 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 phorbol esters; the seeds from one site contained phorbol esters whereas the seeds from the other sites did not contain phorbol esters. Therefore, we wanted to determine phorbol esters 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 phorbol esters in those parts of *J. curcas* plant. Thus we aimed to determine and compare the concentration of phorbol esters in *J. curcas* stems, leaves and latex with that in its seeds.

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

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

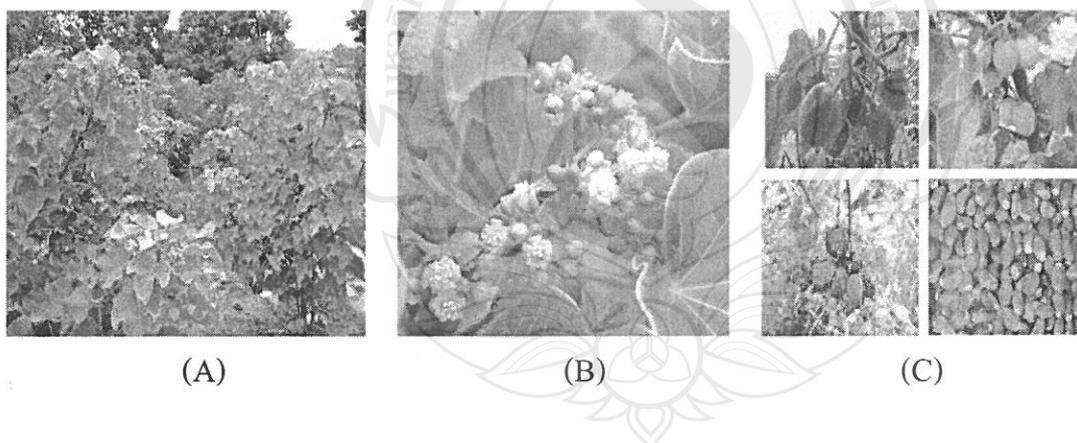


Figure 1 Morphological characteristics of *J. curcas*: (A) branches, (B) flowers and (C) seeds



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	<ul style="list-style-type: none"> - Prevent and control erosion. - Improve land. - Fix atmospheric carbon store in wood and assist in the built of soil carbon. 	Duke <i>et al.</i> , 1985
Cosmetics	Fruit	- Soaps	Duke <i>et al.</i> , 1985
Food	Leaves and stems	- Young leaves after steamed could be eaten	
Insecticide	seed oil	- Inhibit <i>Helicoverpa armigera</i> in cotton.	Chumphonwong and Sripisut, 2007
	Aqueous	- Inhibit <i>Aphis gossypii</i> in cotton.	
		- Inhibit <i>Pectinophra gossypiella</i> in cotton.	
	Seed oil	- Inhibit <i>Empoasca biguttula</i> in cotton (syn. <i>Amrasca biguttula</i>)	
- Inhibit <i>Phthorimaea operculella</i> in potato.			
- Inhibit <i>Callosobruchus maculatus</i> in pulse.			

Table 1 (continued)

Field	Parts	Use	Reference
Fungicide	Shells	<ul style="list-style-type: none"> - Inhibit <i>Pytophthora pamivora</i> - Inhibit pathogen of durian - Inhibit <i>Colletorichum glorosprioides</i> - Inhibit sporangia - Zoospore growth of <i>p. palmivora</i> - Inhibit conidia production and growth of <i>C. gloeorioides</i> 	Chareonsataporn <i>et al.</i> , 2005
Fuel	Seeds	Biodiesel	Chumphonwong and Sripisut, 2007
Medicine	Bark	Laxative, parasiticide and stomachache cures	Duke <i>et al.</i> , 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 Thymelaeaceae. Phorbolesters are bioactive diterpene derivatives that have a multitude of effects in cells (Goel *et al.*, 2007). The fundamental substance of phorbolester is tiglane (**Figure 3**). Tiglane is tetracyclic diterpene that react with hydroxylation and has hydroxyl (OH) group attach with tiglane 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).

Phorbol esters (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 phorbol esters 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 phorbol ester containing feeds (Goel *et al.*, 2007). Carp (*Cyprinus carpio* L.) were found to be highly susceptible to phorbol esters present in *Jatropha*. The threshold level at which phorbol esters 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 phorbol esters 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. Phorbol esters in *J. curcas* have been quantified by a HPLC method (Makkar *et al.*, 1998).

