

ปริมาณสาร Phorbol-12-myristate-13-acetate (PMA) ในลูกสลอดก่อนและหลังการแปลงสภาพด้วยวิธีการทางการแพทย์แผนไทย

Amount of Phorbol-12-myristate-13-acetate (PMA) in *Croton tiglum* L. Seed Before and After Treatment using Methods of Thai Traditional Medicine

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Received: 15 March 2013 ; Accepted: 5 June 2013

บทคัดย่อ

ลูกสลอดเป็นพืชที่มีฤทธิ์ทางชีวภาพหลายชนิด เช่น ฤทธิ์ต้านมะเร็ง ลดความดันโลหิตสูง คลายกล้ามเนื้อเรียบ ต้านเชื้อแบคทีเรีย เชื้อรา และยีสต์ กระตุ้นระบบภูมิคุ้มกัน ลูกสลอดมีฤทธิ์เป็นยาถ่ายอ่างแรง รวมถึงมีองค์ประกอบทางเคมีบางชนิดที่มีความเป็นพิษ เช่น Phorbol-12-myristate-13-acetate (PMA) ลูกสลอดประกอบให้เป็นยาอันตราย ห้ามใช้ดังแต่ปี พ.ศ. 2519 แต่ก็พบว่าลูกสลอดเป็นส่วนประกอบในตำรับยาแผนไทยหลายตำรับมานานแล้ว ซึ่งลูกสลอดที่นำมาใช้ทางการแพทย์แผนไทยจะต้องผ่านการแปรสภาพเสียก่อน โดยเชื่อว่าการแปรสภาพจะช่วยลดความเป็นพิษและผลข้างเคียงของลูกสลอดได้ การวิจัยในครั้งนี้มีวัตถุประสงค์เพื่อพิสูจน์วิธีการแปรสภาพลูกสลอด 4 วิธี คือ วิธีที่ 1 นำเมล็ดลูกสลอดใส่ในหม้อดิน เติมน้ำเปลือก เกลือ และน้ำ ต้มจนน้ำเปลือกبانทั่วทั่ว กว้าน วิธีที่ 2 นำเมล็ดลูกสลอดผสมกับใบมะขาม ใบส้มป่อย ใส่ในหม้อดิน เติมน้ำและต้มจนเดือด วิธีที่ 3 นำเมล็ดลูกสลอดแช่น้ำปลาปักไว้ 1 คืน และเอายอดใส่ในผลมะกรูด ใส่หม้อดินปิดฝา สูญด้วยไฟแกลง วิธีที่ 4 นำเมล็ดลูกสลอดตำให้หลังเอียด คั่วให้เกรียมจนหมัดน้ำมัน⁽¹⁻³⁾ โดยการเปรียบเทียบปริมาณของ PMA และองค์ประกอบทางเคมีอื่นๆ ในลูกสลอดก่อนและหลังการแปรสภาพ ด้วยวิธีクロมาโทกราฟีของเหลวสมรรถนะสูง (HPLC) ผลการศึกษาพบว่าปริมาณของ PMA ในลูกสลอดก่อนและหลังการแปรสภาพทุกวิธีแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) โดยปริมาณ PMA หลังจากการแปรสภาพด้วยวิธีที่ 1, 3 และ 4 ลดลงร้อยละ 44.47, 7.33, 79.76 ตามลำดับ แต่วิธีที่ 2 ทำให้ปริมาณ PMA เพิ่มขึ้น ร้อยละ 13.91 โดยวิธีที่ 4 เป็นวิธีที่สามารถลดปริมาณ PMA ได้มากที่สุด

คำสำคัญ: phorbol-12-myristate-13-acetate (PMA) การแปรสภาพ การแพทย์แผนไทย ลูกสลอด

Abstract

Croton tiglum Linn has been found to possess a variety of beneficial biological activities including anti-cancer, reduction of blood pressure, smooth muscle relaxant, anti-bacteria, fungi and yeast, and immune stimulation. The seed of *C. tiglum* is a powerful drastic purgative, and some chemical constituents are toxic, such as phorbol-12-myristate-13-acetate (PMA). *C. tiglum* has not been allowed to be used as medicine in Thailand since 1976, however it has been used in many Thai traditional preparations for a long time. The seed of *C. tiglum* must be treated by one of many methods in Thai traditional medicine before use. Those methods claimed to reduce toxicity and side effect of *C. tiglum*. The objective of this report was to scientifically prove 4 treatment methods of *C. tiglum*. Method 1: The *C. tiglum* seeds were mixed with unpeeled rice and salt then boiled in a clay pot. Method 2: The *C. tiglum* seeds were

