PHARMACOGNOSTIC SPECIFICATION AND DIOSCORINE CONTENT OF *DIOSCOREA HISPIDA* TUBERS

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ข้อกำหนดทางเภสัชเวทและปริมาณสารไดออสโครีนของหัวกลอย

นางสาวนงลภัส ศศิวัจน์ไพสิฐ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	PHARMACOGNOSTIC SPECIFICATION AND DIOSCORINE CONTENT OF *DIOSCOREA HISPIDA* TUBERS
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Field of Study	Public Health Sciences
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นงลภัส ศศิวัจน์ไพสิฐ : ข้อกำหนดทางเภสัชเวทและปริมาณสารไดออสโครีนของหัวกลอย . (Pharmacognostic Specification and Dioscorine Content of *Dioscorea hispida* Tubers) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : อ. ดร. ชนิดา พลานุเวช , อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร. นิจศิริ เรืองรังษี, 98 หน้า.

กลอย มีชื่อทางวิทยาศาสตร์ว่า *Dioscorea hispida* Dennst. หัวกลอยแห้งเป็นเครื่องยาสมุนไพรในตำรับยาธรณีสันฑะฆาตที่มีสรรพคุณรักษาอาการกระษัยเส้น เถาดาน (อาการแข็งเป็นลำในท้อง) และท้องผูก เนื่องจากกลอยยังไม่มีข้อกำหนดมาตรฐานในตำรามาตรฐานยาสมุนไพรไทย อีกทั้งข้อมูลทางเภสัชวิทยา และพิษวิทยาของหัวกลอยพบว่ามีสารพิษคือไดออสโครีน ซึ่งเป็นสารมีฤทธิ์กระตุ้นระบบประสาทส่วนกลาง การศึกษานี้จึงมีวัตถุประสงค์เพื่อจัดทำข้อกำหนดทางเภสัชเวทรวมทั้งวิเคราะห์หาปริมาณสารไดออสโครีนของหัวกลอย โดยเก็บหัวกลอยจาก 14 แหล่งทั่วประเทศไทย วาดภาพลายเส้นแสดงลักษณะทางพฤกษศาสตร์ของพืชสมุนไพรกลอย นำหัวกลอยมาฝานเป็นแผ่นบาง ตากแดดให้แห้ง ตามการเตรียมเครื่องยากลอย ลักษณะทางมหภาคของเครื่องยามีรูปร่างเป็นแท่งยาวหรือรูปทรงต่าง ๆ สีนวล ขอบสีน้ำตาลอ่อน ลักษณะเด่นทางจุลภาคของ หัวกลอยคือ เม็ดแป้งและผลึกรูปเข็ม การศึกษาเอกลักษณ์ทางเคมี-ฟิสิกส์ของหัวกลอย พบว่า มีปริมาณเถ้ารวม เถ้าที่ไม่ละลายในกรด ความชื้น และปริมาณน้ำ ไม่เกินร้อยละ 3.44, 0.92, 11.50 และ 11.55 โดยน้ำหนัก ตามลำดับ ปริมาณสารสกัดด้วยเอทานอล และปริมาณสารสกัดด้วยน้ำไม่น้อยกว่าร้อยละ 3.00 และ 15.07 โดยน้ำหนัก ตามลำดับ วิเคราะห์ปริมาณไดออสโครีนโดยวิธีทีแอลซีร่วมกับการวิเคราะห์เชิงภาพเปรียบเทียบกับวิธีทีแอลซีเดนซิโทเมทรี เตรียมสารมาตรฐานไดออสโครีนโดยการสกัดหัวกลอยแห้งด้วยเอทานอล ตกผลึกด้วยกรดพิคริก สกัดกลับ และทำให้บริสุทธิ์ โดยวิธีโครมาโทกราฟีแบบคอลัมน์ ตรวจยืนยันโดยวิธีโปรตอน และคาร์บอนนิวเคลียร์แมกเนติกเรโซแนนซ์ สกัดหัวกลอยแห้งทั้ง 14 แหล่งด้วยเอทานอล โดยวิธีสกัดแบบซอกห์เลต นำสารสกัดที่ได้ไปวิเคราะห์หาปริมาณสารไดออสโครีนโดยวิธี TLC เฟสคงที่เป็นอะลูมิเนียมที่ฉาบด้วยอะลูมิเนียม ออกไซด์ ใช้ตัวทำละลาย เมทานอลต่อคลอโรฟอร์ม (97 : 3) เป็น เฟสเคลื่อนที่ บันทึกภาพสารไดออสโครีนภายใต้แสงอัลตราไวโอเลตที่ความยาวคลื่น 254 นาโนเมตร พบว่ามีค่า hRf เท่ากับ 80 วัดปริมาณสารโดยใช้โปรแกรม Scion Image ใน TLC หนึ่งแผ่นประกอบด้วยสารมาตรฐานไดออสโครีน 5 ความเข้มข้น และสารสกัดตัวอย่างหัวกลอยจาก 14 แหล่ง แต่ละแหล่งทำ 3 ซ้ำ วิธีทีแอลซีเดนซิโทเมทรีทำเช่นเดียวกันโดยใช้เครื่อง Camag Linomat syringe และ Camag TLC scanner ร่วมกับ winCATS software ในการดำเนินการแทน พบปริมาณสารไดออสโครีนร้อยละ 0.66 และ 0.72 โดยน้ำหนัก เมื่อวิเคราะห์ด้วยวิธี ทั้งสอง ตามลำดับ วิธีวิเคราะห์สาร ไดออสโครีนทั้งสองวิธี มีค่าสัมประสิทธิ์สหสัมพันธ์เท่ากับ 0.999 ใน ช่วงความเป็นเส้นตรงระหว่าง 2.50 – 12.50 ไมโครกรัมต่อหนึ่งจุด ขีดจำกัดของการตรวจพบและขีดจำกัดของการหาปริมาณของสาร ไดออสโครีนมีค่า 0.28 และ 0.84 ไมโครกรัมต่อหนึ่งจุด เมื่อวิเคราะห์ด้วยวิธีทีแอลซีร่วมกับการวิเคราะห์เชิงภาพ และ 0.37 และ 1.13 ไมโครกรัมต่อหนึ่งจุด เมื่อวิเคราะห์ด้วยวิธีทีแอลซีเดนซิโทเมทรี ตามลำดับ ผลการศึกษาครั้งนี้สามารถจัดทำเป็นข้อกำหนดมาตรฐานของ เครื่องยาสมุนไพรกลอย ซึ่งจะเป็นประโยชน์ในการควบคุมคุณภาพวัตถุดิบ ตลอดจนการวิจัยและพัฒนาตำรับยาที่เข้าตัวยาต่อไป

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต.....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5379308153 : MAJOR PUBLIC HEALTH SCIENCES
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NONGLAPAT SASIWATPAISIT : PHARMACOGNOSTIC SPECIFICATION AND DIOSCORINE CONTENT OF *DIOSCOREA HISPIDA* TUBERS. ADVISOR : CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR : ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 98 pp

Dioscorea hispida Dennst. dried tubers have been used as a crude drug in Thai remedy named Thoraneesanthakhat. It has traditionally been used to treat constipation. The aim of this research is to establish the pharmacognostic specification and determine the content of dioscorine in *D. hispida* tubers. The tubers were collected from 14 different locations throughout Thailand. The drawing of whole plant of *D. hispida* was illustrated in detail. The crude drug was traditionally prepared by slicing the tuber and sun drying. The macroscopic characters were longitudinal pieces or irregularly shaped, off - white colour with some light brown epidermis. The prominent anatomical and histological characteristics were starch granules and raphide crystal. The total ash, acid insoluble ash, loss on drying and water content should be not more than 3.44, 0.92, 11.50 and 11.55 % w/w respectively whereas ethanol-soluble extractive and water-soluble extractive should be not less than 3.00 and 15.07 % w/w respectively. The content of dioscorine in *D. hispida* dried tubers was identified using TLC image analysis compared to TLC-densitometry. The standard dioscorine was prepared from dried tubers by ethanol extraction, picrate crystallization, back extraction and column chromatographic purification. The identification of isolated dioscorine was confirmed by ^{1}H and ^{13}C NMR spectra as well as previously reported spectra prior to be used as dioscorine standard. Dried tuber samples were successively extracted in ethanol by soxhlet apparatus. The extracts were analyzed for dioscorine content by TLC using Aluminium oxide 60 GF_{254} neutral as stationary phase and methanol-chloroform (3 : 97) as mobile phase. The density of dioscorine spot at hRf value of 80 detected under UV254 was analyzed and transformed to peak area by the Scion Image software. Five concentrations of standard and 14 samples were developed on the same TLC plate. Each sample was quantitated in triplicate. For TLC-densitometry, the same protocol was performed using Camag Linomat syringe and Camag TLC scanner with winCATS software instead manual. The dioscorine content of the dried crude drug determined by TLC image analysis and TLC-densitometry were 0.66 and 0.72 % w/w respectively. The polynomial regresstion data of both methods for dioscorine showed good linear relationship with a correlation coefficient of 0.999 in the concentration range of 2.50 – 12.50 µg/spot. The LOD and LOQ were 0.28 and 0.84 µg/spot by TLC image analysis and 0.37 and 1.13 µg/spot by TLC-densitometry respectively. TLC image analysis was valid for quantification of dioscorine in *D. hispida* tuber. This study provided scientific information for the quality control of *D. hispida* tuber ingredient in Thai traditional medicine.

Field of Study : Public Health Sciences ..... Student's Signature .....

Academic Year : 2011 ..... Advisor's Signature .....

Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

AOAC	=	Association of Official Analytical Chemists
°C	=	Degree Celsius
$CDCl_3$	=	Deuterated chloroform
cm	=	Centimeter
^{13}C-NMR	=	Carbon – thirteen nuclear magnetic resonance
D.	=	*Dioscorea*
g	=	Gram
HCl	=	Hydrochloric acid
HPLC	=	High performance liquid chromatography
HPTLC	=	High performance thin layer chromatography
hr	=	Hour
^{1}H-NMR	=	Proton nuclear magnetic resonance
IUPAC	=	International Union of Pure and Applied Chemistry
kg	=	Kilogram
l	=	Liter
LC	=	Liquid chromatography
LD_{50}	=	Lethal dose 50%
LOD	=	Limit of detection
LOQ	=	Limit of quantification
mg	=	Milligram
MHz	=	Megahertz
min	=	Minute
ml	=	Millilitre

mm	=	Millimetre
MS	=	Mass spectrometry
N	=	Normality
ng	=	Nanogram
nm	=	Nanometre
NMR	=	Nuclear magnetic resonance
ppm	=	Parts per million
r^2	=	Correlation coefficients
RSD	=	Relative standard deviation
SD	=	Standard deviation
spp.	=	Species
TLC	=	Thin layer chromatography
UV	=	Ultraviolet
var.	=	Variety
v/v	=	Volume by volume
w/w	=	Weight by weight
%	=	Percent
µg	=	Microgram
µl	=	Microliter
µm	=	Micrometre
δ	=	Chemical shift
α	=	Alpha
β	=	Beta
Δ	=	Delta

CHAPTER I
INTRODUCTION

Background and significance of the study

The World Health Organization express that approximate 85 to 90% of the world's population consumes traditional herbal medicines, while the herbal drug industry has been in high growth due to the growing demand in developing and developed countries [1]. The plant biodiversity has served as the foundation for the development of many traditional system of medicine. Pharmacognosy basically deals with the authentication and standardization. In recent years there has been a rapid increase in the standardization of selected medicinal plants of potential therapeutic significance [2]. Standardization is an essential requirement for the whole plant, plant parts or extracts in order to assess the quality of drugs [3 – 4].

Yams (*Dioscorea* spp.) belong to Dioscoreaceae family. They are climbing plants with a spiny grayish – green stem. Approximately 600 species are eaten in various parts of the world [5]. Of 59 species recorded from Southeast Asia, 8 are known to be bitter and/or poisonous if eaten raw. The local people prepare the tubers before consumption to make them edible [6].

Dioscorea hispida Dennst., commonly known as wild yam or Kloi in Thai , is a staple subsistence food in some tropical regions of the world [7 – 8]. It is one of medicinal plants in Thailand for a long time. Its dried tuber has been used as a crude drug in Thai remedy named Thoraneesanthakhat. It has traditionally been used to treat constipation. The phytochemical investigation of this plant reveals the presence of poisonous alkaloid, dioscorine [9 – 11] which exhibits mydriatic activity, hyperthermia and central nervous system stimulation. The latter effect causes a nervous system paralyze [6, 12].

A review of literatures demonstrates that no pharmacognostic specification has been recorded for this crude drug. Thus the present investigation has been undertaken

with an objective to establish pharmacognostic specification of *D. hispida* dried tubers. The quantitative determination of toxic dioscorine in the tuber crude drug is deeply necessary for standardization and quality control of this medicinal plant material. Thin layer chromatography coupled with image analysis free software can be applied as inexpensive technique for dioscorine content investigation.

Objectives of the study

1. To establish the pharmacognostic specification of *D. hispida* tubers crude drug.
2. To establish the TLC method using image analysis free software for quantification of dioscorine in *D. hispida* tuber crude drug.

Expected benefits

1. This research provides the pharmacognostic specification of *D. hispida* tuber crude drug.
2. This research provides the content variation of dioscorine in *D. hispida* tuber crude drug.
3. This research provides the application of image analysis free software for the quantitative TLC analysis.

CHAPTER II
LITERATURE REVIEWS

Names and synonyms of plant materials

Dioscorea is a genus of the plant family Dioscoreaceae which contains more over 600 different species, of which 25 are edible. In Thailand, the medicinal plant is *Dioscorea hispida* Dennst. (*D. triphylla* Linn. var. *reticulata* Prain and Burkill [13]) also known by the colloquial name of Kloi (general), Mun Kloi or Kloi Hua Neaw (Nakhon Si Thammarat), Kloi Nok or Koi (Northern provinces), Klee (Karen-Mae Hong son). Other names are Wild yam or Asiatic bitter yam (common name), Gado(e)ng or Gadong mabok (Melayu), Maranpash poll, Palidumpa and Pashpoli (Indian) [14 – 16].

Botanical description of *D. hispida* Dennst.

Tubers brown, ovoid or irregularly shaped, variable in size, poisonous; transverse section white. Stem twining, to 30 cm, terete, stout, pubescent when young, glabrescent, prickly. Leaves alternate, palmately 3-foliolate; petiole to 30 cm, hairy; middle leaflet ± ovate to elliptic, 6-12(-17.5) × 4-12 cm, adaxially sparsely hispid, glabrescent, abaxially hispid, palmately veined, apex acuminate; lateral leaflets ovate-elliptic or nearly broadly oblong, oblique, smaller than middle leaflet, margin entire. Male spikes in axillary panicles to 50 cm with 2 levels of branching, most parts densely tomentose. Male flowers: in dense clusters; perianth ca. 1 mm, outer lobes smaller and thinner than inner ones; stamens 6. Female spike solitary, to 40 cm. Capsule long ellipsoid, 3.5 – 7 cm, leathery, densely pubescent; wings 1.2 – 1.5 cm wide. Seeds inserted near apex of capsule; wing pointing toward capsule base [17].

Figure 1 The whole plant of *Dioscorea hispida* Dennst.

Chemical constituents

D. hispida tuber contains carbohydrate, protein, lipid and other substances e.g. inhibitor of amylase, oxalate, phytate, anthocyanin, carotenoid compounds that taste bitter and a poisonous alkaloid, dioscorine [7].

Nutritive analysis

D. hispida, the edible starchy tubers , are of cultural, economic and nutritional importance in the tropical and subtropical regions of the world [18]. They are reported as good sources of essential dietary nutrients [19 – 25].

The nutritive composition in100 g of *D. hispida* tuber is shown in Table 1 – 2.

Table 1 Proximate composition of *Dioscorea hispida* (edible portion) in Thailand [7]

Composition	Unit	Fresh wild yam	Dried wild yam
Moisture	g / 100 g	75.80	12.60
Lipid	g / 100 g	0.30	0.40
Carbohydrate	g / 100 g	21.10	80.30
Fibers	g / 100 g	0.70	2.20
Protein	g / 100 g	2.20	5.70
Calcium	mg / 100 g	22.00	140.00
Phosphorus	mg / 100 g	30.00	31.00
Iron	mg / 100 g	1.20	10.50
Vitamin B1	mg / 100 g	0.04	0.01
Vitamin B2	mg / 100 g	0.02	0.03

Table 2 Nutrition and antioxidant of *Dioscorea hispida* in North Eastern, Thailand [26]

Composition	Amount
Calcium	100.00 mg / 100 g fresh weight
iron	0.40 mg / 100 g fresh weight
zinc	0.70 mg / 100 g fresh weight
copper	0.15 mg / 100 g fresh weight
phosphorus	40.00 mg / 100 g fresh weight
protein	3.50 mg / 100 g fresh weight
antioxidant	90.00 μg / ml

Dioscorine

Dioscorine is an alkaloid presented in *D. hispida* Dennst. and *D. hirsuta* Blume. Boorsma first isolated dioscorine in 1894 from the tubers of *D. hirsuta* Blume. and, later, in 1937 Leyva and Gutierrez isolated this alkaloid from the tubers of *D. hispida* Dennst. [27] Pinder went on to describe the isolation of what he believed to be dioscorine from *D. hispida* in his own laboratory at Oxford. Holmes [28], Fodor [29] and Jones and Pinder [30] discussed the method of extraction and the chemical constitution of dioscorine. Jones and Pinder [30] concluded that 2-oxotropane was a degradation product of dioscorine and described the formula of this alkaloid.

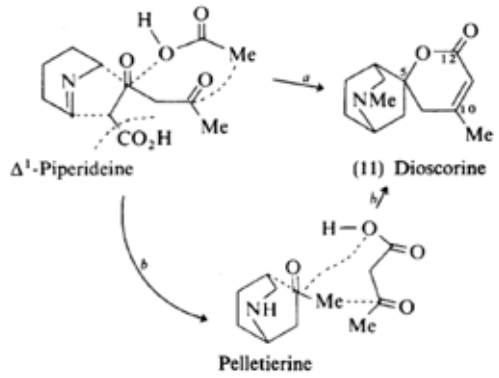
Van Itallie and Bylsma [31] described the following chemical tests for dioscorine:

1. A solution of this alkaloid in sulphuric acid turns yellow when a small amount of iodic acid is added to it from the edge, the yellow colour changes slowly to reddish-violet, which in turn changes to bluish-violet.
2. When a drop of a diluted solution of sodium nitroprusside and a few drops of sodium hydroxide are mixed with dioscorine, a reddish-violet colour appears after a short while.
3. If dioscorine is heated with sulphuric acid on a water-bath, a reddish-violet colour appears slowly.