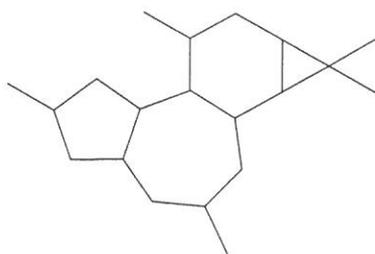


Figure 3 Tiglane

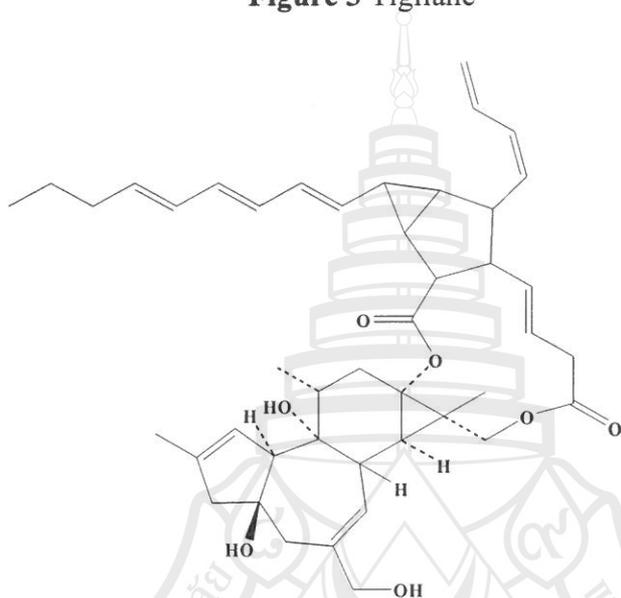


Figure 4 Phorbol esters (12-Deoxy-16-hydroxyphorbol-4'-[12',14'-butadienyl]-6'-[16',18',20'nonatrienyl]-bicyclo[3.1.0]hexane-(13-O)-2'-[carboxylate]-(16-O)-3'-[8'-butenoic-10']ate (DHPB).

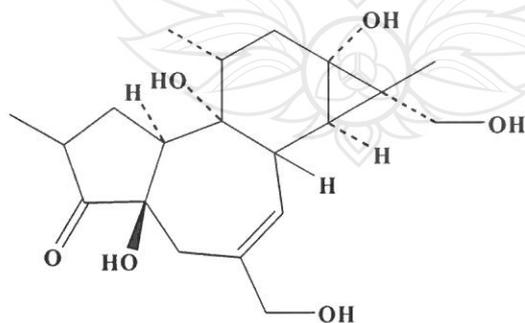


Figure 5 Diterpenoid (12-Deoxy-16-hydroxyphorbol)

High Performance Liquid Chromatography

Reverse-phase high performance liquid chromatography (RP-HPLC) provides high sensitivity for analyzing small amount of composition like phorbolsters. 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. Phorbolsters 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 5 μ m 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 phorbolsters in *Jatropha*. The threshold level in carp at which phorbolsters caused adverse effects was 15 ppm (15 μ g/g) in the diet whereby a level higher than of 31 μ g/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 phorbolsters by using HPLC method. In this project, the method was used for the determination of phorbolsters in *J. curcas* extracts, because HPLC can separate mixture compounds such as phorbolsters 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 pre-concentration. The other fraction was filtered with cotton pads and pre-concentrated 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