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mixed with tamarind leaf and *Acacia concinna* leaf then boiled in a clay pot. Method 3: The *C. tiglum* seeds were soaked in fermented fish sauce (Nam-pla-raa) overnight after that the seeds were put in the Leech lime fruits and boiled in a clay pot. Method 4: The *C. tiglum* seeds were ground to fine powder and roasted in a pan.⁽¹⁻³⁾ The amounts of PMA in *C. tiglum* before and after treatment by 4 methods were determined by HPLC and compared. Results showed that amounts of PMA in the *C. tiglum* seeds before and after treatment in all methods were significantly different ($P<0.05$). The amount of PMA after treatment by method 1, 3 and 4 were decreased 44.47, 7.33, 79.76 %, respectively but that treated by method 2 was increased (13.91%). Method 4 showed the most decrease in the amount of PMA.

Keywords: phorbol-12-myristate-13-acetate (PMA), treatment, Thai traditional medicine, *Croton tiglum*

Introduction

The *Croton tiglum* Linn, a member of family Euphorbiaceae is commonly known as Purging Croton. It is a small tree fifteen to twenty feet high.⁴⁻⁷ This plant has been found to possess a variety of biological activities including anti-cancer, hypertension reduction, smooth muscle relaxant, anti-bacteria, fungi and yeast, and immune stimulation. The seeds of *C. tiglum* are the most powerful drastic purgative.

Many types of compounds were isolated from *C. tiglum* which is composed of oil (30-56%), moisture (6.29%), total ash (3.6%), crude protein (16.15%), fiber (8.25%), and carbohydrate (16.15%). There are many fatty acids including oleic acid, linoleic acid, stearic acid, palmitic acid, myristic acid, lauric acid. The oleic acid and linoleic acid are major fatty acids.⁸⁻¹⁰ Many enzymes and amino acids such as lipase, invertase, amylase, raffinase, proteolytic enzyme, globulin, albumin, arginine and lysine also were found. Other compounds can be found in this plant such as alkaloid ricinine, resin, tiglic acid, oenanthrallic, capronic, valerianic, butyric, isobutyric, acetic, formic acids and tannin. Some compounds of this plant are toxic to many human organs such as skin irritation, carcinogen, promoter tumor and strong laxative. They were identified as in the diterpene ester groups such as phorbol and phorbol derivatives including phorbol-12-myristate-13-acetate (PMA).¹¹⁻¹⁵

Because it is this toxic, the *C. tiglum* has not been allowed in Thailand since 1976.¹⁶ However, it was found that *C. tiglum* was used in many recipes of the Thai traditional texts. From the knowledge of a Thai traditional

doctor, the *C. tiglum* must be treated by some process of Thai traditional medicine such as heating or acid fermentation before use. They call this method as "Sa-tu" or "Kha-rith". This study was to investigate the changing of chemical compounds especially phorbol-12-myristate-13-acetate (PMA) (Figure 1) in *C. tiglum* seeds before and after treatment by 4 different processes.

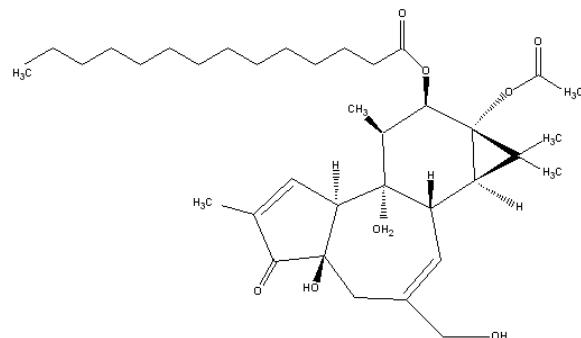


Figure 1 The chemical structure of phorbol-12-myristate-13-acetate (PMA)

Materials and methods

Chemicals

Phorbol-12-myristate-13-acetate (PMA) was purchased from Sigma, methanol (HPLC grade) from CARLO ERBA, methanol (AR grade) from MERCK and trifluoroacetic acid from ACROS.

Plant materials

C. tiglum seeds were collected from i-pun district, Phrasang, Suratthani province and voucher specimen (CT001) was deposited at the Faculty of Pharmacy, Mahasarakham University.



Figure 2 *Croton tiglium* seeds

Treatment method of *C. tiglium* seed

Method 1: The *C. tiglium* seeds 25 g were mixed with unpeeled rice 125 g and salt 50 g then boiled in the clay pot for 5 hr, gently agitating every 15 min, dried at 45°C before extraction.