Holmes [28] wrote: "Dioscorine gives a diagnostic colour reaction with potassium iodate and sulphuric acid. The brownish-yellow colour, first formed, slowly changes to a bluish-violet."

Full details of the mode of incorporation of acetate into dioscorine have been published in 1972 [9]. The results are consistent with either variant, *a* and b, of a pathway involving condensation of four acetate units with a lysine derived unit, plausibly Δ^1-piperideine (Scheme 1**);** as indicated in path ***b,*** pelletierine may be involved. Administration of [2-^{14}C]lysine to the tropical yam, *D. hispida,* however, gave dioscorine with little radioactivity; whilst [6-^{14}C]- Δ^1-piperideine was better used, the labelling pattern was essentially the same as that from [l-^{14}C]acetate, arising presumably by catabolism of the radioactive Δ^1-piperideine to acetate. These poor

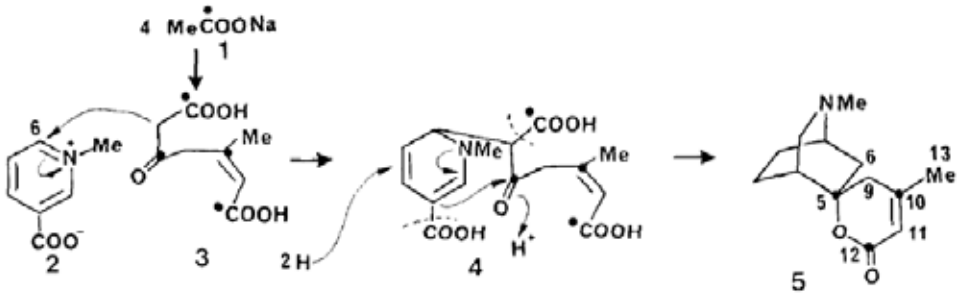
incorporations were rationalized by suggesting that, at the time of feeding, some compound, derivable from lysine, was not being actively synthesized but that it was available for condensation with acetate. This hypothetical compound could not be pelletierine for [l-^{14}C] acetate incorporation would result in labelling of C-10 and C-12 but not C-5, and in fact almost equal labelling of these positions is observed.



Scheme 1

It is worth noting that incorporation of acetate into dioscorine was only achieved with considerable difficulty and it seems possible that the administered lysine and Δ^1-piperideine are not reaching the site of alkaloid synthesis, in which case pelletierine may yet prove to be a precursor for dioscorine.

In 1971, Leete and Pinder were discovered that the administration of sodium [1-^{14}C] acetate to this plant, afforded dioscorine labelled at its C-5, C-10, and C-12 positions, the activity being equally divided between these positions [9, 32]. Six years later, they made the unexpected discovery that nicotinic acid serves as a precursor of the isoquinuclidine moiety of dioscorine [33]. In 1988, they had shown that trigonelline was a precursor of part of the isoquinuclidine ring of the alkaloid and Scheme 2 illustrated, the hypothesis for the biosynthesis of dioscorine from acetate and trigonelline. In this Scheme the branched eight-carbon compound, derived from four acetate units condenses with trigonelline at its C-6 position to form the intermediate 4. Two decarboxylations, bond formations and reductions, as illustrated, then afford dioscorine [34].



Scheme 2

The chemical shifts of ^{1}H and ^{13}C NMR spectra of dioscorine was recorded in Table 3. The assignments of both the ^{1}H and ^{13}C NMR were examined by its 2D-HETCOR MNR. The 2D-HETCOR spectrum was illustrated in Figure 2.

Table 3 Chemical shifts (ppm from TMS) of carbons and their attached hydrogens of dioscorine ($CDCl_3$) [34]

C	^{1}H-NMR (ppm)	^{13}C-NMR (ppm)
1	2.36	52.23 (1) *
3	2.23 (3b), 2.73 (3a)	53.65 (2)
4	1.70	35.00 (1)
5	-	81.40 (0)
6	1.55 (6 endo), 1.86 (6 exo)	40.77 (2)
7	1.31 (7 endo), 1.74 (7 exo)	20.14 (2)
8	1.25 (8 exo), 1.96 (8 endo)	19.37 (2)
9	2.41	39.34 (2)
10	-	155.74 (0)
11	5.59	116.20 (1)
12	-	164.88 (0)
13	1.79	23.27 (3)
N-Me	2.10	42.52 (3)

* Number of attached hydrogens determined by the DEPT pulse sequence.

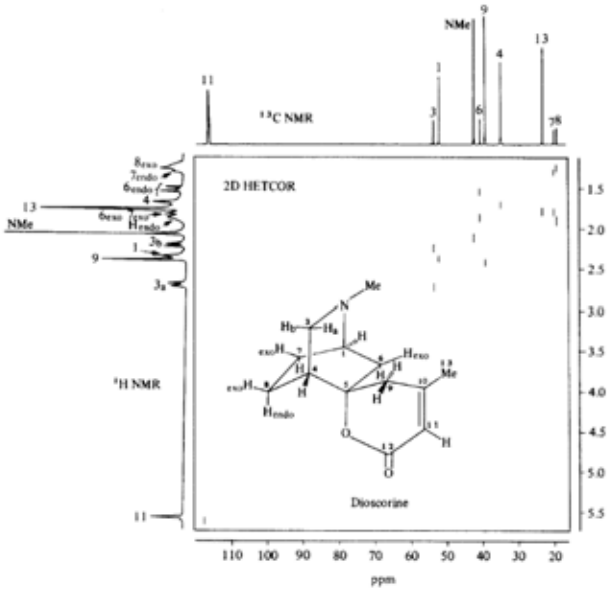
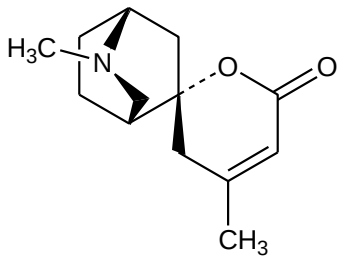


Figure 2 2D-HETCOR NMR spectrum of dioscorine in $CDCl_3$ [34]

The structural formula of dioscorine (stereochemistry) is shown in Figure 3. The molecular formula of dioscorine is $C_{13}H_{19}NO_2$. It is greenish-yellow prisms from ether, melting point 54-55°C. Soluble in water, alcohol, acetone, chloroform; slightly soluble in ether, benzene, petroleum ether [35].



Dioscorine

Figure 3 The chemical structure of dioscorine

Extraction and identification of dioscorine

Bhandari and Kawabata [5] reported the method for extraction and identification of toxic alkaloid, dioscorine. Forty grams of peeled and sliced yam tuber was extracted with 200 ml of 0.5N HCl in an electric blender. After standing for 2 days, the mixture was filtered and made alkaline with potassium carbonate and extracted with three portion of ether using a separating funnel. All the extracts were combined and dried overnight with sodium sulfate. Dried extract was filtered and concentrated under reduced pressure to a final volume of about 5 ml.

The concentrated extract was spotted on a 20 × 20 cm TLC plate (Silica gel G, 60 F_{254}, 0.5mm thickness, Merck). The compounds were separated by an ascending method with a solvent mixture of chloroform: ethanol: ammonia (100: 10: 0.5). The plates were air-dried and were sprayed with Dragendorff reagent. The calculated R_f value was compared with the literature R_f value. The compound having R_f value of 0.3 was isolated and further subjected for MS and NMR analysis. Field desorption mass spectra were obtained with a mass spectrometer and ^{13}C-NMR spectra were observed using chloroform-*d* as a solvent [5].

Pharmacological activities

Biological activities of dioscorine from *D. hispida* have been studied. Broadbent and Schnieden reported some pharmacological activities of dioscorine. For analeptic activity, when dioscorine 40 mg/kg was administered intravenously, it increased the respiratory rates of anaesthetized rats. For local anaesthetic effects, when a 5% solution of dioscorine was applied to the corneae of guinea-pigs, it did not prevent the corneal reflex, in fact some blepharospasm was noted. However, when dioscorine was injected intradermally into 12 guinea-pigs, it had local anaesthetic activity, dioscorine in 0.5% solution having approximately the same activity as 0.05% cocaine. For effects on isolated guinea-pig ileum, dioscorine also showed slight anti-acetylcholine activity at a concentration of 10^{-6}. Two milligram of dioscorine had no effect on the isolated rabbit heart set up in the manner of Langendorff. But in this dose, they diminished the responses of the heart to a subsequent injection of acetylcholine. For antidiuretic actions, one milligram of dioscorine had the activity of approximately 100 μU of Pitressin [12]. Moreover, Coursey reported that dioscorine triggered the fatal paralysis of the nervous system when 100 g of *D. hispida* tuber was ingested [5].

Toxicology of dioscorine

Dioscorine, $C_{13}H_{19}NO_2$ has been isolated from the tubers of *D. hirsuta* Blume and *D. hispida* Dennst. When injected into monkeys, it has a mydriatic action, and in certain respects it resembles the pharmacological action of picrotoxin and cardiac glycosides (vagal stimulation) [28]. Screening tests on *D. hispida* proved positive result for alkaloids and a negative one for cyanide. The toxicity of *D. hispida* in a wide range of animals has been demonstrated by Leyva and Gutierrez [36].

Dioscorine caused convulsions in rats and mice. These were at first clonic but became tonic and death usually occurred in extensor spasm. The convulsions closely resembled those produced by picrotoxin. The LD_{50} value in mice was 60 mg/kg. One percent solution of dioscorine was injected intraperitoneally into mice at the estimated LD_{100} of 110 mg/kg. The solution killed 10/10 mice. The solution was left overnight in a refrigerator, and tested on the following day. It killed 10/10 mice. After two days, dioscorine killed 10/10 mice. After a week, dioscorine solution killed 9/10 animals [12]. It is thus apparent that aqueous solutions of dioscorine slowly lost convulsant properties.

High performance thin layer chromatography (HPTLC)

The basic thin layer chromatography procedure has largely remained unchanged over the last fifty years. In l973, Halpaap was one of the first to recognize the advantage of using a smaller average particle size of silica gel (about 5 – 6 μm) in the preparation of chromatographic plates. He compared the effect of particle size on development time, retardation factor (R_f) and plate height. By the mid 1970s HPTLC added a new dimension to thin layer chromatography, as precision could be improved ten – fold, analysis time could be reduced by a similar factor, less mobile phase was required, and the development distances on the layers could be reduced [37].

The technique could now be made fully instrumental to give accuracy comparable with HPLC. In 1977 the first major high performance thin layer chromatography publication appeared, simply called HPTLC high performance thin layer chromatography [38].

The 1980s show improvements in spectrodensitometric scanners with full computer control becoming possible, including options for peak purity and the measurement of full UV/visible spectra for all separated components. Automated multiple development made its appearance in 1984. This improvement enabled a marked increase in number and resolution of the separated components [39].

At the present time all steps of thin layer chromatography can be computer controlled. The use of highly sensitive charge coupled device cameras which have high resolution has enabled the chromatographer to electronically store images of chromatograms for future use and for direct entry into reports at a later date. When one considers the latest technical and methodological developments modern HPTLC, is a reliable and powerful analytical technique, which can be the method of choice when many samples have to be analyzed, flexibility is of importance and rapid quantitative and semi-quantitative data are needed at low cost per sample [40].

As smaller particles improved efficiencies in LC columns and resulted in HPLC. Small particles (about 5 – 6 μm) were also tried in thin layer chromatography.

As in column in LC, these plates resulted in HPTLC. Some characteristics of two types of plates are included in Table 4 [41].

Table 4 Comparison between TLC and HPTLC

	TLC	HPTLC
Mean particle size	10 – 15 μm	3 – 7 μm
Size distribution	wide	narrow
Layer thickness	≥ 250 μm	100 – 200 μm
Number of samples	12	36 – 72
Migration distance	100 – 150 mm	30 – 70 mm
Migration time	30-200 min	3 – 20 min
Solvent use	≥ 50 ml	5 – 10 ml
Detection limit		
Absorption measurement	100 – 1000 ng	10 – 100 ng
Fluorescence measurement	1 – 100 ng	0.1 – 10 ng

Nowadays, HPTLC is involved in a lot of applications, analysis in pharmaceuticals and drugs, clinical chemistry, forensic chemistry, biochemistry, cosmetology, food analysis, environmental analysis and other areas [42].

Unlike TLC, HPTLC uses automatic micro syringe for sample application and scanner for quantification.

1. Sample application

   Successful quantitative thin layer chromatography is strongly dependent on the quality of sample application. Reproducibility of sample amount and spot (band like) size are quite important. To achieve good chromatographic resolution and sensitivity of detection, the shape of the spots of the applied sample is also great importance. Micro syringes are commonly used in HPTLC. They are equipped with a micrometer screw for precise control of the position of the syringe plunger. One advantage of a micro syringe over a pipette is that it delivers the sample solution by displacement rather than by capillary action [43].

2. Detection, quantification and documentation

The simplest method of detecting substances on HPTLC is by visual detection of the spots caused by substances with a color of their own. Inspection of the chromatogram under UV light is also a non-destructive detection method. Spots of fluorescent compounds can be seen at 254 nm or at 366 nm but spots of non fluorescent compounds require fluorescent stationary phase (silica gel GF) to be seen using UV light. Non UV absorbing compounds can be detected by dipping the plates in iodine vapour. But when individual component does not respond to UV, derivatization is required for detection [44]. *In situ* densitometry offers a simple way of quantifying directly on the plate. A definition of direct densitometry is resolving the compounds to be separated on the chromatoplate and measuring the optical density of the separated spots directly on the plate. The amounts of compounds are determined by comparing them to a standard curve from reference materials chromatographed simultaneously under the same conditions [45]. After an evaluation by scanner, the complete data is recorded in the form of a number of hard-copy pages, representing the main part of the whole documentation. This documentation system is useful to recall the photo at any time easily and include it in a printed text document, for easy archiving and retrieval and easy reference to previous work [40].

CHAPTER III
MATERIALS AND METHODOLOGY

Materials

Cover slips	(Menzel. Glazer)
Filter paper No.40 (Ashless)	(Whatman, England)
Filter paper No.4	(Whatman, England)
Microscope slide	(Sail Brand, China)
TLC Aluminium oxide 60 GF_{254} neutral	(Merck, Germany)
TLC Silica gel 60 GF_{254}	(Merck, Germany)

Chemicals and reagents

Acetic acid	(Analytical grade, B. H. Chemicals, England)
Butanol	(Analytical grade, B. H. Chemicals, England)
Chloroform	(Analytical grade, J. T. Baker Chemical, USA)
Ammonia	(Analytical grade, B. H. Chemicals, England)
Dichloromethane	(Analytical grade, Labscan, Thailand)
Hydrochloric acid	(Analytical grade, Labscan, Thailand)
Methanol	(Analytical grade, B. H. Chemicals, England)
Sulphuric acid	(Analytical grade, B. H. Chemicals, England)
Silica gel 60 column chromatography	(Merck, Germany)
Toluene	(Analytical grade, Labscan, Thailand)

Equipments and instruments

Ashing furnace	(Carbolite, UK)
Balance readability 0.01 g	(Pioneer™ Ohaus Crop. Pine Brook, NJ, USA)
Balance readability 0.0001 g	(Adventurer™ Ohaus Crop. Pine Brook, NJ, USA)
Centrifuge	(Sorvall® Primo R, UK)
Digital camera	(Cannon Power shot A640)
Hot air oven	(WTC Binder tuttlingen, Germany)
TLC-densitometry instrument	(Camag, Switzerland) with winCATS software
Microscope	(Carl Zeiss model Axio Lab, Germany) with AxioVision40 V4.6.3.0 software
NMR Spectrometer	(500 MHz Varian INOVA, USA)
Rotary vacuum evaporator	(Buchi, Switzerland)
Scion Image Software	(Scion Corporation, USA)
Shaker	(Adolf Kuhner AG, Switzerland)
Soxhlet apparatus	
Syringe	(Hamilton Company, USA)
TLC Chamber	(Camag, Switzerland)
Ultrasonic bath	(Analytical Lab Science Co., LTD, Bangkok)
Water bath	(Brinkmann, USA)

Methods

Collection of plant materials

Fourteen samples of *Dioscorea hispida* tubers were collected from 12 different location throughout Thailand as follows: Chiang Mai, Nong Khai, Kalasin, Nakhon Sawan, Uthai Thani, Lop Buri, Nakhon Pathom, Bangkok (3 areas), Rayong, Ratchaburi, Surat Thani, and Nakhon Si Thammarat. All of crude drugs were authenticated by Ruangrungsi, N. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. The tubers were sliced and sun dried in a hot air oven.

Determination of pharmacognostic specification

Macroscopic examination, microscopic examination and standardization parameters due to quality of crude drug were examined according to World Health Organization (WHO) guideline quality control methods for medicinal plant materials [3].

Macroscopic examination

D. hispida morphology that including stem, leaf, flower, infructescence, root and tuber was botanically illustrated by hand – drawing with the scale relative to the actual size.

Microscopic examination

Transverse section by a razor blade was determined for anatomical characteristics of *D. hispida* tuber. Powders by grinding and sifting through 250 micron sieve were determined for histological characteristics. The tissue section and powders were mounted onto a slide in water for microscopical observation under objective lens with a 10X, 20X and 40X magnifications. Photographs were taken with the help by a digital camera. The microscopic characters were drawn in the proportion size related to the original.

Determination of total ash

Dried slices of tubers were pulverized. Placed 3.000 g of the ground crude drug, in a previously tared crucible. Dried on gas stove until it was smokeless then ignited it by gradually increasing the heat to 500 ^{O}C until it was white. Cooled in a desiccator and weighed without delay.

Determination of acid-insoluble ash

For the crucible containing the total ash, add 25.0 ml of hydrochloric (70 g/l), covered with a watch-glass and boiled gently for 5 minutes. Rinsed the watch-glass with 5.0 ml of hot water and added this liquid to the crucible. Collected the insoluble matter on an ashless filter paper and washed with hot water until the filtrate was neutral. Transfered the filter paper containing the insoluble matter to the original crucible, dried on a hot plate and ignited to constant weight. Allowed the residue to cool in a desiccators and weighed without delay.