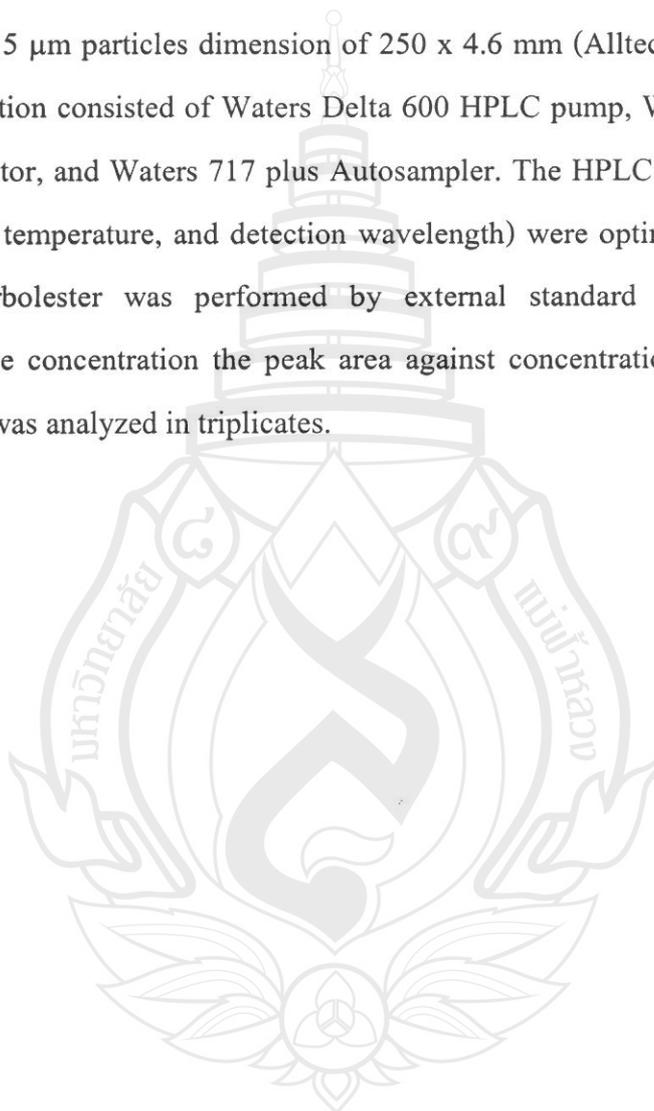
Phorbol-12-myristate-13-acetate (TPA) used as standard stock solution (5,000 ppm), was prepared by dissolving 5 mg of TPA and diluting with 100 % methanol in a 100 mL volumetric flask. The TPA standard stock solution was stored in a refrigerator at a temperature between 2-8 °C.

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 phorbol ester 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, phorbolsters 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 ₃ CN/H ₃ PO ₄ (80:20 v/v)	1.3	280	No	25
2	CH ₃ CN/H ₂ O (80:20 v/v)	1.0	280	No	20
3	CH ₃ CN/H ₃ PO ₄ (80:20 v/v)	1.0	250	No	20
4	100% CH ₃ CN	1.0	254	30	20
5	100% CH ₃ CN	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 found at 10.88 min.

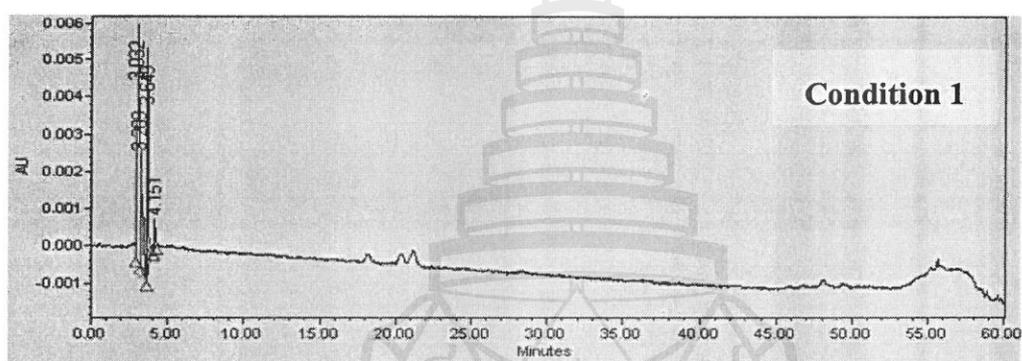


Figure 6 Chromatogram of standard solution containing 500 ppm of TPA,
mobile phase: gradient acetonitrile:85% *o*-phosphoric acid (80:20 v/v)