Method 2: The *C. tiglium* seeds 25 g were mixed with tamarind leaf 50 g and *Acacia concinna* leaf 50 g in 1,000 ml of DI water and boiled for 2 hr in the clay pot, gently agitating every 15 min, dried at 45°C before extraction.

Method 3: The *C. tiglium* seeds were soaked in the fermented fish sauce (Nam-pla-raa) overnight followed by putting in the Leech lime fruits and boiled in the clay pot for 5 hr, gently agitating every 15 min, dried at 45°C before extraction.

Method 4: The *C. tiglium* seeds 25 g were ground to fine powder and roasted in the pan for 40 min.

Extraction of *C. tiglium*

The seed of *C. tiglium* both before and after treatment by 4 methods were extracted in the same manner. The seed of *C. tiglium* was ground to powder. An amount of 25 g of the powder was added to volumetric flask followed by adding methanol 150 ml and sonicated for 30 min. The solution was filtered and the filtrate was evaporated to dryness to get a crude extract.

HPLC analysis of *C. tiglium*

The *C. tiglium* extracts collected from before and after treatment by methods of Thai traditional medicine were dissolved in HPLC grade methanol and subjected to HPLC for qualitative and quantitative analysis of PMA and other compounds. The HPLC system consist of Shimadzu LC-10A which was equipped with photodiode

array detector (Shimadzu SPD-M 20 A), Phenomenex Column (RP, Kromasil 5u 100A C-18, 250x4.60 mm), Guard column (Phenomenex®) and data were integrated by Shimadzu Class VP series software. Separation was achieved with a two pump linear gradient program for pump A (Water containing 0.05% trifluoroacetic acid) and pump B (Methanol). Initially starting with a gradient of 40% B and then changing to 50, 75, 90, 90, 100 and 100 % at 0, 10, 30, 60, 70, 90 and 120 min. The flow rate was 1 ml/min and determined at wave length 254 nm. Results were obtained by comparison of peak areas of the samples with the calibration curve of referent standard. Every process was repeated 3 times.

Validation method

Linearity

The following concentration of PMA 0.07, 0.15, 0.31, 0.62, 1.25, 2.50 and 5.00 mg/ml were prepared and analysis by HPLC. Graph between concentration of the PMA and area under curve was plotted and calculated for linear regression.

Accuracy and precision

The PMA concentration of 0.156, 0.625 and 2.5 mg/ml was analysis by HPLC for intraday and interday precision. The percent recovery and relative standard deviation were calculated.

Limit of detection and limit of quantitation

The 0.07 mg/ml PMA was dilute and analysis by HPLC step by step to the concentration that could not be detected. The limit of detection and limit of quantitation were three times and ten times higher than the lowest concentration that could be detected, respectively.

Statistical analysis

Statistical analysis, one way-ANOVA was applied followed by Newman Keuls Multiple Comparison test. Experimental results were given as mean \pm SEM to show variations in groups. Differences were considered statistically significant at the value of probability less than 5% ($p < 0.05$).

Results and discussion

The *C. tiglum* extract before treatment gave dark yellow oil (11.56 % w/w dried seed) as same as the extract treated by methods 1-3 while the extract treated by method 4 gave dark brown oil. The amount of extract treated by method 1-4 were 9.16, 10.04, 12.24, and 11.88 % w/w dried seed, respectively.

This HPLC analysis method was accepted for determination of PMA in *C. tiglum* which its validation method showed the coefficient of determination (R^2) 0.9998 (Figure 3) and the percent recovery between 95-105 %. The HPLC chromatogram of referent standard (PMA) was showed in the Figure 2. The HPLC chromatogram of *C. tiglum* extract before and after treatment by 4 different methods were showed in the Figure 3-4. The retention time of PMA appeared at 71.25 min. The position of PMA in HPLC chromatogram of extract was identified by comparison of retention time and UV spectrum of PMA including spiking. The amount of PMA in *C. tiglum* extract was determined by comparison of the area under curve with calibration curve of PMA. The amounts of PMA in *C. tiglum* extract before treatment was 57.57 ± 0.99 $\mu\text{g/g}$ and after treatment by method 1-4 were 31.97 ± 1.07 , 65.58 ± 1.74 , 53.35 ± 1.11 and 11.65 ± 2.66 $\mu\text{g/g}$, respectively (Table 1).