Determination of ethanol-soluble extractive value

Macerated 5.000 g of the ground sample with 100.0 ml of 95% ethanol in a closed conical flask for 6 hours in shaking bath then allowed to stand for 18 hours. Filtered rapidly to avoid loss of ethanol, evaporated 20.0 ml of the filtrate to dryness in a tared small beaker and dried with heat to constantly weight.

Determination of water-soluble extractive value

Macerated 5.000 g of the ground sample with 100.0 ml of distilled water in a closed conical flask for 6 hours in shaking bath then allowed to stand for 18 hours. Filtered rapidly to avoid loss of water, evaporated 20.0 ml of the filtrate to dryness in a tared small beaker and dried with heat to constantly weight.

Determination of loss on drying

Weighed 5.000 g of the ground sample in a tared small beaker and dried with heating at 105 ºC to constantly weight.

Determination of water content

Weighed 50.00 g of the ground sample, add 200.0 ml of water-saturated toluene and distilled by azeotropic distillation. As soon as water was completely distilled, rinsed the inside of the condenser tube with toluene and continue the distillation for 5 more minutes. Allow the receiving tube to cool to room temperature. Allow the water and toluene layers to separate and read off the volume of water.

Determination of volatile oil content

Weighed 100.00 g of ground sample, added 600.0 ml of water and distilled by Clevenger apparatus then read off the volume of volatile oil.

TLC fingerprint analysis

Extracted 1.000 g of the ground sample with 10.0 ml of 95% ethanol for 6 hours, then filtered through filtered paper (Whatman No.4), evaporated the filtrate to dryness and kept in well-closed container. Redissolved the residue in 1.0 ml of 95% ethanol, applied 10 µl on TLC Silica gel 60 F_{254} by micropipette, allowed to dry in the air. Developed the chromatogram in the chamber with the solvent (butanol: acetic acid: water, 4: 1: 1). Removed the plate, allowed it to dry in air and observed the produced spot under UV light 254 nm, UV light 365 nm and sprayed the spots with 10% sulphuric acid in methanol.

Preparation of standard dioscorine

Extraction and purification of dioscorine from *D. hispida* tubers

Two kg of dried *D. hispida* tubers were ground to coarse powder. These were continuously macerated with 95% ethanol (8000 ml) until it was exhausted, filtered, and the filtrates of maceration were combined. The combined filtrate was concentrated under reduced pressure to syrupy mass. The syrupy mass was dissolved in 5% acetic acid and filtered. The acid solution was adjusted to alkaline with ammonia and followed by extraction with dichloromethane until the base was exhausted. The combined dichloromethane extract was evaporated under reduced pressure to give a syrupy crude base. Examination of the crude base by TLC indicated that at least two alkaloids were present. Crude alkaloid was dissolved in a small amount of absolute ethanol and saturated picric acid (in water) was added to form yellow crystalline picrate. This crystalline picrate was designated subsequently identified as alkaloidal picrate. The alkaloidal picrate was made alkaline by ammonia and followed by extraction with dichloromethane until the base was exhausted. The crude base was fractionated using a silica gel column chromatography (6×5 cm) with a mixture of acetone: water: ammonia (90: 7: 10) as eluent. Seventeen fractions in the volume of 20 ml were collected. Each fraction was evaporated on water bath to syrupy mass. Each syrupy mass was dissolved in 1 ml of chloroform. Two µl of each sample was spotted on TLC plates coated with silica gel GF_{254} and aluminium oxide GF_{254} and allowed to dry in the air. The plates were developed in chambers saturated with solvent system I (acetone: water: ammonia, 90: 7: 10) and solvent system II (chloroform: methanol, 97: 3) respectively. After the solvent ascended 8 cm, the plates were removed from the chambers, allowed to dry in the air and determined under UV 254 nm. Fractions number 11 – 14 which showed the quenching spot were combined and concentrated to syrupy mass on water bath. The syrupy mass was cooled in desiccator for further identification by nuclear magnetic resonance (NMR).

Identification of isolated dioscorine by NMR

Thirty mg of syrupy mass previously described in aforementioned was identified for chemical structure using proton as well as carbon NMR. Spectra of ^{1}H and ^{13}C-NMR were determined in deuterated chloroform, operating at 500 MHz using a Varian INOVA spectrometer (Scientific and Technological Research Equipment Centre, Chulalongkorn University). Identification of the compound was compared with previously reported [46].

Preparation of standard solutions

A stock solution of dioscorine was prepared by dissolving 10.000 mg of standard dioscorine in 4.0 ml methanol. The standard solution of dioscorine was prepared by diluting the stock solution to obtain the concentration ranges of 0.5 – 2.5 mg/ml and used for preparation of the calibration curves.

Determination of dioscorine content in *D. hispida* tubers

Preparation of crude extract for dioscorine determination

For the analysis of dioscorine content of 14 samples of *D. hispida* tubers, the crude extract was prepared by weighing 20.00 g of dried powdered tubers and subjecting to be extracted with 95% ethanol (200 ml) using soxhlet apparatus until the extract was colorless. The solvents were completely removed under reduced pressure by rotary vacuum evaporator. The dried extracts were weighed and kept in desiccator.

For sample solutions, each extract was dissolved in methanol to a concentration of 20.0 mg/ml. Five µl of each sample solution was applied in triplicate on a TLC plate and analyzed by TLC image analysis. Dioscorine content was calculated from the calibration curve. The sample with dioscorine content over than 2.5 mg/ml was diluted and re-analyzed. The content of dioscorine was expressed as gram per 100 gram of dried tubers.

Chromatographic conditions

TLC analysis was performed on TLC Aluminium oxide 60 F_{254} neutral. Five microliters of 5 standard solutions (0.5 – 2.5 mg/ml) and 14 sample solutions (5 – 20 mg/ml) were spotted as 6.0 mm bands in length onto a same TLC plate by using a Camag Linomat 5 syringe. A distance between each spot was 9.4 mm. The plate was then developed to a distance of 8.0 cm in a TLC chamber previously saturated with methanol-chloroform (3 : 97, *v/v*) for at least 30 minutes.

TLC image analysis

Quantification of dioscorine in the TLC image was carried out by Scion image software. An image of the TLC chromatogram under UV 254 nm was taken using a digital camera. The image file which saved as in .tiff format was opened with Scion Image for Windows version Alpha 4.0.3.2. The natural colour was converted to grayscale by photoshop software. A profile plot along the chromatogram was generated using the macro Gelplot2. The peak corresponding to dioscorine was selected by the wand tool for measuring the area under the curve.

TLC-densitometry

Five microliters of each sample solution was spotted as 6.0 mm band length on a precoated silica gel aluminium plate 60 F_{254} using a Camag Linomat 5 syringe. A constant application rate of 150 nl/s was employed while a space between each band was 9.4 mm. The slit dimension was kept at 4.00 mm × 0.30 mm while 20 mm/s scanning speed was employed. The mobile phase consisting of chloroform : methanol (97:3) was used. Linear ascending development was carried out in 20 × 10 cm twin trough glass chamber saturated with the mobile phase. The length of each chromatogram run was 8 cm. After developing, the TLC plate was dried using an air dryer. Densitometric scanning was performed on Camag TLC scanner 3 in the

absorbance mode at 254 nm, operated by winCATS software. The source of radiation utilized was a deuterium lamp. The TLC image documentation was carried out with CAMAG Visualizer.

Method validation

Calibration curve and linearity

The standard solution of dioscorine were prepared to a concentration range of 0.5 – 2.5 mg/ml. Each standard solution was spotted 5 µl on the TLC plate to obtain final concentration 2.5 – 12.5 µg/spot. Each concentration was spotted six times on the TLC plate. The plate was developed on mobile phase (methanol : chloroform, 3 : 97 v/v). The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs using Excel.

Accuracy

The accuracy of the method was determined by using the standard addition method [47]. The three different concentrations (0.375, 0.75, 1.50 µg/µl) of standard solution were added to the sample of *D. hispida* tuber, known amounts of dioscorine (0.635 % dry weight). The percentage recovery was calculated by the following equation:

$$\% \text{ Recovery} = \frac{(C_s - C) \times 100}{C_a}$$

C_s : the amount of dioscorine that found after adding of standard solution

C : the amount of dioscorine that found before adding

C_s : the amount of reference standard actually added to the sample

Precision

Precision of this method was determined by analyses the measurement of area under peak of five different concentrations (2.50, 5.00, 7.50, 10.00, 12.50 µg/spot) of standard solutions in triplicates on the same day (repeatability) and on 6 different days (intermediate precision) [48]. The relative standard deviation (RSD) was calculated by the following formula:

$$\% \text{ RSD} = \frac{\text{SD} \times 100}{\text{X}}$$

SD : standard deviation

X : mean

Limit of detection and limit of quantification (LOD and LOQ)

The LOD and LOQ were determined based on the standard deviation of the response and the slope [48]. The slope was estimated from the calibration curve of the analytic and the estimate of the standard deviation was carried out from the residual standard deviation of a regression line. The LOD and LOQ were calculated by the following formula:

$$\text{LOD} = 3.3\delta / \text{S}$$

$$\text{LOQ} = 10\delta / \text{S}$$

δ : the standard deviation of the response

S : the slope of the calibration curve

Statistic analysis

For pharmacognostic specification, the data will be calculated as grand mean and pooled standard deviation (Grand mean ± Pooled SD). For determination of dioscorine content, the area under peak will be analyzed using Scion Image software and TLC densitometry. The dioscorine content was statistically analyzed using paired t-test by SPSS 16.0 for windows program for analyzing of significant difference.

CHAPTER IV
RESULTS

Pharmacognostic specification

The drawing of whole plant of *Dioscorea hispida* was illustrated in detail (Figure 4). *D. hispida* crude drug was traditionally prepared by slicing the tuber and sun drying. The crude drugs were either longitudinal pieces or irregularly shaped, variable in size, off- white with some light brown epidermis (Figure 5).

The anatomical characterization showed hypodermis, periderm, raphide crystal, parenchyma containing starch granules and vessel (Figure 6).

The histological characteristics was composed of parenchyma, starch granule, parenchyma containing starch granules, brownish mass, raphide crystal, reticulate vessel and fiber (Figure 7).

The constant numbers due to quality of *D. hispida* dried tubers were shown in Table 5. The total ash, acid insoluble ash, loss on drying and water content should be not more than 3.44, 0.92, 11.50 and 11.55 % of dry weight respectively whereas ethanol – soluble extractive and water – soluble extractive values should be not less than 3.00 and 15.07 % of dry weight respectively.

Thin layer chromatography fingerprint of methanolic extract of *D. hispida* dried tubers were shown in Figure 8.

Macroscopic characters (Whole plant)

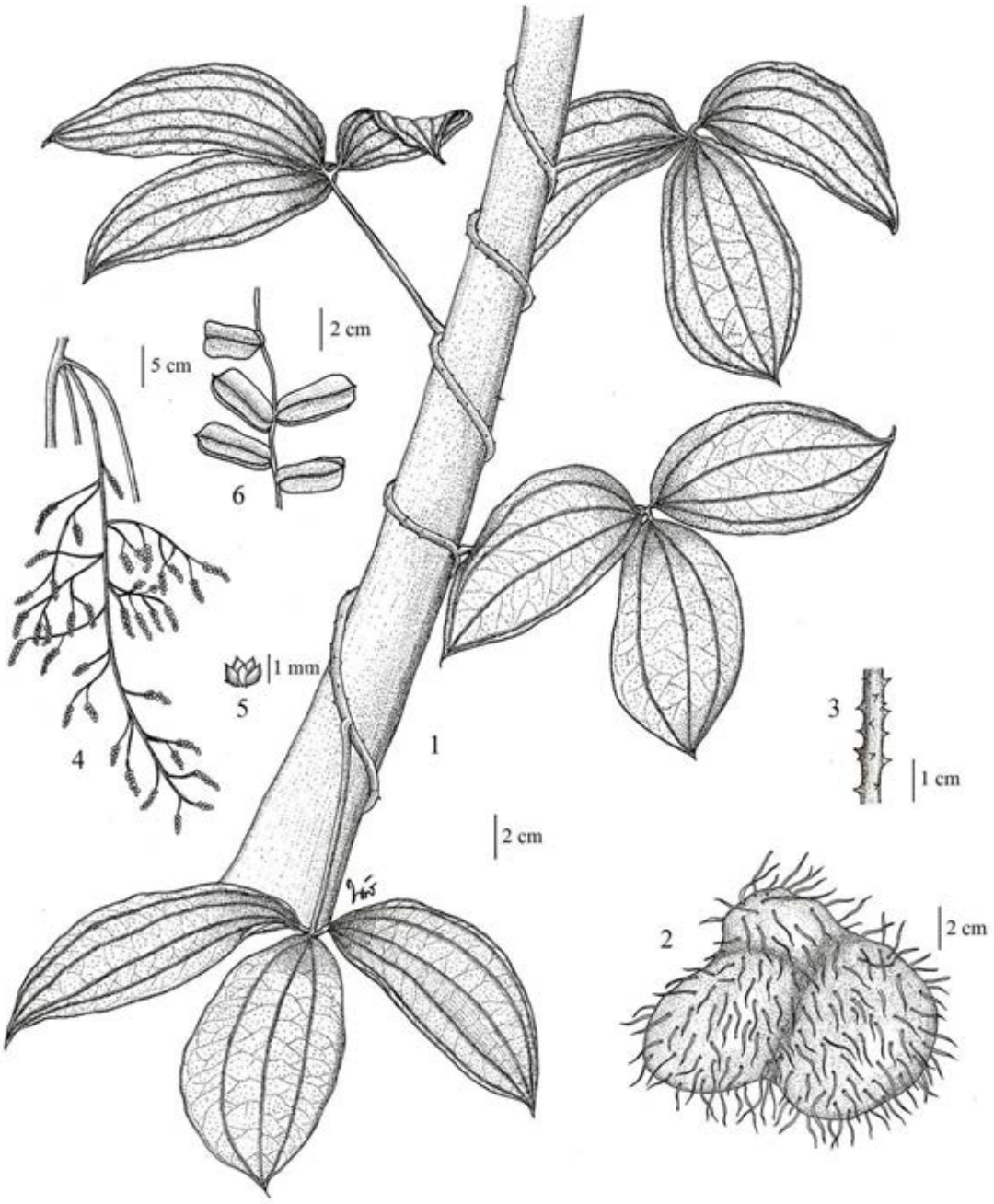


Figure 4 Whole plant of *Dioscorea hispida* Dennst.
1. twining vine with leaves
2. tuber
3. stem
4. flowering branch showing male inflorescence
5. male flowers
6. fruits (capsules)

Macroscopic characters (Crude drug)

Figure 5 Crude drug of *Dioscorea hispida* Dennst.

Microscopic characters (Anatomical Characters)

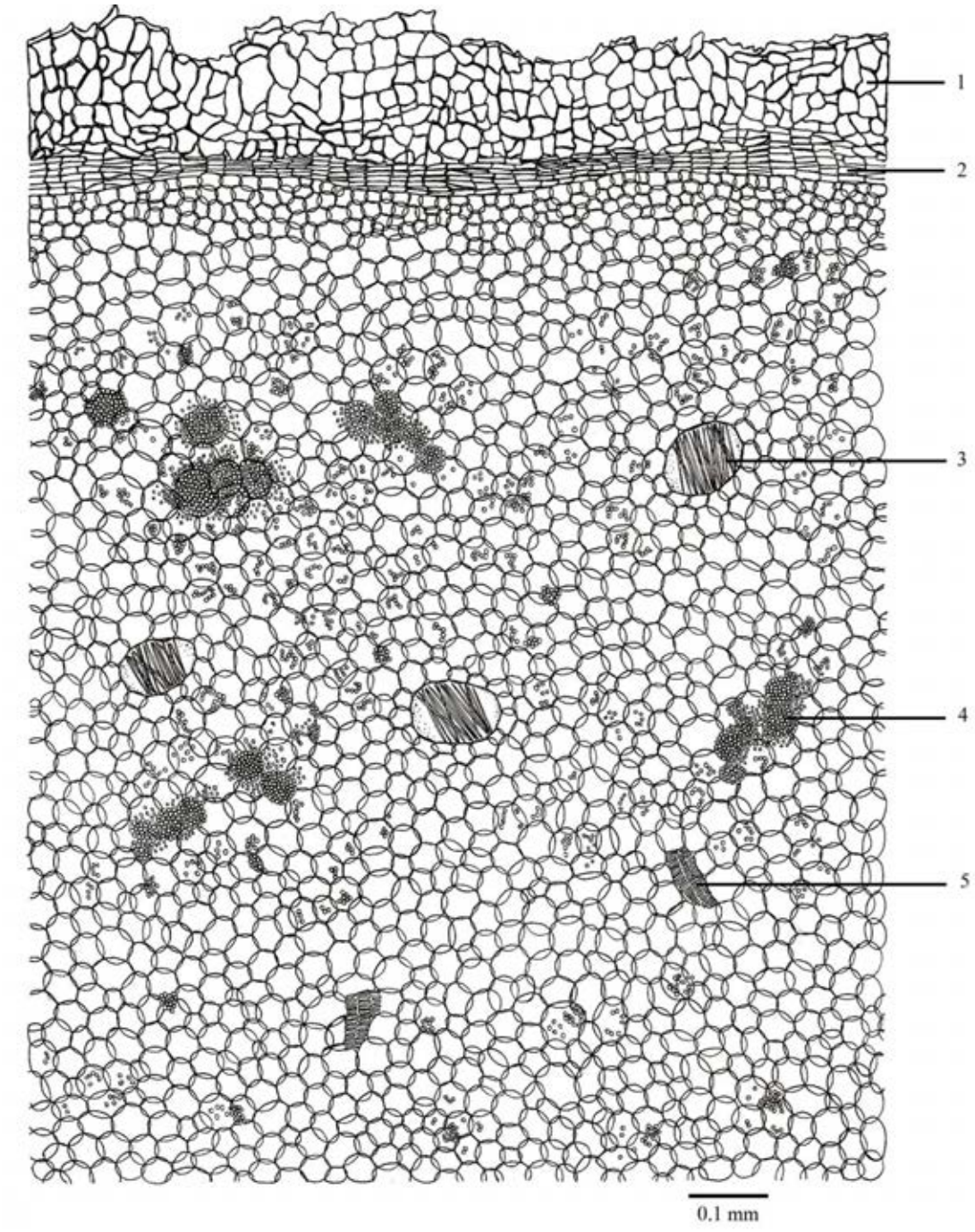


Figure 6 Transverse section of *Dioscorea hispida* Dennst. tuber:

1. hypodermis
2. periderm
3. raphide crystal
4. parenchyma containing starch granules
5. vessel

Microscopic characters (Histological Characters)

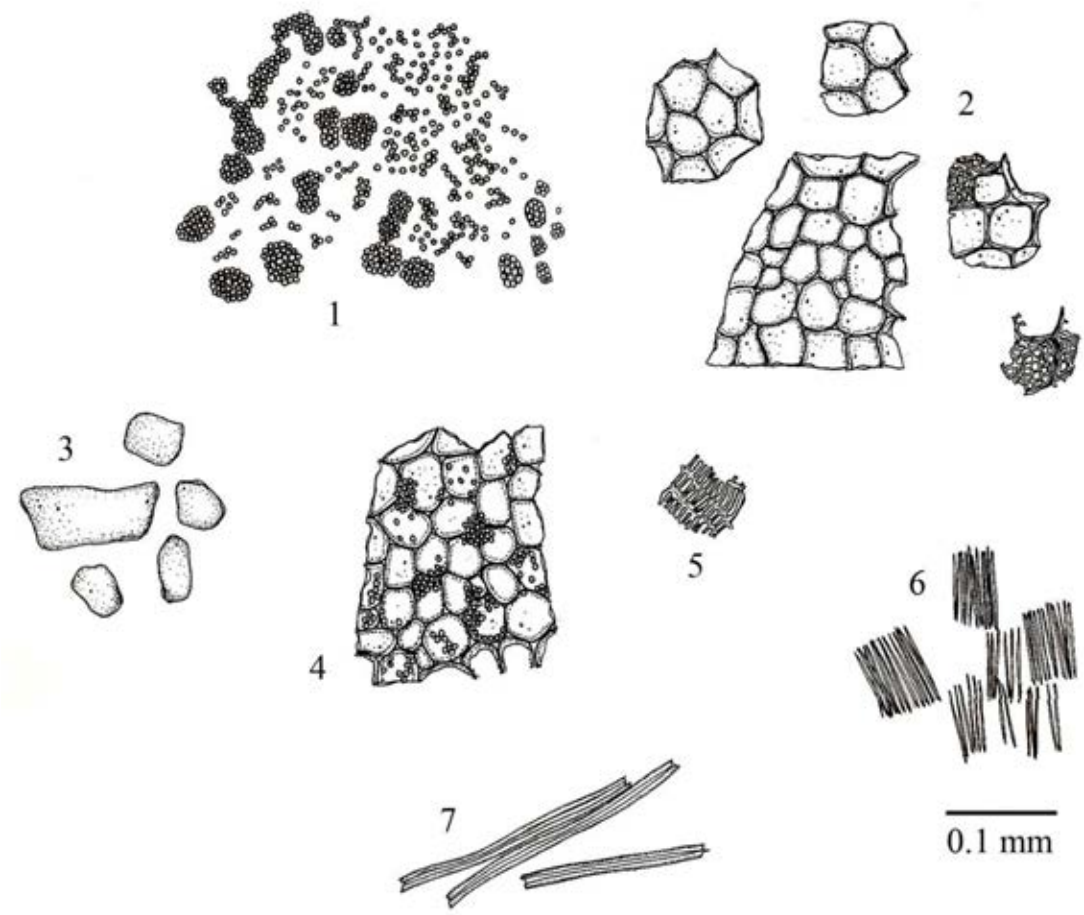


Figure 7 Powdered of *Dioscorea hispida* Dennst. tuber:

1. starch granule
2. parenchyma, transverse view
3. brownish mass
4. parenchyma containing starch granule
5. fragment of reticulated vessel
6. raphide crystal
7. fragment of fiber

Table 5 The constant numbers due to quality of *Dioscorea hispida* tubers

Content (% by weight)	**Mean ± SD**	**Min – Max**	**N**
Loss on drying	11.50 ± 0.33	8.33 – 12.97	14
Total ash	3.44 ± 0.08	2.28 – 4.50	14
Acid-insoluble ash	0.92 ± 0.13	0.36 – 2.01	14
Ethanol–soluble extractive	3.00 ± 0.15	1.16 – 8.39	14
Water–soluble extractive	15.07 ± 0.25	11.52 – 19.77	14
Water content	11.55 ± 0.38	9.00 – 13.00	14
Volatile oil content	0	0	14

N = 14, each sample was done in triplicate

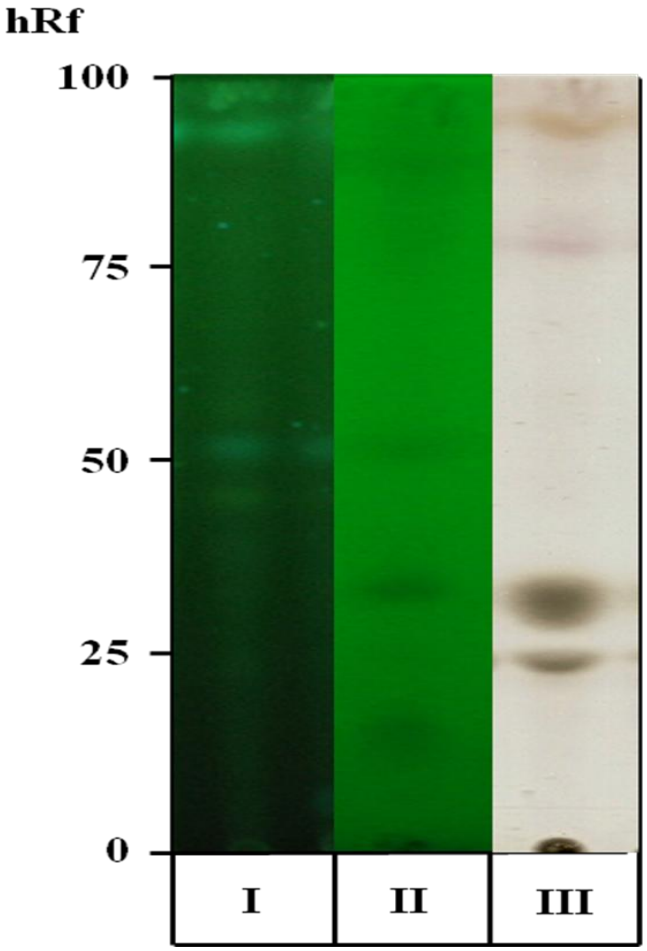


Figure 8 Thin-layer chromatographic of methanolic extract of *Dioscorea hispida* tubers

Detection

I = detection under UV light 365 nm

II = detection under UV light 254 nm

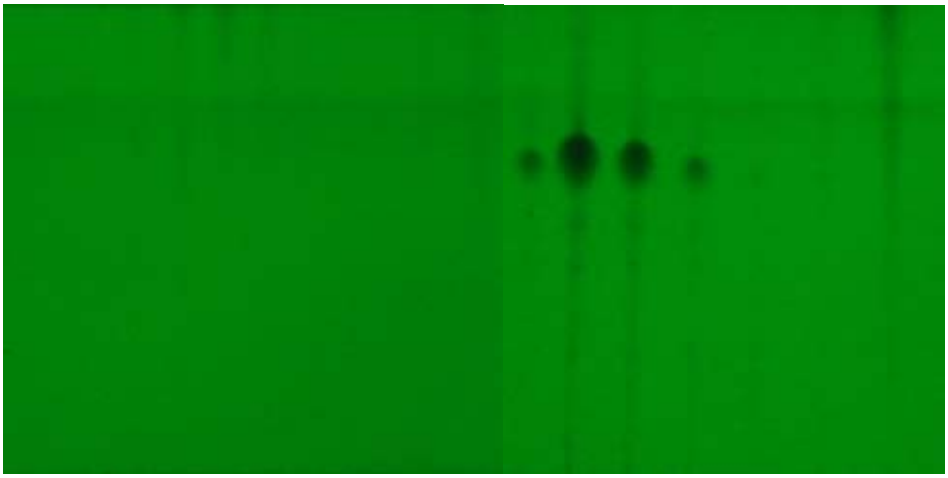
III = detection with 10% sulphuric acid in methanol

Preparation of standard dioscorine

Extraction and purification of dioscorine from *D. hispida* tubers

Dried *D. hispida* tubers were powdered and extracted by maceration in 95% ethanol. The ethanolic extract was dried under reduce pressure. The yield of crude ethanolic extract was 5.23 %w/w.

The crude ethanolic extract was dissolved in 5% acetic acid and filtered. The acid solution was made alkaline with ammonia and followed by extraction with dichloromethane until the base was exhausted. The combined dichloromethane extract was evaporated under reduced pressure to give a syrupy crude base. Crude alkaloid was dissolved in a small amount of absolute ethanol and saturated picric acid (in water) was added to form yellow crystalline picrate. This crystalline picrate was designated subsequently identified as alkaloidal picrate. The alkaloidal picrate was made alkaline by ammonia and followed by extraction with dichloromethane until the base was exhausted. Dioscorine was isolated from the crude base by a silica gel column chromatography (6 x 5 cm). The mixture of acetone: water: ammonia (90: 7: 10) was used as eluent. Fraction number 11 to 14 showed single spot on both TLC systems (silica gel 60 GF_{254}, acetone : water : ammonia 90 : 7 : 10 under UV 254 and Aluminium oxide 60 GF_{254}, chloroform : methanol 97:3 under UV 254) (Figure 9 and 10). Therefore these fractions were pooled, evaporated to dryness and weighed. One hundred grams of the crude ethanolic extract yielded 30.64 mg of isolated dioscorine by this method.



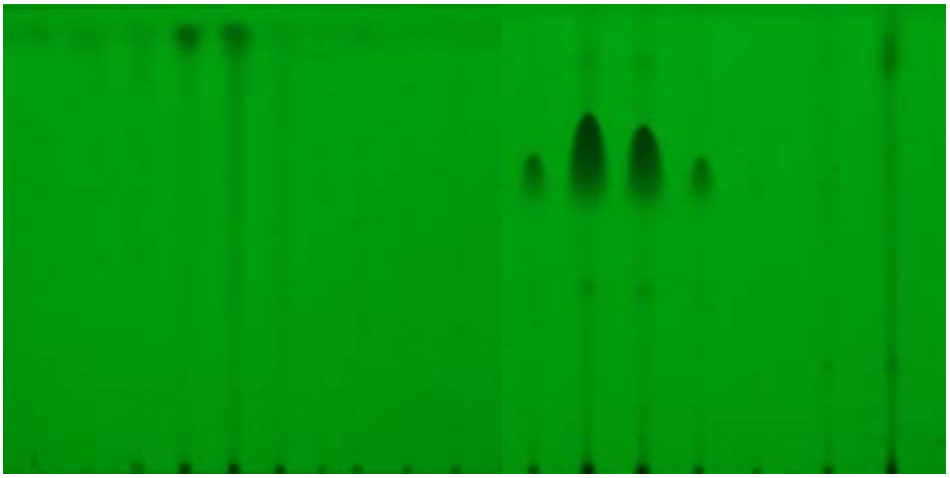
F_1 F_2 F_3 F_4 F_5 F_6 F_7 F_8 F_9 F_{10} F_{11} F_{12} F_{13} F_{14} F_{15} F_{16} F_{17}

Figure 9 TLC system I of 17 fractions from column chromatography

Adsorbent : silica gel 60 GF_{254}

Solvent system : acetone : water : ammonia (90:7:10)

Detection : under UV 254



F_1 F_2 F_3 F_4 F_5 F_6 F_7 F_8 F_9 F_{10} F_{11} F_{12} F_{13} F_{14} F_{15} F_{16} F_{17}

Figure 10 TLC system II of 17 fractions from column chromatography

Adsorbent : Aluminium oxide 60 GF_{254}

Solvent system : chloroform : methanol (97:3)

Detection : under UV 254

Identification of isolated dioscorine by NMR

The chemical structure of isolated dioscorine was confirmed by ^{1}H-NMR and ^{13}C-NMR using deuterated chloroform as a solvent. The ^{1}H-NMR and ^{13}C-NMR spectra were shown in Figures 16 – 22. The chemical shifts of this compound were shown in Table 6. Carbon-13 nuclear magnetic resonance revealed 13 carbons, 2 methyl, 5 methylene, 3 quaternary carbon and 3 methine. There were similar to ^{13}C-NMR of dioscorine **(**Table 3**)**. These data confirmed this compound as dioscorine.

Table 6 ^{1}H-NMR spectral and ^{13}C-NMR spectral assignment of isolated dioscorine

C	^{1}H-NMR (ppm)	^{13}C-NMR (ppm)
1	2.561	52.40
3	2.407 (3β), 2.932 (3α)	53.70
4	1.880	35.10
5	-	81.30
6	1.753 (6 endo), 2.063 (6 exo)	39.40
7	1.514 (7 endo), 2.035 (7 exo)	19.90
8	1.469 (8 exo), 2.120 (8 endo)	19.30
9	2.574	40.90
10	-	155.60
11	5.784	116.40
12	-	165.00
13	1.947	23.30
N-Me	2.294	42.60

Determination of dioscorine content in *D. hispida* tubers

1. Preparation of crude extract for dioscorine determination

The dried powder of *D. hispida* tubers from 14 different locations were extracted with 95 % ethanol to obtain crude ethanolic extract by soxhlet apparatus. The yield of crude ethanolic extract from each location was shown in Table 7. Average yield of crude ethanolic extract from 100 grams of dried powder was 6.91 ± 1.03 grams. The highest yield (8.59 % w/w) was found in the samples from Nong Khai. The lowest yield (5.24 % w/w) was found in the samples from Kalasin.

Table 7 Yield of ethanolic extracts of *Dioscorea hispida* tuber from 14 different locations in Thailand (% dry weight)

No.	Location	Ethanolic extract (% dry weight)
1	Bangkok 1	6.48
2	Bangkok 2	8.53
3	Bangkok 3	8.00
4	Chiang Mai	7.05
5	Kalasin	5.24
6	Lop Buri	5.44
7	Nakhon Pathom	7.04
8	Nakhon Sawan	6.39
9	Nakhon Si Thammarat	6.88
10	Nong Khai	8.59
11	Ratchaburi	6.94
12	Rayong	6.60
13	Surat Thani	5.89
14	Uthai Thani	7.63
	Average	**6.91 ± 1.03**

2. Determination of dioscorine content in ethanolic extract by TLC image analysis

The dioscorine content in ethanolic extract of *D. hispida* tubers from 14 different locations were evaluated by TLC image analysis using Scion image software.

The yield of dioscorine from each location was shown in Table 8. Average yield of dioscorine from 100 grams of dried powder was 0.661 ± 0.074 grams. The highest dioscorine (1.386 % w/w) was found in the samples from Nong Khai. The lowest dioscorine (0.355 % w/w) was found in the samples from Bangkok 2.

Table 8 Yield of dioscorine from 14 different locations of Thailand (% dry weight)

No.	Location	Dioscorine content in dried powder (% dry weight)			
		No.1	**No.2**	**No.3**	**Average**
1	Bangkok 1	0.746	0.698	0.608	0.684
2	Bangkok 2	0.342	0.409	0.315	0.355
3	Bangkok 3	0.448	0.394	0.392	0.411
4	Chiang Mai	0.458	0.410	0.367	0.412
5	Kalasin	0.456	0.486	0.432	0.458
6	Lop Buri	1.359	1.232	1.194	1.262
7	Nakhon Pathom	0.527	0.581	0.401	0.503
8	Nakhon Sawan	0.882	0.738	0.729	0.783
9	Nakhon Si Thammarat	0.400	0.349	0.370	0.373
10	Nong Khai	1.294	1.564	1.300	1.386
11	Ratchaburi	0.763	0.632	0.632	0.676
12	Rayong	0.475	0.531	0.404	0.470
13	Surat Thani	0.844	0.740	0.748	0.777
14	Uthai Thani	0.699	0.775	0.639	0.704
Average					**0.661 ± 0.074**

2.1 Method validation

Linearity

The *Rf* values and peak area of standard dioscorine (2.5, 5.0, 7.5, 10.0, and 12.5 μg/spot) were shown in Table 9. Five concentrations of dioscorine were plotted against the response (peak area in pixel^2) for a polynomial calibration curve. The correlation coefficient (r^2) of the curve was 0.999 (Figure 11) and polynomial equation was $y = 0.342x^2 + 108.4x + 25.45$

Table 9 The polynomial data of dioscorine by TLC image analysis

Concentration (μg/spot)	Injection No.	Peak area (pixel^2)	Average	SD
2.5	1	132.0		
	2	489.0		
	3	336.0		
	4	269.0		
	5	253.0		
	6	340.3	303.217	118.299
5.0	1	194.0		
	2	1001.0		
	3	603.0		
	4	513.0		
	5	480.0		
	6	616.0	567.833	261.405
7.5	1	283.0		
	2	1659.0		
	3	844.0		
	4	741.0		
	5	770.0		
	6	845.3	857.050	445.872
10.0	1	382.0		
	2	2325.0		
	3	1113.0		
	4	1045.0		
	5	977.0		
	6	1084.7	1154.450	635.125
12.5	1	517.0		
	2	2833.0		
	3	1368.0		
	4	1258.0		
	5	1182.0		
	6	1421.7	1429.950	761.181