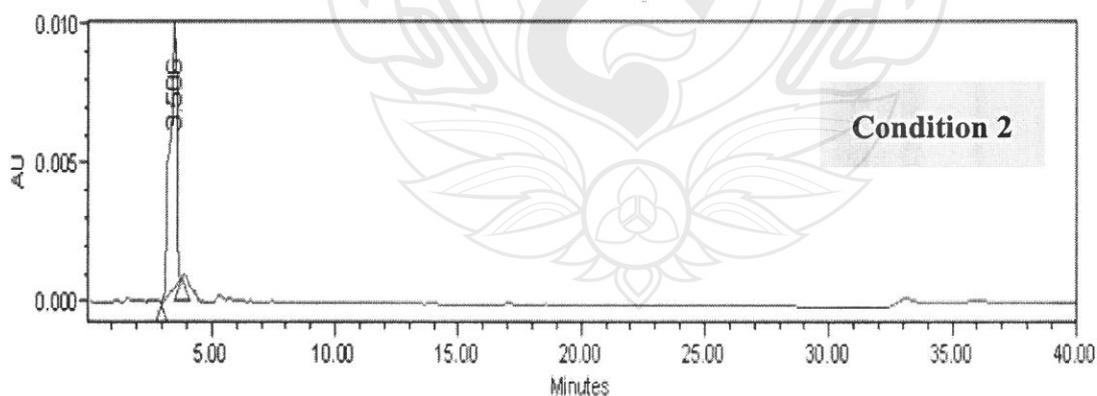


Figure 7 Chromatogram of standard solution containing 500 ppm of TPA,
mobile phase: acetonitrile:water (80:20 v/v)

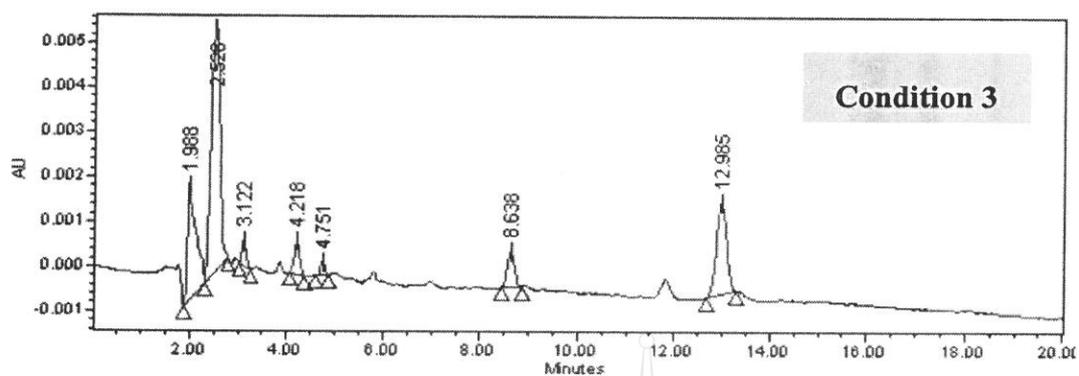


Figure 8 Chromatogram of standard solution containing 500 ppm of TPA,
mobile phase: acetonitrile:85% *o*-phosphoric acid (80:20 v/v)

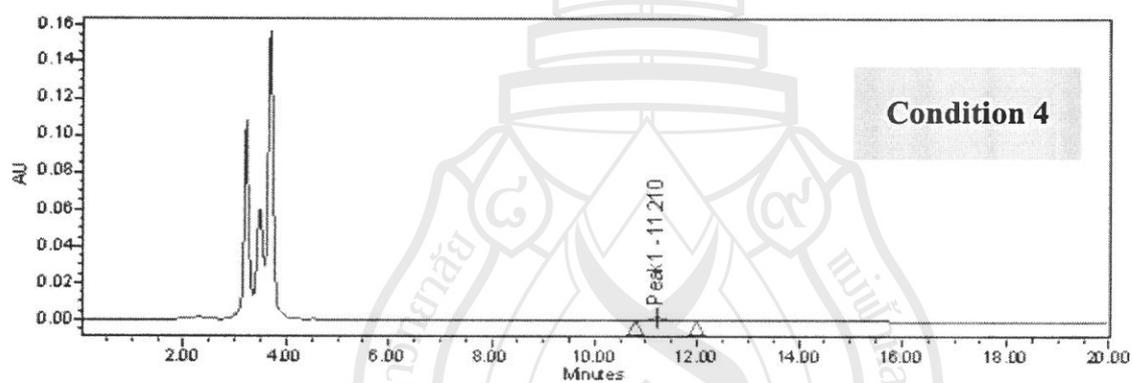


Figure 9 Chromatogram of standard solution containing 500 ppm of TPA,
mobile phase:100% acetonitrile