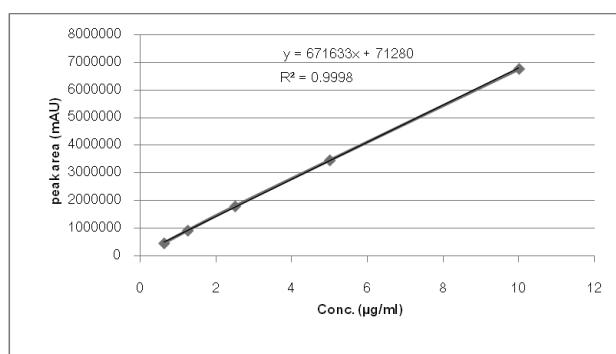


Figure 3 Calibration curve of a PMA

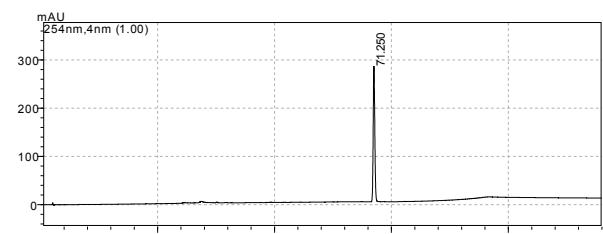


Figure 4 HPLC chromatogram of a PMA

The amounts of PMA in the *C. tiglum* seeds before and after treatment by 4 methods showed significantly different ($p < 0.05$). The amounts of PMA in *C. tiglum* obtained from most methods were decreased after treatment except method 2 which was increased (Table 1). Method 4 showed the most decrease of PMA (79.76%) followed by method 1 (44.47%) and method 3 (7.33%), respectively. In contrast, the amounts of PMA in *C. tiglum* after treatment by method 2 was increased (13.91%). The changing of chemical compounds in the *C. tiglum* seeds after treatment might be from the effects of heating, reaction with acid or base, size of sample and duration of treatment. The method 1 was treated by heating only, method 2 by heating and reaction with acid, method 3 by heating and reaction with base and method 4 by heating and small size of sample. In this study, the effect of heating and small size of sample in method 4 showed as the most powerful for reducing amount of PMA. However, treatment by reducing of *C. tiglum* seeds to small size and direct heat in method 4 might be not suitable for treatment of *C. tiglum* because other chemical compounds might be decreased as well. In the case of treatment by heating and reaction with acid, the amount of PMA was not decreased but increased. Method 3 which was macerated in the base condition and followed by heating was correspond to the study of Mantana Kaewma¹⁷ on reducing of phorbol ester in *Jatropha curcas*. The result shows that soaking *J. curcas* with NaOH and heat by autoclave could reduce phorbol ester to 80.16%. The study of detoxifications of phorbol ester by changing some conditions such as acid-base, temperature, bleaching, refining, method of extraction, solvent extraction also found in many reports.^{8, 14, 17-20} Decreasing of PMA in *C. tiglum* might be from base hydrolysis reaction of phorbol ester (PMA) to phorbol alcohol and form a new esterification with other compounds in *C. tiglum*. This hypothesis was confirmed by previous report which studied the effect of hydrolysis of phorbol ester by NaOH and HClO_4 .²¹ The result showed that only hydrolysis by NaOH was successful.

Surprisingly, a peak appeared at the retention time about 19.2 min in all HPLC chromatogram of *C. tiglum* after treatment. This compound might have occurred from some interaction between phorbol derivatives

and other chemical compounds in *C. tiglium*. It needs further isolation and purification of this peak to clarify the chemical compound and mechanism of this occurring.

In conclusion, method 1, 3 and 4 which were treated by heating or heating together with base reaction could reduce PMA in *C. tiglium* seed, which support the treatment by method of traditional medicine. For method

2, treatment by acid reaction followed by heating could not reduce PMA. It needs further study to clarify.

Acknowledgement

The authors wish to express their gratitude to the Faculty of Pharmacy, Mahasarakham University for supporting the study.