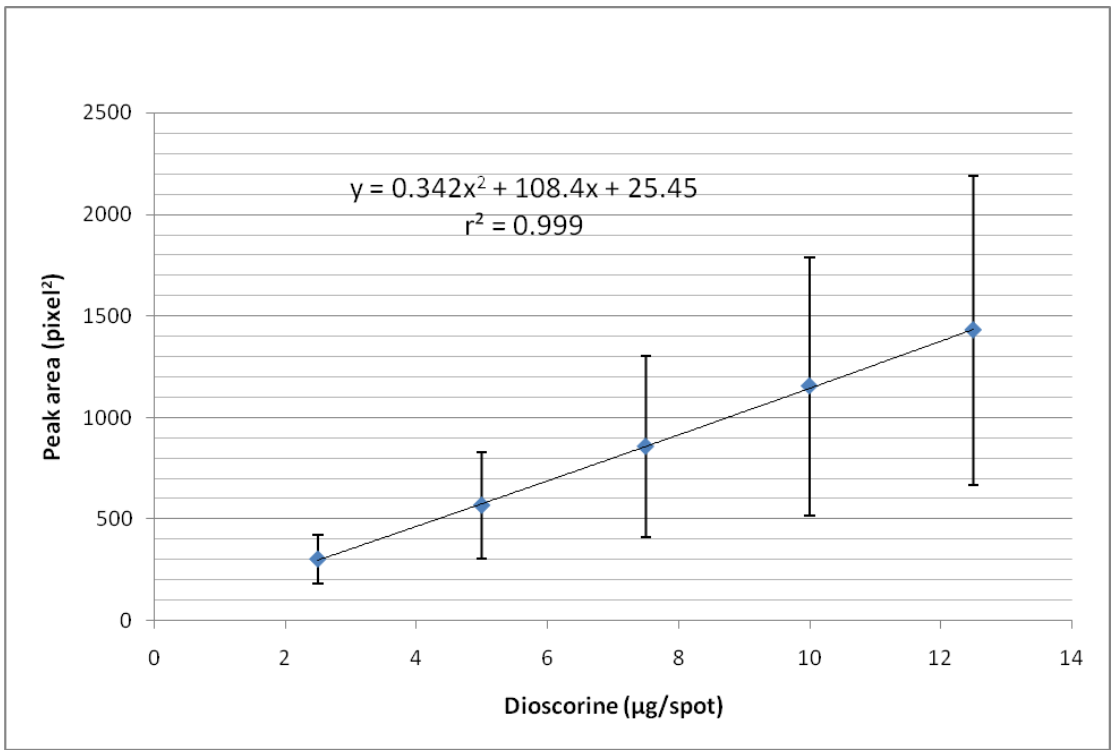


Figure 11 The calibration curve of dioscorine by TLC image analysis

Accuracy

The recovery of dioscorine from the extract was performed on samples spiked with three different concentrations of dioscorine standard (0.375, 0.75, 1.50 µg/µl). The accuracy of dioscorine was determined and the average of % recovery was found to be 90.31 ± 9.96. (Table 10)

Table 10 Recovery study of dioscorine by TLC image analysis (n = 3)

Amount of dioscorine added (µg/spot)	**Amount of dioscorine detected (µg/spot)**	**Recovery (%)**
0	3.175	
1.875	5.051	100.05
3.750	6.531	89.49
7.500	9.279	81.39
	Average	**90.31 ± 9.96**

Precision

Repeatability (within day) was evaluated by assaying each standard at 2.50, 5.00, 7.50, 10.00, 12.50 µg/spot on the same day. The intermediate precision (between days) was studied by comparing the assays on different days (6 days). The %RSD of repeatability of dioscorine were 2.21, 1.70, 1.70, 1.42, and 0.40, respectively (Table 11). The %RSD of intermediate precision of dioscorine were 3.96, 4.06, 1.96, 2.13, and 0.74, respectively (Table 12).

Table 11 The repeatability (within day) of dioscorine by TLC image analysis (n = 3)

Concentration (µg/spot)	No.	Concentration calculated from peak area (µg/spot)
2.50	1	2.54
	2	2.44
	3	2.53
	Average	2.50
	SD	0.06
	%RSD	2.21
5.00	1	4.94
	2	5.11
	3	5.05
	Average	5.04
	SD	0.09
	%RSD	1.70
7.50	1	7.48
	2	7.48
	3	7.26
	Average	7.41
	SD	0.13
	%RSD	1.70
10.00	1	10.07
	2	9.94
	3	10.23
	Average	10.08
	SD	0.14
	%RSD	1.42
12.50	1	12.47
	2	12.53
	3	12.43
	Average	12.48
	SD	0.05
	%RSD	0.40

Table 12 The intermediate precision (between days) of dioscorine by TLC image analysis (n = 6)

Concentration (µg/spot)	Day	Concentration calculated from peak area (µg/spot)
2.50	1	2.49
	2	2.63
	3	2.48
	4	2.54
	5	2.59
	6	2.35
	Average	2.51
	SD	0.10
	%RSD	3.96
5.00	1	4.98
	2	4.75
	3	5.08
	4	5.00
	5	4.75
	6	5.28
	Average	4.97
	SD	0.20
	%RSD	4.06
7.50	1	7.60
	2	7.49
	3	7.42
	4	7.28
	5	7.71
	6	7.53
	Average	7.50
	SD	0.15
	%RSD	1.96
10.00	1	9.91
	2	10.26
	3	10.03
	4	10.29
	5	10.00
	6	9.73
	Average	10.04
	SD	0.21
	%RSD	2.13

Table 12 The intermediate precision (between days) precision of dioscorine by TLC image analysis (n = 6) (cont.)

Concentration (ng/spot)	Day	Concentration (µg/spot)
12.50	1	12.53
	2	12.37
	3	12.50
	4	12.39
	5	12.46
	6	12.62
	Average	12.48
	SD	0.09
	%RSD	0.74

LOD and LOQ

For this study, LOD and LOQ values were determined based on estimated standard deviation of the response and the slope. The slope and standard deviation of the response were estimated from 6 calibration curves. The slope value and standard deviation of the response were 113.57 and 9.51, respectively. The LOD value was 0.28 µg/spot which was the lowest amount of analyzing in sample that can be detected but not necessary quantitated as an exact value. LOQ for dioscorine was 0.84 µg/spot which was the lowest concentration of sample, accurately detected and integrated by TLC image analysis using Scion image software.

3. Determination of dioscorine content in ethanolic extract by TLC-densitometry

The dioscorine content in ethanolic extract of *D. hispida* tubers from 14 different locations were evaluated by TLC-densitometry.

The yield of dioscorine from each location was shown in Table 13. Average yield of dioscorine from 100 grams of dried powder was 0.717 ± 0.070 grams. The highest dioscorine (1.378 % w/w) was found in the samples from Nong Khai. The lowest dioscorine (0.393 % w/w) was found in the samples from Bangkok 2.

Table 13 Yield of dioscorine from 14 different locations of Thailand (% dry weight)

No.	Location	Dioscorine content in dried powder (% dry weight)			
		No.1	**No.2**	**No.3**	**Average**
1	Bangkok 1	0.756	0.797	0.748	0.767
2	Bangkok 2	0.353	0.441	0.385	0.393
3	Bangkok 3	0.425	0.454	0.411	0.430
4	Chiang Mai	0.429	0.473	0.465	0.456
5	Kalasin	0.462	0.538	0.499	0.500
6	Lop Buri	1.493	1.315	1.311	1.373
7	Nakhon Pathom	0.644	0.628	0.562	0.611
8	Nakhon Sawan	0.807	0.889	0.845	0.847
9	Nakhon Si Thammarat	0.460	0.431	0.391	0.427
10	Nong Khai	1.238	1.581	1.316	1.378
11	Ratchaburi	0.697	0.806	0.758	0.754
12	Rayong	0.466	0.571	0.467	0.501
13	Surat Thani	0.919	0.858	0.843	0.873
14	Uthai Thani	0.646	0.811	0.727	0.728
Average					**0.717 ± 0.070**

3.1 Method validation

Linearity

The *Rf* values and peak area of dioscorine standard (2.5, 5.0, 7.5, 10.0, and 12.5 µg/spot) were shown in Table 14. Five concentration levels of dioscorine were plotted against for a polynomial calibration curve which its correlation coefficient (r^2) was 0.999 (Figure 12) and polynomial equation was $y = -12.90x^2 + 943.5x + 192.9$

Table 14 The polynomial data of dioscorine by TLC-densitometry

Concentration (µg/spot)	Injection No.	Peak area (pixel^2)	Average	SD
2.5	1	1148.7		
	2	3250.8		
	3	2740.7		
	4	2527.0		
	5	2445.2		
	6	2913.7	2504.350	724.212
5.0	1	1700.4		
	2	6093.2		
	3	5233.6		
	4	4542.6		
	5	4611.3		
	6	4940.9	4520.333	1491.330
7.5	1	2343.9		
	2	8932.0		
	3	7531.8		
	4	6591.7		
	5	6772.5		
	6	7117.1	6548.167	2222.699
10.0	1	3024.0		
	2	11326.7		
	3	9938.6		
	4	8337.1		
	5	8571.0		
	6	9203.5	8400.150	2846.508
12.5	1	3856.7		
	2	13956.3		
	3	11697.0		
	4	9740.7		
	5	9823.2		
	6	10563.9	9939.633	3366.508

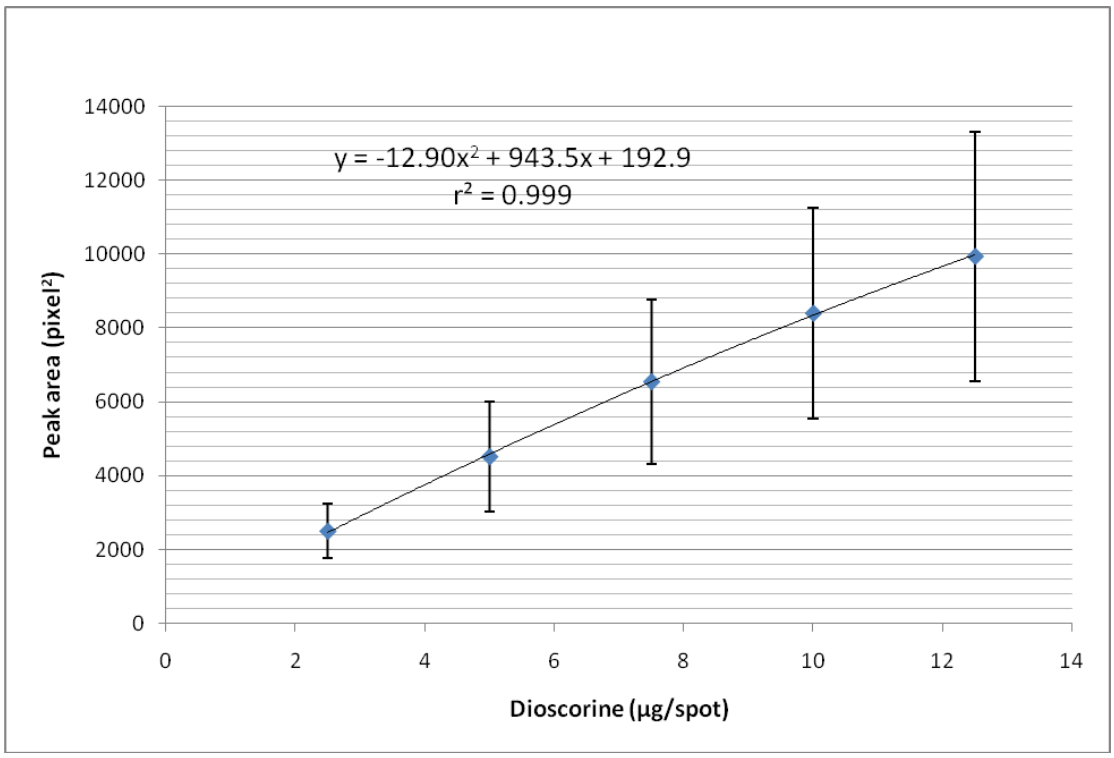


Figure 12 The calibration curve of dioscorine by TLC-densitometry

Accuracy

The recovery of dioscorine from the extract was performed on samples spiked with three different concentrations of dioscorine standard (0.375, 0.75, 1.50 µg/µl). The accuracy of dioscorine was determined and the average of % recovery was found to be 90.31 ± 9.96. (Table 15)

Table 15 Recovery study of dioscorine by TLC-densitometry (n = 3)

Amount of dioscorine added (µg/spot)	**Amount of dioscorine detected (µg/spot)**	**Recovery (%)**
0	2.922	
1.875	4.800	100.16
3.750	6.213	87.76
7.500	9.490	87.57
	Average	**91.83 ± 7.21**

Precision

Repeatability (within day) was evaluated by assaying each standard at 2.50, 5.00, 7.50, 10.00, 12.50 µg/spot and during the same day. The intermediate precision (between days) was studied by comparing the assays on different days (6 days). The %RSD of repeatability of dioscorine were 1.49, 3.74, 0.65, 0.81, and 0.38, respectively (Table 16). The %RSD of intermediate precision of dioscorine were 1.57, 1.52, 0.92, 1.52, and 0.59, respectively (Table 17).

Table 16 The repeatability (within day) of dioscorine by TLC-densitometry (n = 3)

Concentration (µg/spot)	No.	Concentration calculated from peak area (µg/spot)
2.50	1	2.59
	2	2.55
	3	2.62
	Average	2.59
	SD	0.04
	%RSD	1.49
5.00	1	4.70
	2	5.03
	3	4.74
	Average	4.83
	SD	0.18
	%RSD	3.74
7.50	1	7.49
	2	7.40
	3	7.47
	Average	7.45
	SD	0.05
	%RSD	0.65
10.00	1	10.23
	2	10.24
	3	10.38
	Average	10.28
	SD	0.08
	%RSD	0.81
12.50	1	12.35
	2	12.39
	3	12.29
	Average	12.34
	SD	0.05
	%RSD	0.38

Table 17 The intermediate precision (between days) of dioscorine by TLC-densitometry (n = 6)

Concentration (µg/spot)	Day	Concentration calculated from peak area (µg/spot)
2.50	1	2.48
	2	2.49
	3	2.53
	4	2.55
	5	2.56
	6	2.59
	Average	2.53
	SD	0.04
	%RSD	1.57
5.00	1	5.01
	2	4.99
	3	4.97
	4	4.89
	5	4.87
	6	4.82
	Average	4.92
	SD	0.07
	%RSD	1.52
7.50	1	7.55
	2	7.59
	3	7.40
	4	7.54
	5	7.52
	6	7.46
	Average	7.51
	SD	0.07
	%RSD	0.92
10.00	1	9.93
	2	9.89
	3	10.18
	4	10.09
	5	10.16
	6	10.28
	Average	10.09
	SD	0.15
	%RSD	1.52

Table 17 The intermediate precision (between days) of dioscorine by TLC-densitometry (n = 6) (cont.)

Concentration (µg/spot)	Day	Concentration (µg/spot)
12.50	1	12.53
	2	12.54
	3	12.42
	4	12.44
	5	12.40
	6	12.35
	Average	12.45
	SD	0.07
	%RSD	0.59

LOD and LOQ

For this study, LOD and LOQ values were determined based on estimated standard deviation of the response and the slope. The slope and standard deviation of the response were estimated from 6 calibration curves. The slope value and standard deviation of the response were 749.85 and 84.51, respectively. The LOD value was 0.37 µg/spot which was the lowest amount of analyzing in sample that can be detected but not necessary quantitated as an exact value. LOQ for dioscorine was 1.13 µg/spot which was the lowest concentration of sample, accurately detected and integrated by TLC image analysis using Scion image software.

4. Method comparison between TLC image analysis and TLC-densitometry

Dioscorine in 14 *Dioscorea hispida* samples were analysed by TLC image analysis using Scion image software and compared to the TLC-densitometry. The analytical data of both methods were shown in Table 18.

Table 18 Paired samples t – test

1. Paired samples statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	TLC image analysis	.6610	14	.31883	.08521
	TLC-densitometry	.7170	14	.32309	.08635

Paired samples statistics shows for each variable, the number of cases, the mean, the standard deviation, and the standard error of the mean

2. Paired samples correlations

		N	Correlation	Sig.
Pair 1	TLC image analysis & TLC-densitometry	14	.994	.000

Paired samples correlations shows the correlation between the two variables. The two variables are positively correlated, $r\ (N = 14) = 0.994$, $p = 0.000$.

3. Paired samples test

	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
Pair 1 TLC image analysis – TLC-densitometry	-.05600	.03551	.00949	-.07650	-.03550	-5.901	13	.000

Paired samples test shows the *t* statistics for the paired differences. Compare between content of dioscorine determine by TLC image analysis using Scion image software and TLC-densitometry. The mean was less difference, -0.056, t (13) = -5.901, p = 0.000.

TLC image analysis = dioscorine content determine by TLC image analysis using Scion image software

TLC-densitometry = dioscorine content determine by TLC-densitometry

CHAPTER V
DISCUSSIONS AND CONCLUSIONS

The quality of herbal medicine is implication of safety and efficacy, which is profile of constituents present in it. According to the WHO [3], determinations of macroscopic and microscopic characteristics are the first steps towards estrablishing the identity and the purity of such materials, and these step should be carried out before any further tests are undertaken.

This study dealt with the investigation of pharmacognostic specification of *Dioscorea hispida* tubers. The data generated from the present studies would help in the authentication of this crude drug both in slice and powder forms. The revealed macroscopic examination in this study had illustration in detail as previous report in Flora of China (Figure 13).

The thin layer chromatographic fingerprinting was performed to identify the individual substances in the mixture and to determine the purity of these substances. The TLC chromatogram showed characteristic fingerprint profiles that could be use as markers for quality evaluation and standardization of crude drug.

The physical constant evaluation of the crude drugs is an important parameter in detecting adulteration or improper handling of drugs. *D. hispida* tubers from 14 different locations throughout Thailand were determined and concluded the data as an estimated percentage values. The physicochemical parameters could be used to form the standardization of this drug as shown in Table 5. The water content was employed to control the water in crude drug. On the other hand, loss on drying controlled the loss in weight (due to water and other volatile materials) of crude drug.

Figure 332. 1–5. **Dioscorea hispida** Dennstedt, 白薯莨 bai shu liang. —1. Tuber. —2. Flowering branch showing male inflorescence. —3. Stem. —4. Male flower. —5. Infructescence. (FOC 290; FRPS 16(1): 100, pl. 31. 1985. —蒋亦炯 Jiang Xingqiang; redrawn by 蔡淑琴 Cai Shuqin).