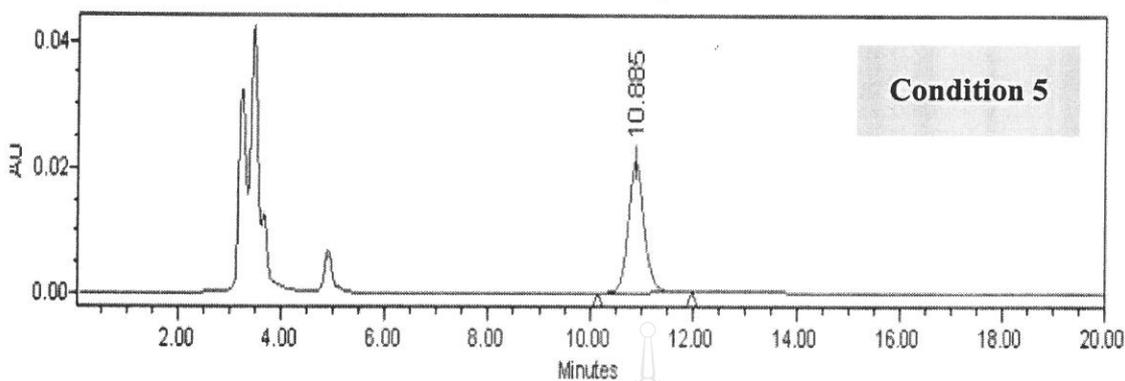


Figure 10 Chromatogram of standard solution containing 500 ppm of TPA,
mobile phase: 100% acetonitrile.

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).

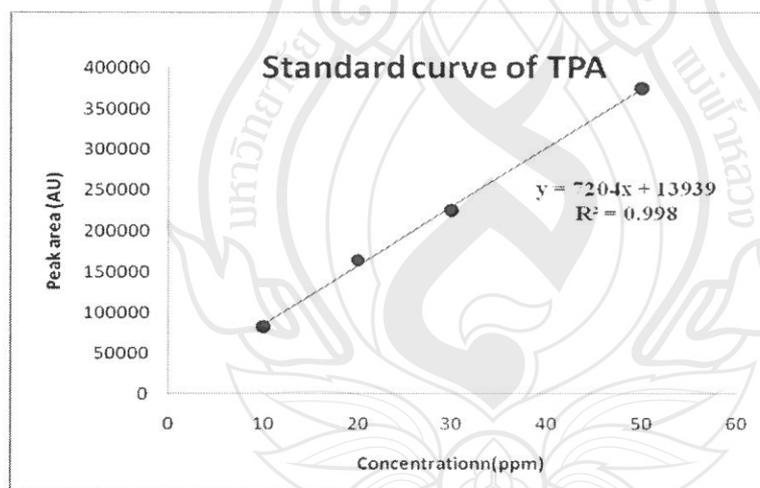


Figure 11 Standard curve of TPA

4.2 Determination of phorbolsters

The HPLC method using the optimum condition was applied to determine phorbolsters 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 phorbolsters. However, the peak nearby might be other forms or isomers of phorbolsters. This is needed to be investigated further.

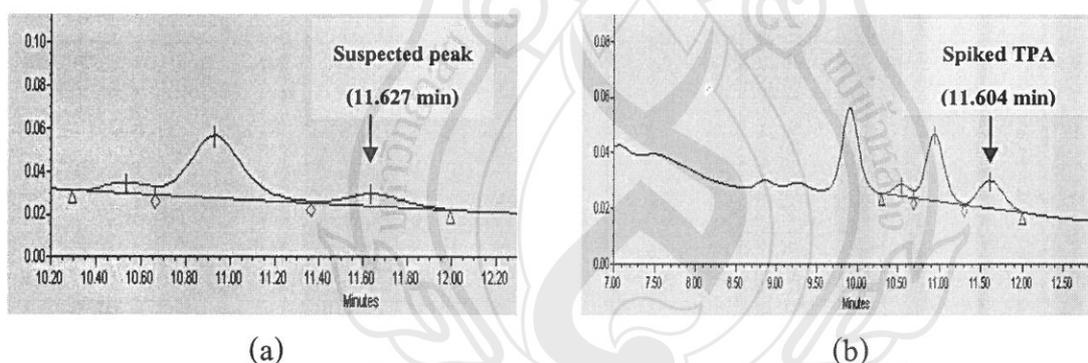


Figure 12 Chromatogram of (a) non spiked samples and (b) TPA standard spiked samples in Pak Chong 32 (Leaf samples)