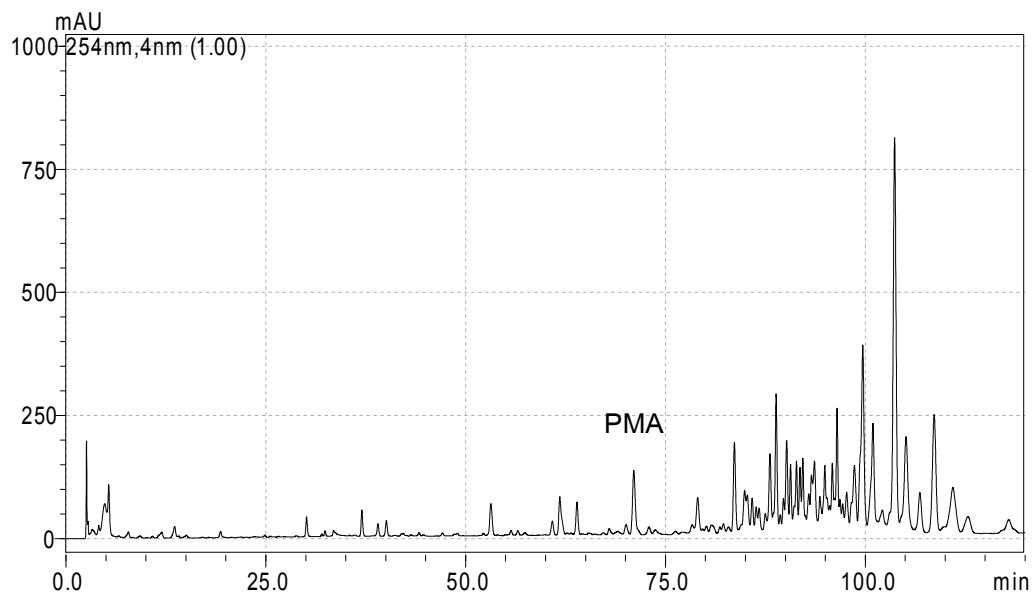


Figure 4 HPLC chromatogram of the *C. tiglium* seeds extract before treatment

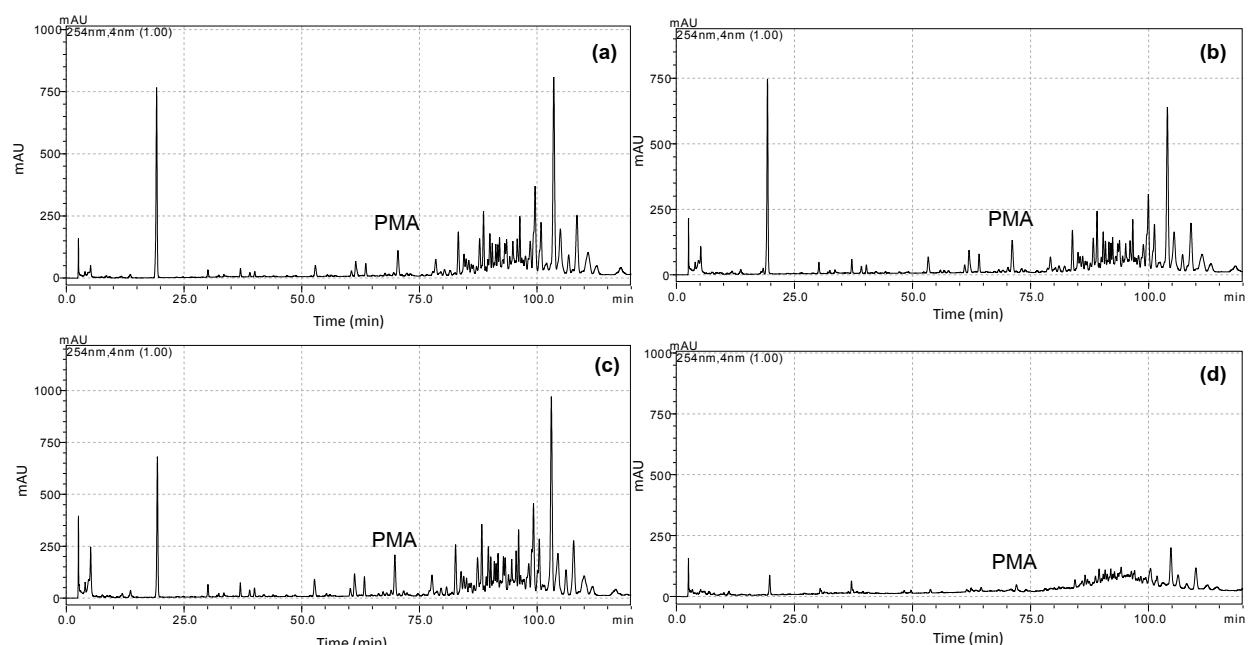


Figure 5 HPLC chromatogram of the *C. tiglium* seeds extract after treatment (a) Method 1 (b) Method 2 (c) Method 3 (d) Method 4

Table 1 The amount of PMA in *C. tiglium* seed extracts before and after treatment by 4 methods of Thai traditional medicine

Method	Amounts of PMA		
	µg/g	% decrease	% increase
Before treatment	57.57±0.99	-	-
After treatment with			
Method 1	31.97±1.07	44.47	-
Method 2	65.58±1.74	-	13.91
Method 3	53.35±1.11	7.33	-
Method 4	11.65±2.66	79.76	-

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