Figure 13 Whole plant of *Dioscorea hispida* Dennst. in Flora of China [49]

Phytochemical screening was used to detect principle compounds in the plants. Various species of *Dioscorea* are known to be poisonous [50 – 51]. Previous study reported that only *D. hispida* and *D. dumetorum* [52] contained dioscorine whereas most of yam species were free from such toxic alkaloid [6]. The principal alkaloid of *D. hispida* grown in Thailand was found to be dioscorine [53]. Qualitative chemical examination of *D. hispida* tubers revealed the presence of isoquinuclidine alkaloid, dioscorine. It is proved that the tuber of *D. hispida* contains dioscorine as a main alkaloid. The limitation of this study may be due to the standard dioscorine purity. Dioscorine compound is not commercially available, so the standard dioscorine was prepared from dried tubers by ethanol extraction, picrate crystallization, back extraction and column chromatographic purification. Nevertheless, the identification of isolated dioscorine from *D. hispida* was examined by ^{1}H NMR and ^{13}C NMR and shown to be dioscorine after comparison with previous reported [9, 33 – 34].

Making known that dioscorine is a toxic principle in *D. hispida* tuber [54 – 55]. There are toxicities from consuming raw *D. hispida* tubers [52, 56 – 57]. The amount of dioscorine content from the tubers had a much higher specific activity than that found in the leaves and stems, influentially indicating that the tubers was probably the primary site of alkaloid [58]. The dioscorine content of the dried tuber crude drugs was around 0.66 – 0.72 % by weight as shown in Table 8 and 13. The dioscorine content of fresh tubers of *D. hispida* were 0.12% [6] and 0.017 [9] – 0.060 % [36] which previously reported in and out of Thailand respectively. The different values of the dioscorine content from literatures may be due to the extraction process, the analytic method, the origin of *D. hispida* tuber and crude drug forms.

For development of the optimum mobile phase, several trials for TLC silica gel 60 GF_{254} were done to separate dioscorine using lots of developing systems. The thin layer chromatography mobile phase initially employed was chloroform: methanol: ammonia (100: 10: 5, v/v/v) based on the method in the previous report [5]. However, the distance moved by the dioscorine usually showed a long tail. The mobile phase was changed to acetone: water: ammonia and adjusted ratio to 90: 10: 7 (v/v/v). The resolution was satisfactory for giving suitable R_f values of dioscorine. Also the development of the spots in saturated chamber more than 30 minutes

produced better spot shapes and separation than using an unsaturated one. The best separation of spots was obtained upon TLC aluminium oxide 60 GF_{254} neutral using chloroform: methanol (97: 3 v/v) as a developing system because silica gel has less adsorptive power than alumina. However, the isolation of pure alkaloid dioscorine by using silica gel column chromatography can be made because the separating band of alkaloids was sharp enough for purification.

To our knowledge, no article related to the TLC-densitometry determination of dioscorine has ever been mentioned in literature. Nowadays TLC-densitometry is becoming a routine analysis technique due to advantage of low operating cost, high simple throughput and need for minimum sample clean up. The major advantage of TLC-densitometry is that several samples can be run simultaneous using a small quantity of mobile phase, thus lowering analysis time and cost per analysis.

This study was attempted to develop the TLC method for determining dioscorine content. Both TLC-densitometry and TLC image analysis using Scion image software were used to determined dioscorine in *D. hispida*. These methods were suitable for dioscorine determination. The Scion image software was easy to use and low cost. It is a public domain software that can be downloaded from www.scioncorp.com [59]. Whereas, TLC-densitometry was required long procedure with many steps and long time for analysis.

The image analysis software – Scion Image was used for quantitative evaluation of dioscorine from TLC images and compared to TLC-densitometry method. From data aforementioned, TLC image analysis using Scion image software could be further applied for rapid determination of dioscorine and might be used as alternative to more expensive quantitative chromatographic methods, which could not be afforded by small laboratory. The developed TLC image analysis using Scion image software and TLC-densitometry technique are precise and accurate for quantitation of dioscorine in the extract of *D. hispida* tuber. These methods have several advantages over the other analytical procedures such as low cost, simple pretreatment of samples, and a large number of samples which can be screened in

parallel [60]. The statistical analysis proves that both methods are reproducible and selective for the simultaneous analysis of the content of dioscorine.

By TLC image analysis, the content of dioscorine in ethanolic extract of 14 *D. hispida* tubers ranged from 0.335 – 1.386 % w/w. The grand average content of dioscorine among all ethanolic extract was 0.661 ± 0.074 % w/w (see Table 8).

By TLC-densitometry, the content of dioscorine in ethanolic extract of 14 *D. hispida* tubers ranged from 0.393 – 1.378 % w/w. The grand average content of dioscorine among all ethanolic extract was 0.717 ± 0.070 % w/w (see Table 13).

Validation of TLC image analysis and TLC-densitometry for determination of dioscorine content of *D. hispida* tubers exhibited good linear relationship with $r^2 >$ 0.99 in the concentration range of 2.5 – 12.5 μg/spot. Accuracy and precision of the methods have shown satisfactory results.

Moreover, TLC image analysis was compared to TLC-densitometry. In this study, the content of dioscorine in *D. hispida* tuber samples from 14 different locations throughout Thailand by two methods were analyzed for their dioscorine content and the results were compared. It can be seen in Table 18 that the values of dioscorine content of 14 *D. hispida* tubers determined by TLC image analysis (Mean = 0.6610, SD = 0.31883) were closed to values of dioscorine content determined by TLC-densitometry (Mean = 0.7170, SD = 0.32309). The two variables are positive correlated, r (N = 14) = 0.994, p = 0.000 and there are significantly different (t (13) = -5.901, $p < 0.05$).

The present study on pharmacognostic specification and dioscorine content of *D. hispida* tubers will provide useful information. Dioscorine could be used as a marker for quantitative analysis in standardization of *D. hispida* tubers crude drug.

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APPENDICES

APPENDIX A

Data of Pharmacognostic characters (% by weight)

of *Dioscorea hispida* tuber

Table 19 Acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, water content (% by weight) of *Dioscorea hispida* tubers from Bangkok 1.

Parameter	**Crude drug sample**	**Amount (% by weight)**	**Mean**	**SD**
Acid-insoluble ash	No. 1	0.92		
	No. 2	0.90		
	No. 3	0.92	0.91	0.01
Total ash	No. 1	3.19		
	No. 2	3.21		
	No. 3	3.13	3.18	0.04
Ethanol-soluble extractive	No. 1	1.42		
	No. 2	1.59		
	No. 3	1.59	1.53	0.10
Water-soluble extractive	No. 1	13.93		
	No. 2	13.60		
	No. 3	13.62	13.72	0.19
Loss on drying	No. 1	10.73		
	No. 2	11.39		
	No. 3	11.54	11.22	0.43
Water content	No. 1	11.50		
	No. 2	11.50		
	No. 3	11.50	11.50	0.00

Table 20 Acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, water content (% by weight) of *Dioscorea hispida* tubers from Bangkok 2.

Parameter	**Crude drug sample**	**Amount (% by weight)**	**Mean**	**SD**
Acid-insoluble ash	No. 1	0.73		
	No. 2	0.71		
	No. 3	0.95	0.80	0.14
Total ash	No. 1	3.22		
	No. 2	3.18		
	No. 3	3.24	3.21	0.03
Ethanol-soluble extractive	No. 1	2.59		
	No. 2	2.39		
	No. 3	2.87	2.62	0.24
Water-soluble extractive	No. 1	16.37		
	No. 2	16.35		
	No. 3	16.51	16.41	0.09
Loss on drying	No. 1	11.17		
	No. 2	10.98		
	No. 3	10.83	10.99	0.17
Water content	No. 1	11.00		
	No. 2	11.00		
	No. 3	10.00	10.67	0.58

Table 21 Acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, water content (% by weight) of *Dioscorea hispida* tubers from Bangkok 3.

Parameter	**Crude drug sample**	**Amount (% by weight)**	**Mean**	**SD**
Acid-insoluble ash	No. 1	0.79		
	No. 2	0.73		
	No. 3	0.86	0.79	0.06
Total ash	No. 1	3.24		
	No. 2	3.19		
	No. 3	3.19	3.21	0.03
Ethanol-soluble extractive	No. 1	3.03		
	No. 2	3.34		
	No. 3	2.94	3.10	0.21
Water-soluble extractive	No. 1	13.98		
	No. 2	13.24		
	No. 3	13.48	13.57	0.38
Loss on drying	No. 1	11.45		
	No. 2	11.43		
	No. 3	11.90	11.59	0.27
Water content	No. 1	12.00		
	No. 2	12.00		
	No. 3	12.00	12.00	0.00

Table 22 Acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, water content (% by weight) of *Dioscorea hispida* tubers from Chiang Mai

Parameter	**Crude drug sample**	**Amount (% by weight)**	**Mean**	**SD**
Acid-insoluble ash	No. 1	0.67		
	No. 2	0.78		
	No. 3	0.72	0.72	0.06
Total ash	No. 1	3.07		
	No. 2	3.05		
	No. 3	3.00	3.04	0.04
Ethanol-soluble extractive	No. 1	2.70		
	No. 2	2.71		
	No. 3	2.84	2.75	0.08
Water-soluble extractive	No. 1	16.33		
	No. 2	16.56		
	No. 3	16.03	16.31	0.27
Loss on drying	No. 1	13.15		
	No. 2	12.97		
	No. 3	12.79	12.97	0.18
Water content	No. 1	13.00		
	No. 2	12.00		
	No. 3	13.00	12.67	0.58

Table 23 Acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, water content (% by weight) of *Dioscorea hispida* tubers from Kalasin.

Parameter	**Crude drug sample**	**Amount (% by weight)**	**Mean**	**SD**
Acid-insoluble ash	No. 1	0.81		
	No. 2	0.78		
	No. 3	0.70	0.76	0.06
Total ash	No. 1	4.13		
	No. 2	4.29		
	No. 3	4.13	4.18	0.10
Ethanol-soluble extractive	No. 1	3.96		
	No. 2	4.41		
	No. 3	4.29	4.22	0.23
Water-soluble extractive	No. 1	16.57		
	No. 2	16.83		
	No. 3	17.07	16.82	0.25
Loss on drying	No. 1	10.48		
	No. 2	10.38		
	No. 3	11.42	10.76	0.57
Water content	No. 1	11.00		
	No. 2	11.00		
	No. 3	11.00	11.00	0.00

Table 24 Acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, water content (% by weight) of *Dioscorea hispida* tubers from Lop Buri.

Parameter	**Crude drug sample**	**Amount (% by weight)**	**Mean**	**SD**
Acid-insoluble ash	No. 1	0.64		
	No. 2	0.65		
	No. 3	0.54	0.61	0.06
Total ash	No. 1	2.76		
	No. 2	2.79		
	No. 3	2.79	2.78	0.02
Ethanol-soluble extractive	No. 1	1.18		
	No. 2	1.03		
	No. 3	1.26	1.16	0.12
Water-soluble extractive	No. 1	11.52		
	No. 2	11.51		
	No. 3	11.52	11.52	0.01
Loss on drying	No. 1	12.01		
	No. 2	12.08		
	No. 3	12.52	12.20	0.28
Water content	No. 1	12.40		
	No. 2	12.40		
	No. 3	12.60	12.47	0.12

Table 25 Acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, water content (% by weight) of *Dioscorea hispida* tubers from Nakhon Pathom.

Parameter	**Crude drug sample**	**Amount (% by weight)**	**Mean**	**SD**
Acid-insoluble ash	No. 1	0.51		
	No. 2	0.57		
	No. 3	0.56	0.55	0.03
Total ash	No. 1	2.82		
	No. 2	2.84		
	No. 3	2.88	2.85	0.03
Ethanol-soluble extractive	No. 1	3.23		
	No. 2	3.04		
	No. 3	3.17	3.15	0.09
Water-soluble extractive	No. 1	13.10		
	No. 2	13.04		
	No. 3	12.91	13.02	0.10
Loss on drying	No. 1	12.33		
	No. 2	11.92		
	No. 3	12.24	12.16	0.21
Water content	No. 1	11.00		
	No. 2	10.00		
	No. 3	11.00	10.67	0.58

Table 26 Acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, water content (% by weight) of *Dioscorea hispida* tubers from Nakhon Sawan.

Parameter	**Crude drug sample**	**Amount (% by weight)**	**Mean**	**SD**
Acid-insoluble ash	No. 1	2.09		
	No. 2	1.87		
	No. 3	2.08	2.01	0.12
Total ash	No. 1	4.18		
	No. 2	4.09		
	No. 3	4.24	4.17	0.08
Ethanol-soluble extractive	No. 1	1.97		
	No. 2	2.12		
	No. 3	2.22	2.10	0.13
Water-soluble extractive	No. 1	15.53		
	No. 2	15.14		
	No. 3	15.32	15.33	0.20
Loss on drying	No. 1	11.31		
	No. 2	11.19		
	No. 3	11.54	11.35	0.18
Water content	No. 1	11.00		
	No. 2	11.00		
	No. 3	10.00	10.67	0.58

Table 27 Acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, water content (% by weight) of *Dioscorea hispida* tubers from Nakhon Si Thammarat.

Parameter	**Crude drug sample**	**Amount (% by weight)**	**Mean**	**SD**
Acid-insoluble ash	No. 1	1.60		
	No. 2	1.52		
	No. 3	1.51	1.54	0.05
Total ash	No. 1	4.21		
	No. 2	4.18		
	No. 3	4.14	4.18	0.03
Ethanol-soluble extractive	No. 1	2.33		
	No. 2	2.32		
	No. 3	2.42	2.37	0.07
Water-soluble extractive	No. 1	13.70		
	No. 2	13.98		
	No. 3	14.01	13.90	0.17
Loss on drying	No. 1	8.23		
	No. 2	8.70		
	No. 3	8.05	8.32	0.34
Water content	No. 1	9.00		
	No. 2	9.00		
	No. 3	9.00	9.00	0.00

Table 28 Acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, water content (% by weight) of *Dioscorea hispida* tubers from Nong Khai.

Parameter	**Crude drug sample**	**Amount (% by weight)**	**Mean**	**SD**
Acid-insoluble ash	No. 1	1.04		
	No. 2	1.81		
	No. 3	1.20	1.35	0.40
Total ash	No. 1	4.24		
	No. 2	4.71		
	No. 3	4.54	4.50	0.24
Ethanol-soluble extractive	No. 1	8.35		
	No. 2	8.62		
	No. 3	8.21	8.39	0.21
Water-soluble extractive	No. 1	18.50		
	No. 2	18.32		
	No. 3	18.27	18.36	0.12
Loss on drying	No. 1	12.37		
	No. 2	12.50		
	No. 3	12.14	12.34	0.18
Water content	No. 1	12.00		
	No. 2	12.00		
	No. 3	13.00	12.33	0.58

Table 29 Acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, water content (% by weight) of *Dioscorea hispida* tubers from Ratchaburi.

Parameter	**Crude drug sample**	**Amount (% by weight)**	**Mean**	**SD**
Acid-insoluble ash	No. 1	1.08		
	No. 2	1.15		
	No. 3	1.05	1.09	0.05
Total ash	No. 1	4.19		
	No. 2	4.05		
	No. 3	4.06	4.10	0.08
Ethanol-soluble extractive	No. 1	2.96		
	No. 2	2.96		
	No. 3	3.15	3.02	0.11
Water-soluble extractive	No. 1	19.64		
	No. 2	19.81		
	No. 3	19.85	19.77	0.11
Loss on drying	No. 1	10.16		
	No. 2	10.86		
	No. 3	9.85	10.29	0.52
Water content	No. 1	10.00		
	No. 2	10.00		
	No. 3	10.00	10.00	0.00

Table 30 Acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, water content (% by weight) of *Dioscorea hispida* tubers from Rayong.

Parameter	**Crude drug sample**	**Amount (% by weight)**	**Mean**	**SD**
Acid-insoluble ash	No. 1	0.39		
	No. 2	0.33		
	No. 3	0.37	0.36	0.03
Total ash	No. 1	2.27		
	No. 2	2.28		
	No. 3	2.29	2.28	0.01
Ethanol-soluble extractive	No. 1	3.07		
	No. 2	2.89		
	No. 3	3.15	3.04	0.13
Water-soluble extractive	No. 1	13.99		
	No. 2	13.92		
	No. 3	13.79	13.90	0.10
Loss on drying	No. 1	12.28		
	No. 2	12.14		
	No. 3	12.50	12.31	0.18
Water content	No. 1	13.00		
	No. 2	12.00		
	No. 3	13.00	12.67	0.58

Table 31 Acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, water content (% by weight) of *Dioscorea hispida* tubers from Surat Thani.

Parameter	**Crude drug sample**	**Amount (% by weight)**	**Mean**	**SD**
Acid-insoluble ash	No. 1	0.73		
	No. 2	0.83		
	No. 3	0.64	0.74	0.10
Total ash	No. 1	3.27		
	No. 2	3.28		
	No. 3	3.27	3.27	0.01
Ethanol-soluble extractive	No. 1	1.90		
	No. 2	1.74		
	No. 3	1.85	1.83	0.08
Water-soluble extractive	No. 1	13.59		
	No. 2	14.12		
	No. 3	14.07	13.93	0.29
Loss on drying	No. 1	12.11		
	No. 2	12.84		
	No. 3	12.83	12.59	0.42
Water content	No. 1	13.00		
	No. 2	13.00		
	No. 3	13.00	13.00	0.00

Table 32 Acid-insoluble ash, total ash, ethanol-soluble extractive, water-soluble extractive, loss on drying, water content (% by weight) of *Dioscorea hispida* tubers from Uthai Thani.