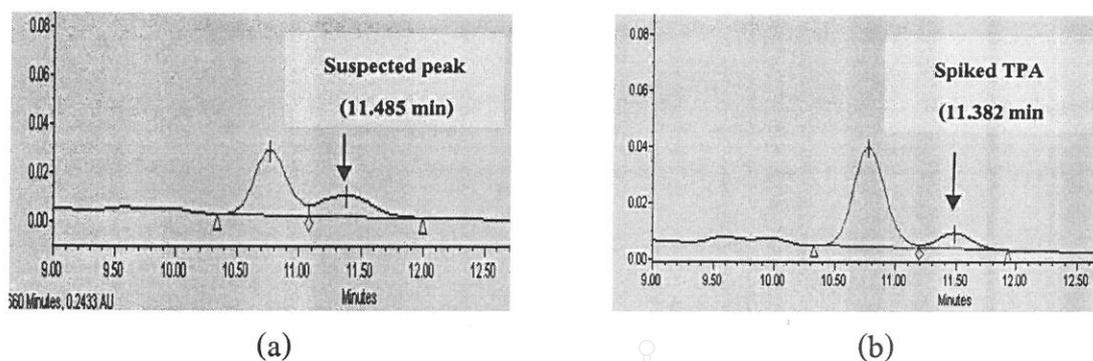


Figure 13 Chromatogram of (a) non spiked samples and (b) TPA standard spiked samples in Korat (Leaf samples)

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

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

Part	Sample	Concentration of phorbolsters (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 phorbolsters 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 evaporation might lead to more concentrated extracts. In addition, more volume of extract used for the LPCE and LKRE samples might dissolve more phorbolsters into the extracts. Thus, concentrations of phorbolsters 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 evaporation process.

Table 5 Recovery of extraction procedure

Part	Sample	Concentration of phorbolsters (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 phorbolsters, 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 phorbolsters concentration was determined by using the peak area of this peak. The concentrations of phorbolsters 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.

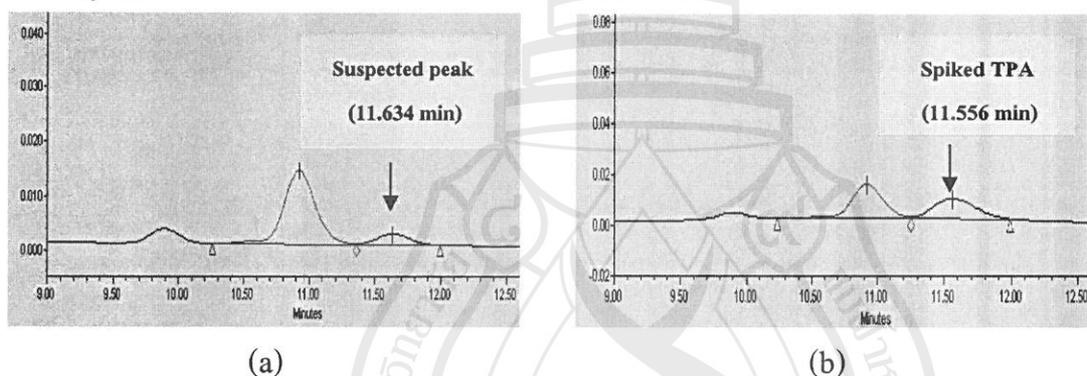


Figure 14 Chromatogram of (a) non spiked samples and (b) TPA standard spiked samples in Pak Chong 32 (Stem samples)

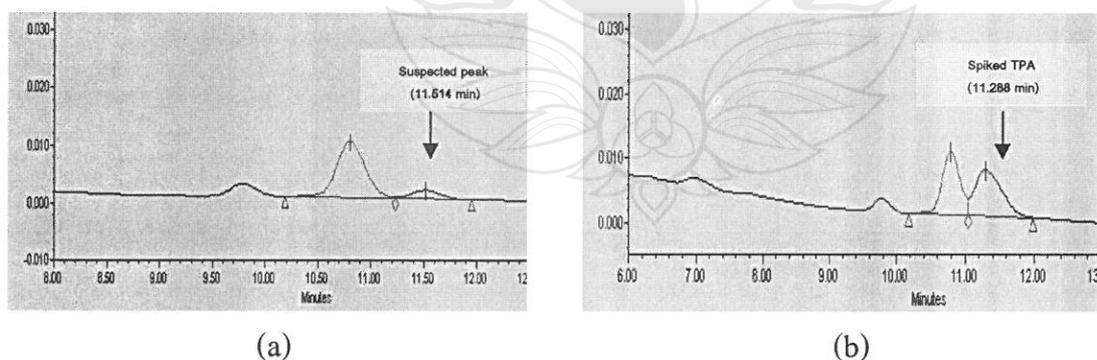


Figure 15 Chromatogram of (a) non spiked samples and (b) TPA standard spiked samples in Korat (Stem samples)