Parameter	**Crude drug sample**	**Amount (% by weight)**	**Mean**	**SD**
Acid-insoluble ash	No. 1	0.77		
	No. 2	0.63		
	No. 3	0.51	0.64	0.13
Total ash	No. 1	3.15		
	No. 2	3.17		
	No. 3	3.21	3.17	0.03
Ethanol-soluble extractive	No. 1	2.70		
	No. 2	2.96		
	No. 3	2.67	2.78	0.16
Water-soluble extractive	No. 1	14.19		
	No. 2	15.21		
	No. 3	14.08	14.49	0.62
Loss on drying	No. 1	12.08		
	No. 2	11.61		
	No. 3	12.01	11.90	0.25
Water content	No. 1	13.00		
	No. 2	13.00		
	No. 3	13.00	13.00	0.00

APPENDIX B

^{1}H NMR and ^{13}C NMR spectra of isolated dioscorine in $CDCL_3$

Figure 14 The 500 MHz ^{1}H NMR spectrum of isolated dioscorine in $CDCl_3$

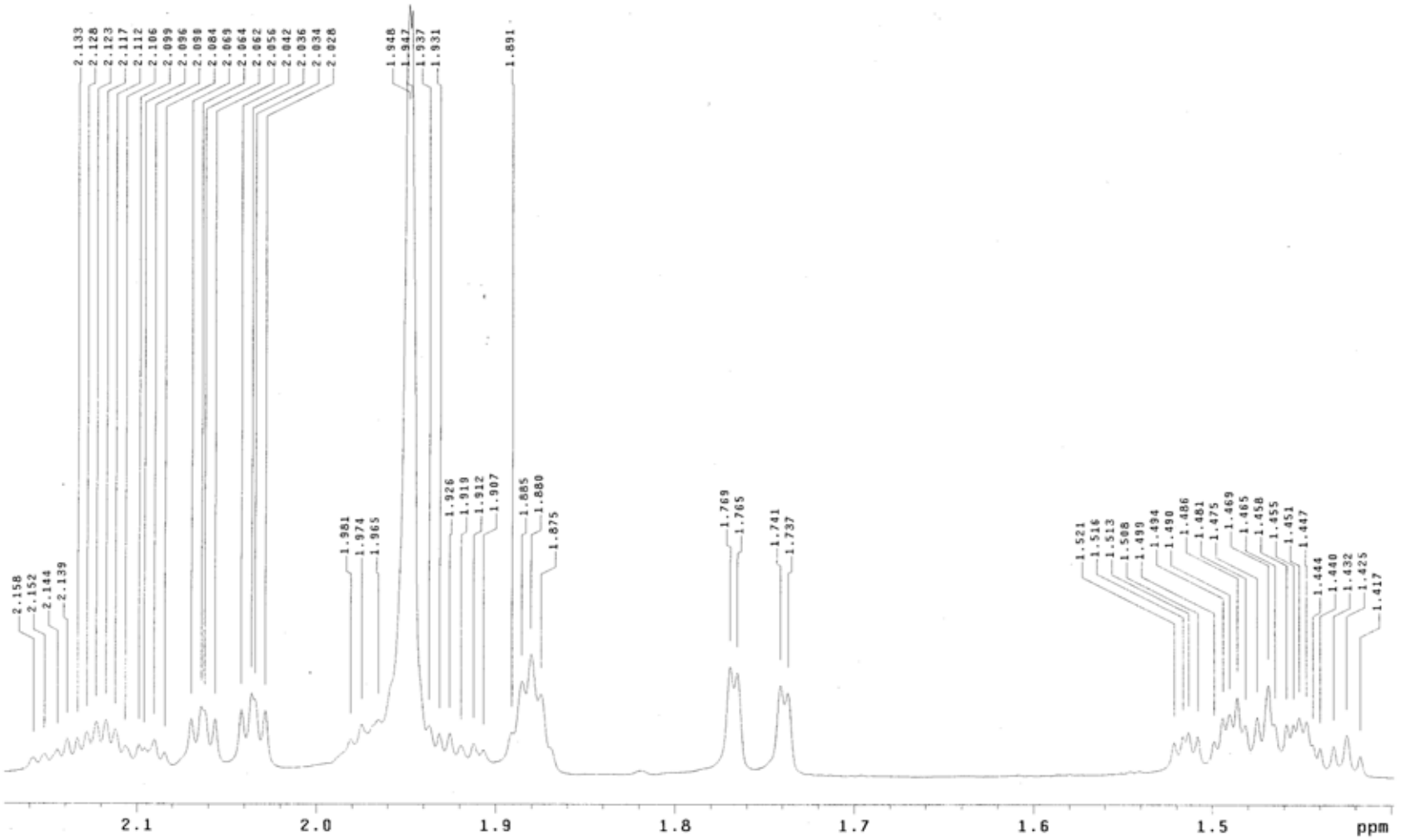

Figure 15 The 500 MHz ^{1}H NMR spectrum of isolated dioscorine in $CDCl_3$ (Continued)

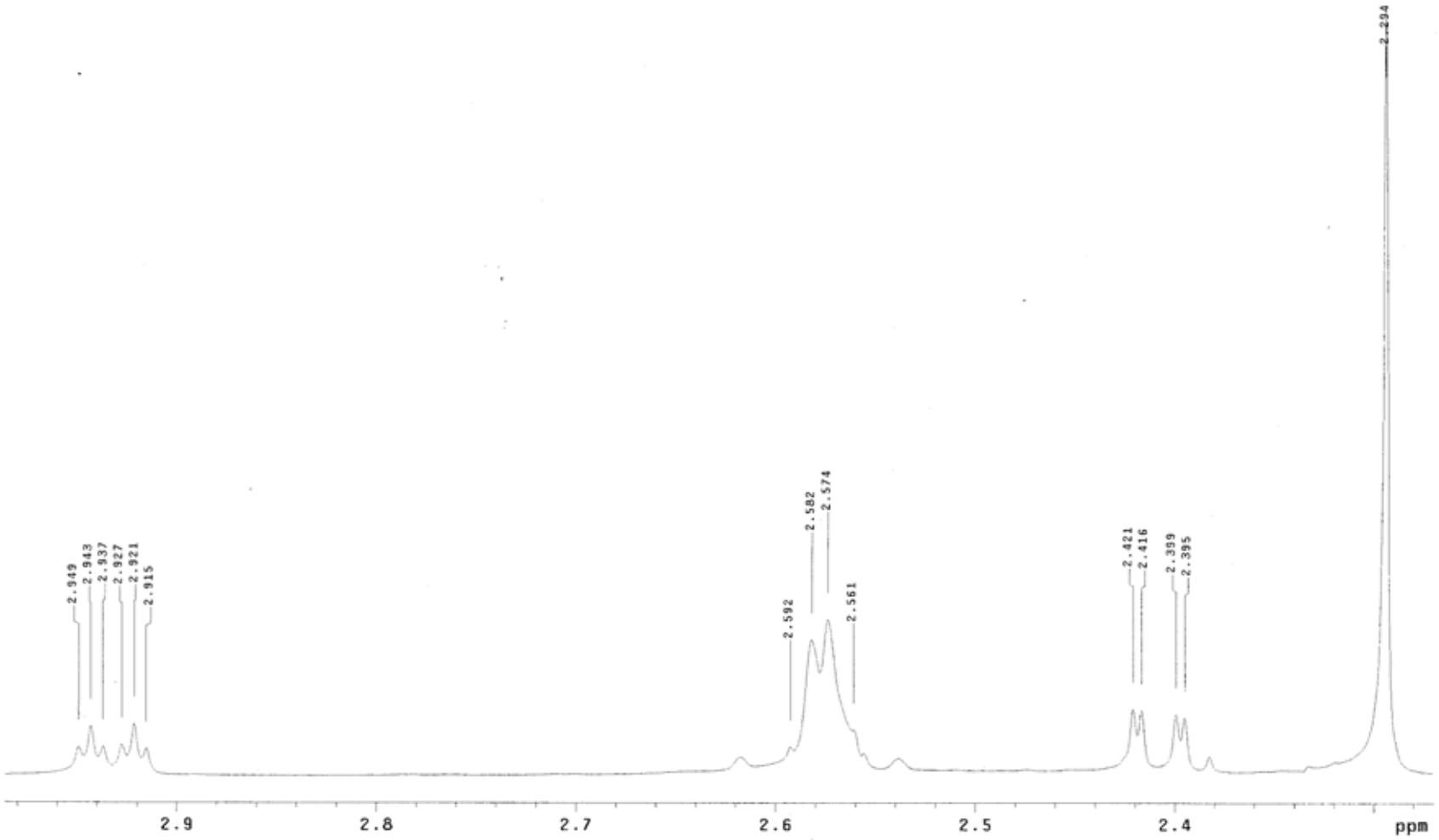

Figure 16 The 500 MHz ^{1}H NMR spectrum of isolated dioscorine in $CDCl_3$ (Continued)

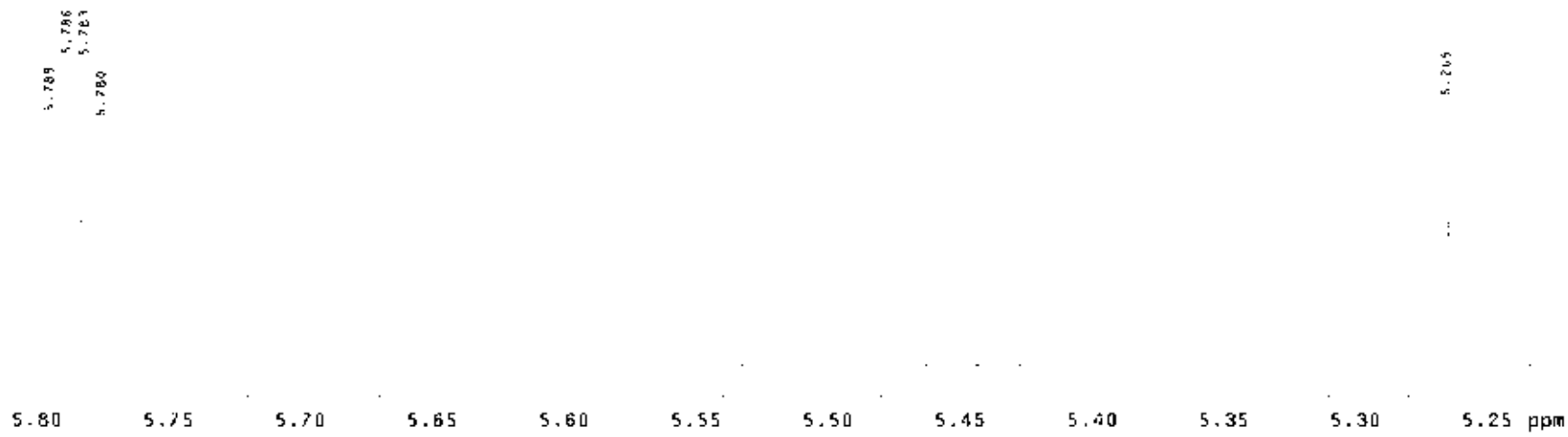

Figure 17 The 500 MHz ^{1}H NMR spectrum of isolated dioscorine in $CDCl_3$ (Continued)

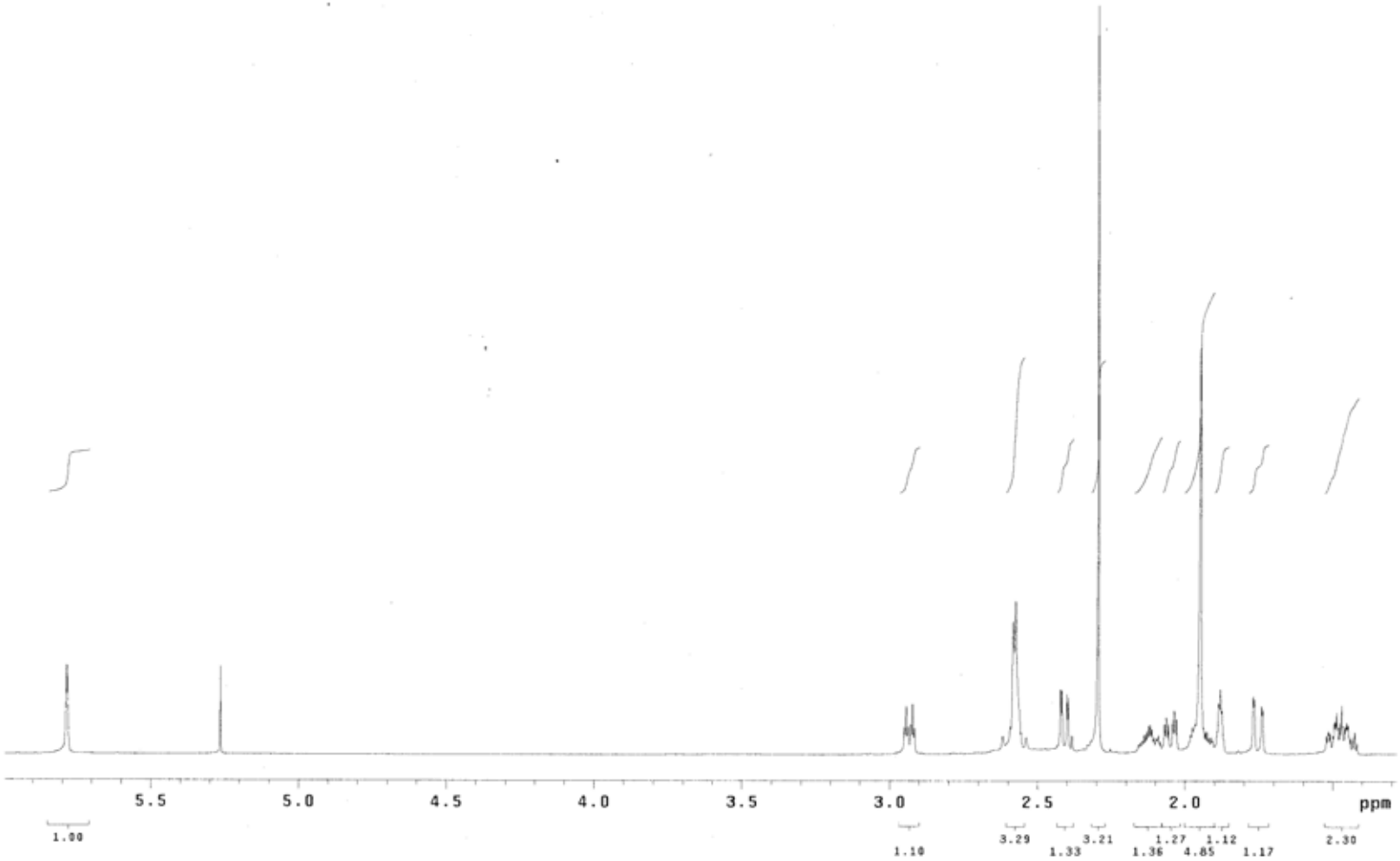

Figure 18 The 500 MHz ^{1}H NMR spectrum and integration of isolated dioscorine in $CDCl_3$