However, from **Table 4**, the phorbol ester 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 phorbol esters may be limited by its concentration level. This might lead to the insignificant difference of phorbol ester concentration in the evaporized and unevaporized stem extracts. The little difference of concentration was assumed to be from the evaporation process.

The phorbol ester 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 phorbol ester (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 phorbol esters 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 phorbol esters 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 phorbol esters in 20 min. It may be because the sample preparation method was not suitable to separate phorbol esters in latex samples. The samples preparation method for analysis of phorbol esters in latex samples should be optimized in further work.

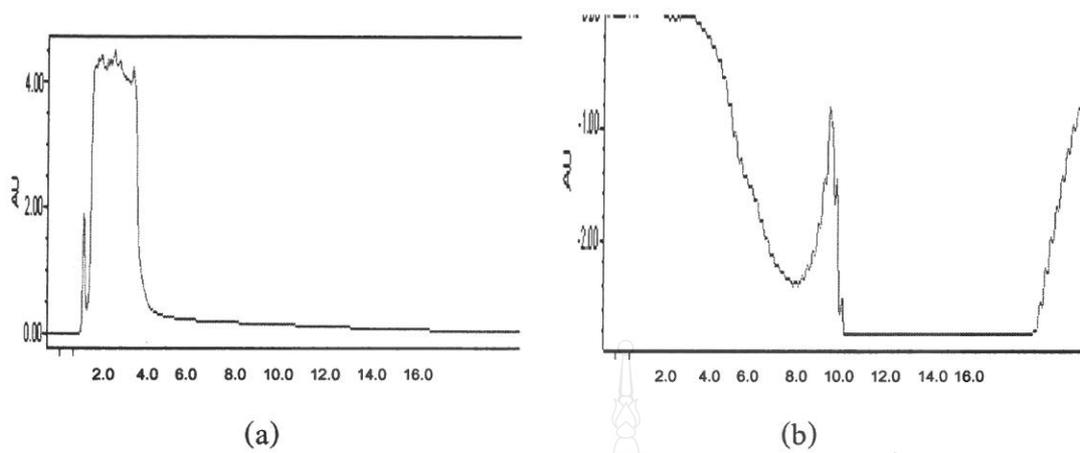
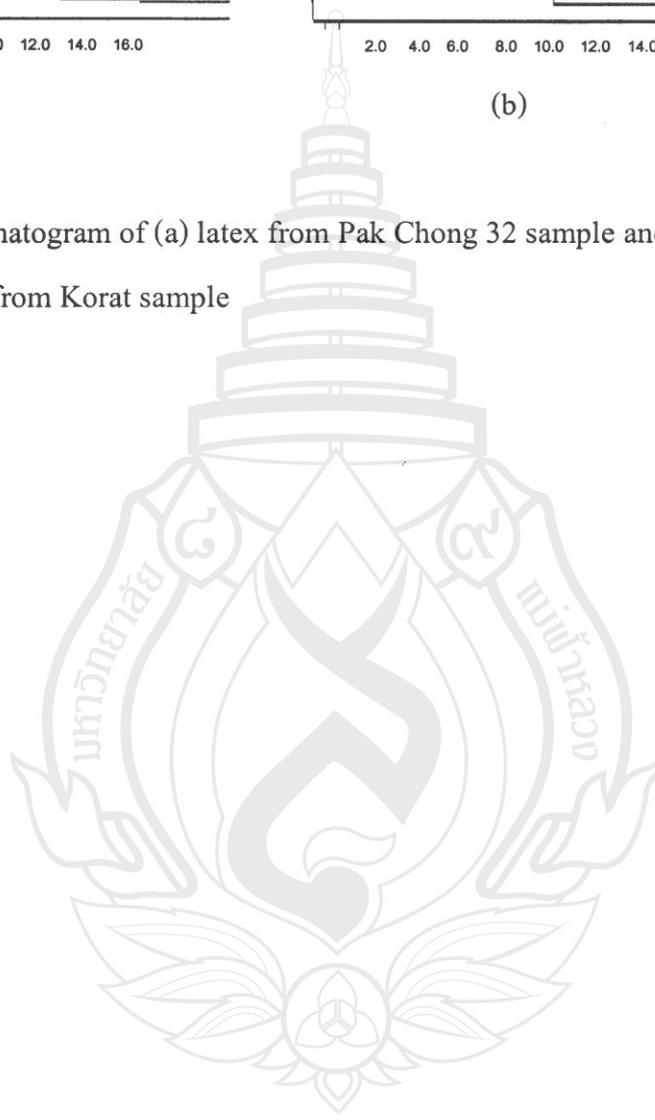


Figure 16 Chromatogram of (a) latex from Pak Chong 32 sample and (b) latex from Korat sample



CHAPTER 5

CONCLUSION

In this experiment, determination of phorbol esters 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 phorbol esters, 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 range of 0.38 % to 3.57 %. The accuracy was tested by determination of recovery. Recoveries for phorbol ester determination in *Jatropha* were found to be ranging from 32.23 to 107.99 %.

The concentration of phorbol esters in the stem samples from Pak Chong 32 and Korat were found at 21.75 and 24.63 ppm, respectively. The phorbol esters 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 phorbol esters 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 phorbol esters. Thus, they should be detoxified before used as animal feed.

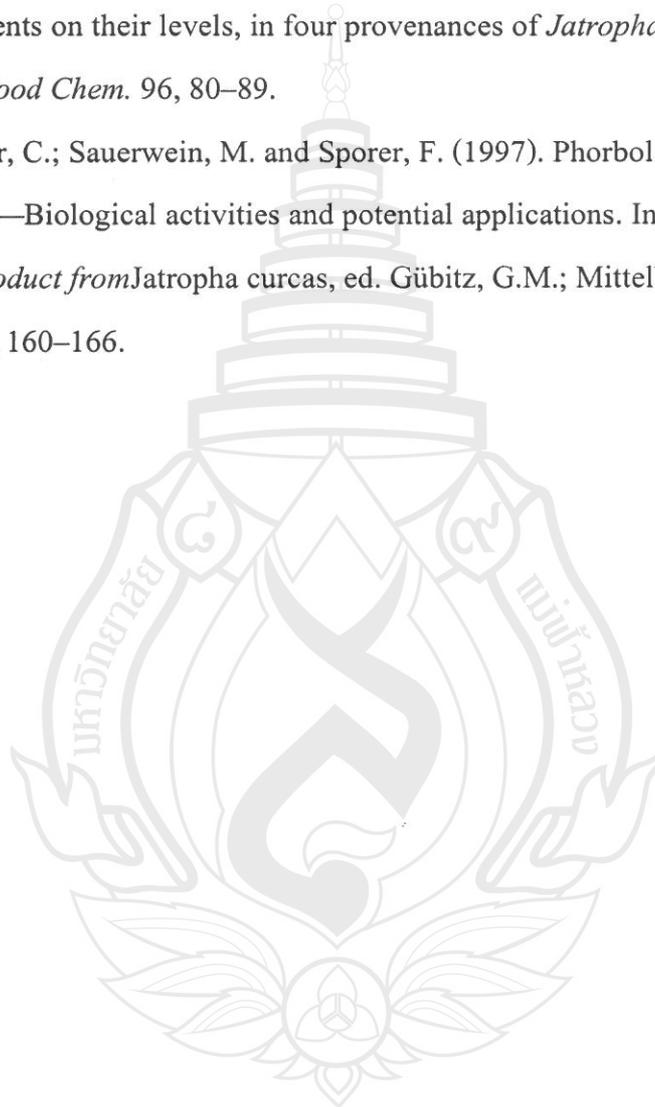
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Education background

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2006	Ph.D.	Organic Chemistry	Prince of Songkla University
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1995-1999 Bachelor of Science in Chemistry. Prince of Songkla University.

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Education background

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1983	B.Sc.	เกษตรศาสตร์	Kasetsart University

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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.
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