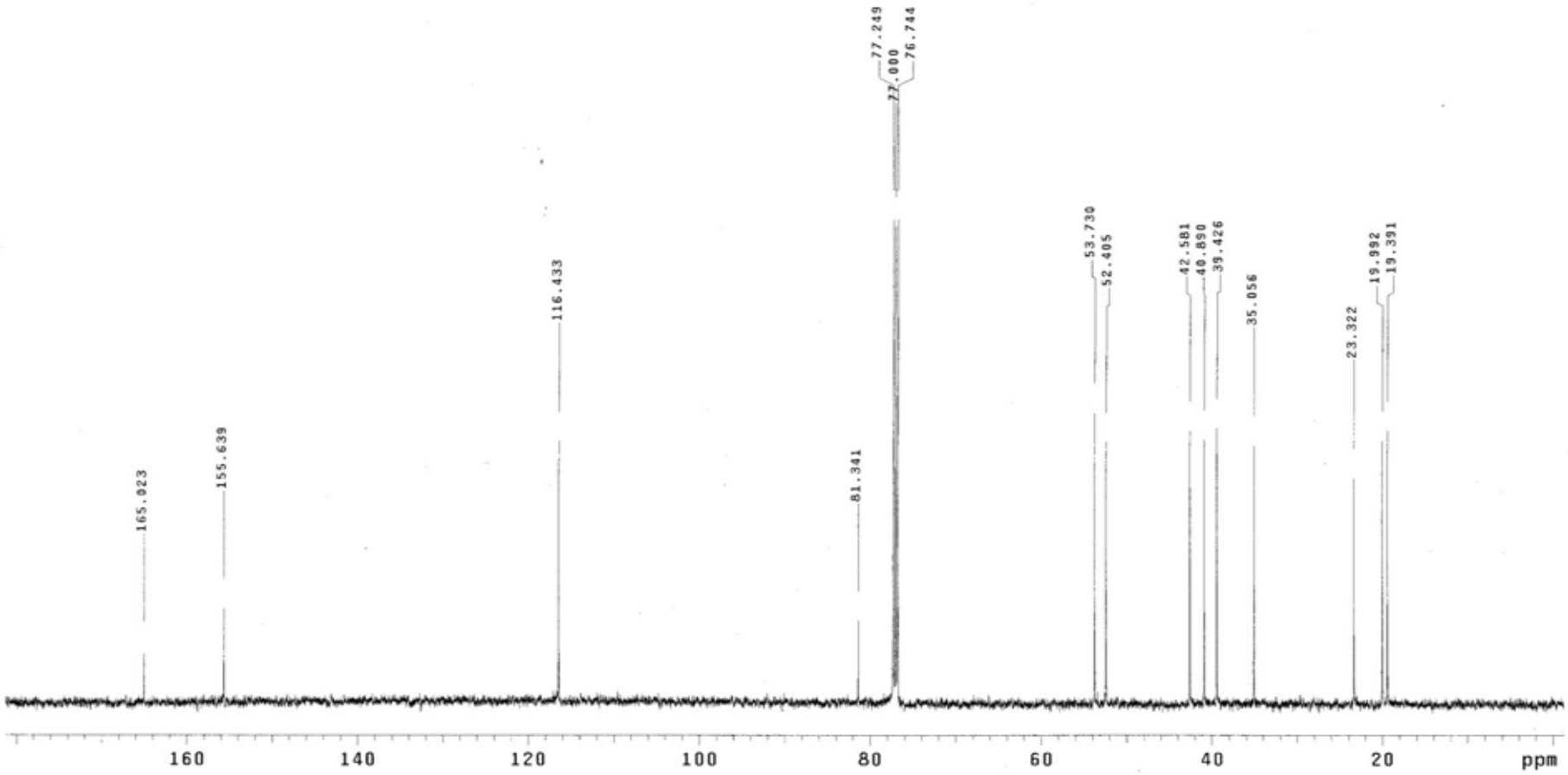

Figure 19 The 125 MHz ^{13}C NMR spectrum of isolated dioscorine in $CDCl_3$

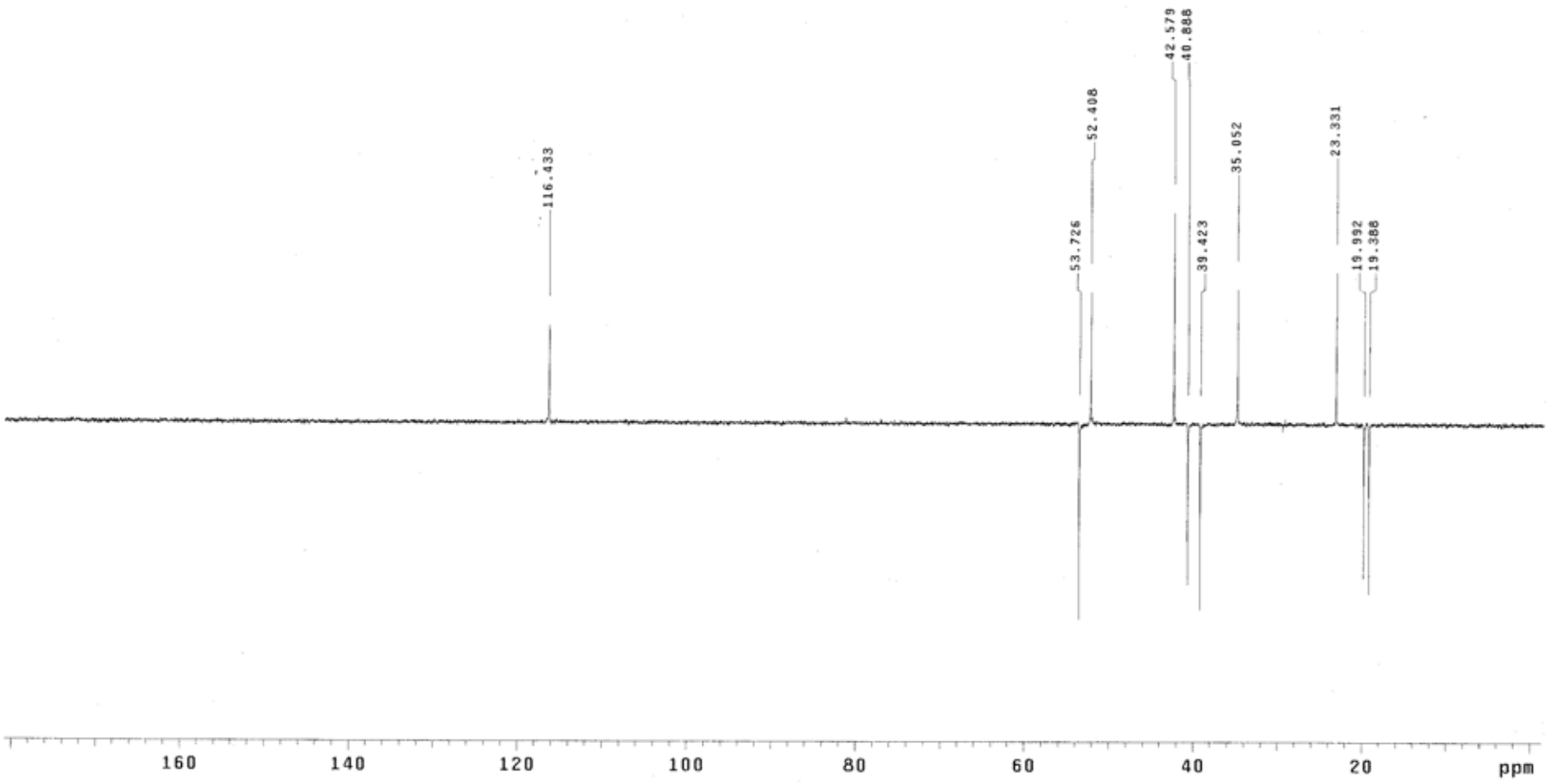

Figure 20 The 125 MHz ^{13}C NMR spectrum with DEPT of isolated dioscorine in $CDCl_3$

APPENDIX C

Processing of TLC image analysis by Scion image software

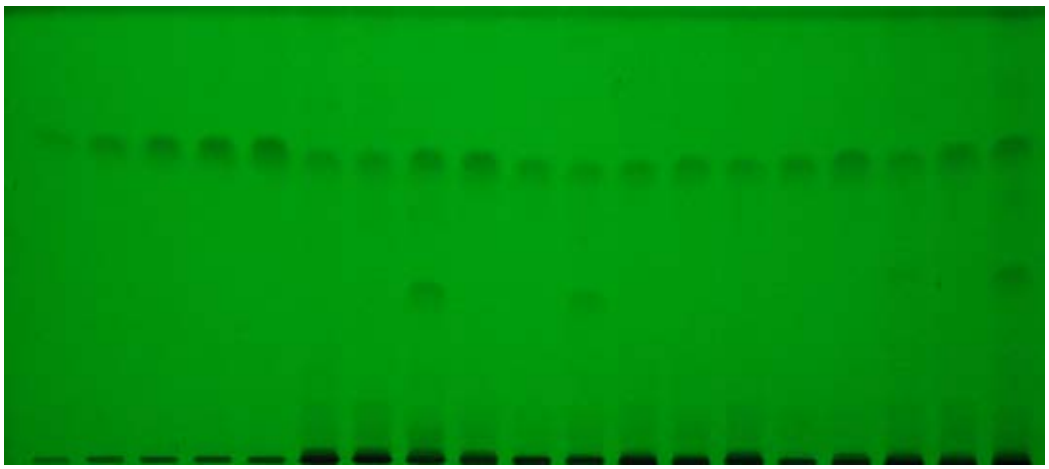

Figure 21 Thin-layer chromatography of standard dioscorine (2.5 – 12.5 µg/spot) and *Dioscorea hispida* tuber from 14 different locations detecting under UV 254 nm

From the left to right lanes:

1. Standard No.1 : Standard dioscorine (2.5 µg/spot)
2. Standard No.2 : Standard dioscorine (5.0 µg/spot)
3. Standard No.3 : Standard dioscorine (7.5 µg/spot)
4. Standard No.4 : Standard dioscorine (10.0 µg/spot)
5. Standard No.5 : Standard dioscorine (12.5 µg/spot)
6. Sample No.1 : *Dioscorea hispida* tuber from Bangkok 3
7. Sample No.2 : *Dioscorea hispida* tuber from Nakhon Si Thammarat
8. Sample No.3 : *Dioscorea hispida* tuber from Kalasin
9. Sample No.4 : *Dioscorea hispida* tuber from Bangkok 1
10. Sample No.5 : *Dioscorea hispida* tuber from Nakhon Sawan
11. Sample No.6 : *Dioscorea hispida* tuber from Ratchaburi
12. Sample No.7 : *Dioscorea hispida* tuber from Chiang Mai
13. Sample No.8 : *Dioscorea hispida* tuber from Uthai Thani
14. Sample No.9 : *Dioscorea hispida* tuber from Rayong
15. Sample No.10 : *Dioscorea hispida* tuber from Lop Buri
16. Sample No.11 : *Dioscorea hispida* tuber from Nong Khai
17. Sample No.12 : *Dioscorea hispida* tuber from Bangkok 2
18. Sample No.13 : *Dioscorea hispida* tuber from Nakhon Pathom
19. Sample No.14 : *Dioscorea hispida* tuber from Surat Thani

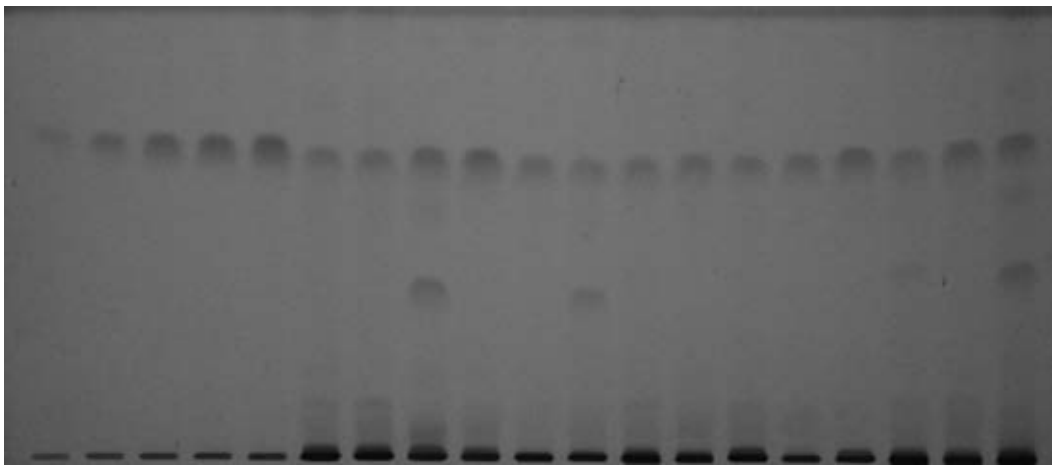

Figure 22 Thin-layer chromatography of standard dioscorine (2.5 – 12.5 µg/spot) and *Dioscorea hispida* tuber from 14 different locations detecting under UV 254 nm and convert to grayscale (From the left to right lanes as previous described in Figure 21)

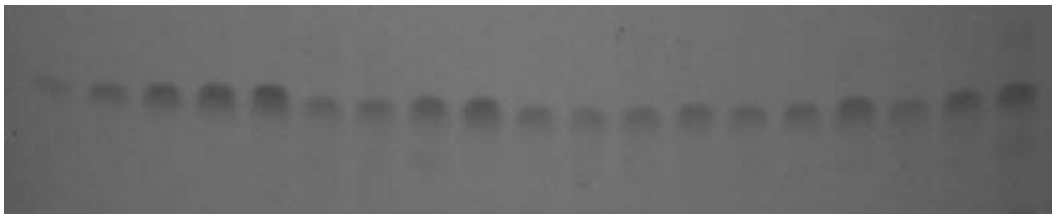

Figure 23 Thin-layer chromatography of standard dioscorine (2.5 – 12.5 µg/spot) and *Dioscorea hispida* tuber from 14 different locations in the selected area by the wand tool (From the left to right lanes as previous described in Figure 21)

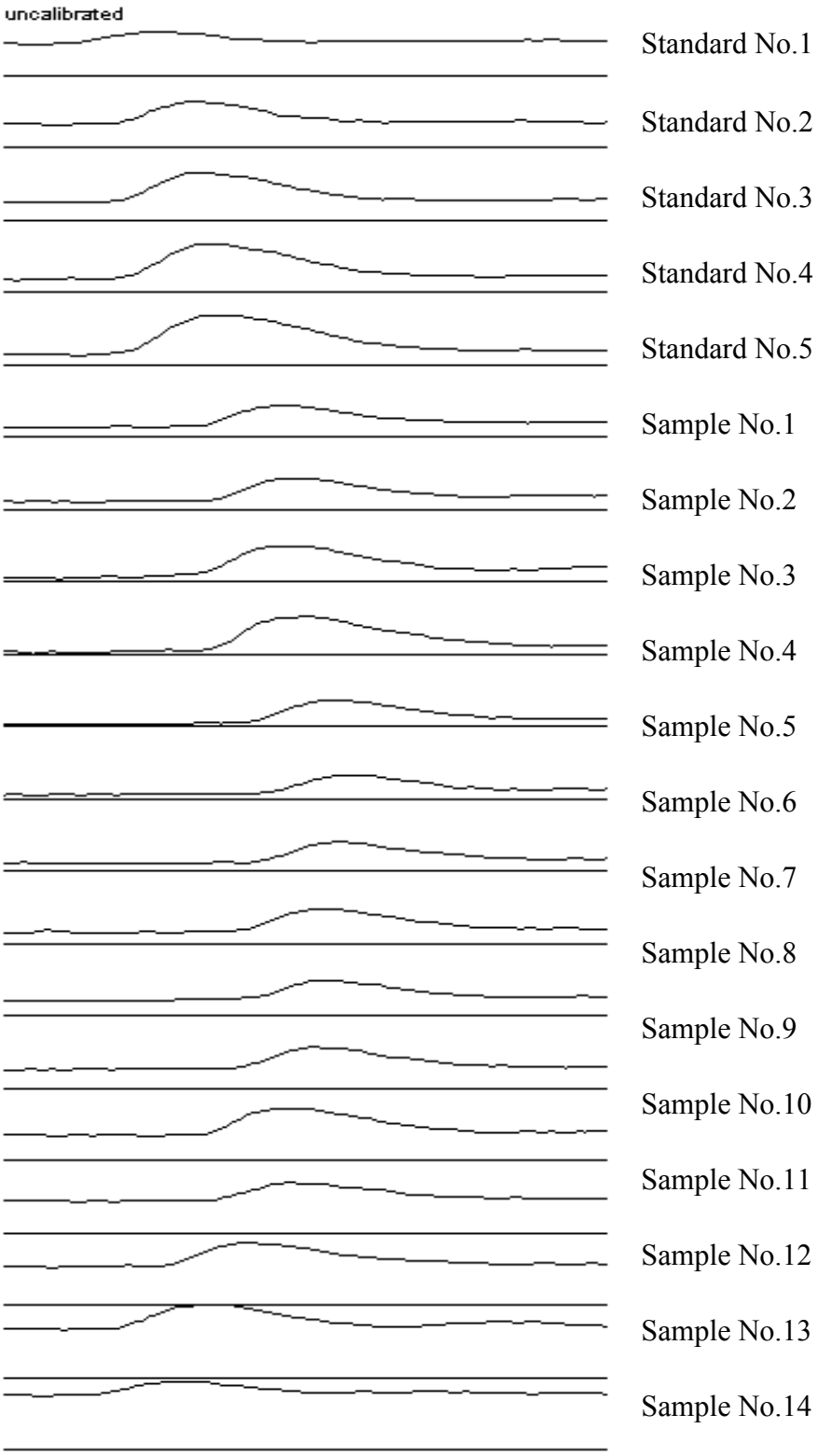

Figure 24 TLC image analysis chromatogram by Scion Image software of standard dioscorine (2.5 – 12.5 µg/spot) and dioscorine content in *Dioscorea hispida* tuber from 14 different locations (From the up to down lanes as previous described in Figure 21)

APPENDIX D

TLC-densitometry chromatogram

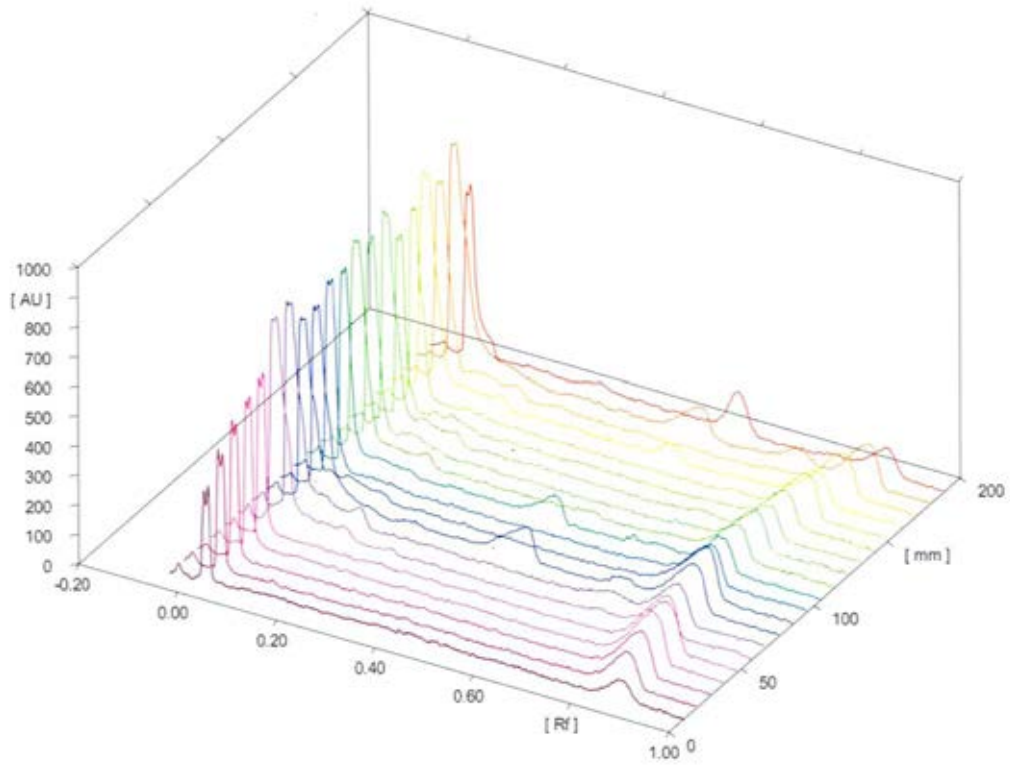

Figure 25 The TLC-densitometry chromatogram of standard dioscorine (2.5 – 12.5 µg/spot) and dioscorine content in *Dioscorea hispida* tuber from 14 different locations (From the left to right lanes as previous described in Figure 21)

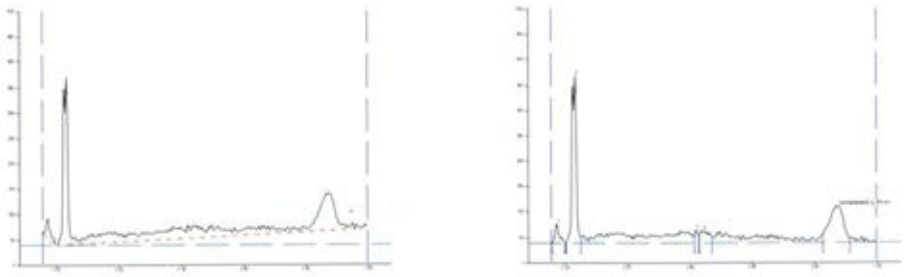

Figure 26 TLC-densitometry chromatogram of standard dioscorine (2.5 µg/spot)

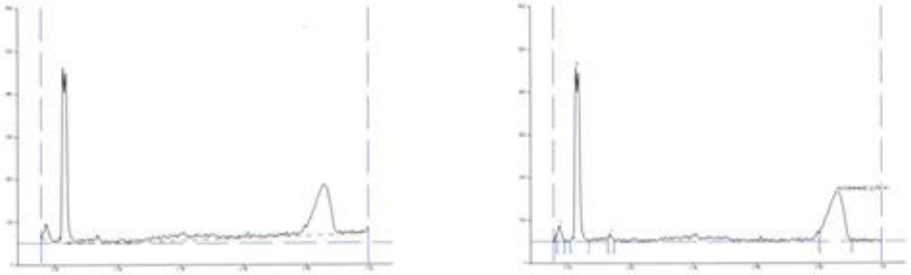

Figure 27 TLC-densitometry chromatogram of standard dioscorine (5.0 µg/spot)

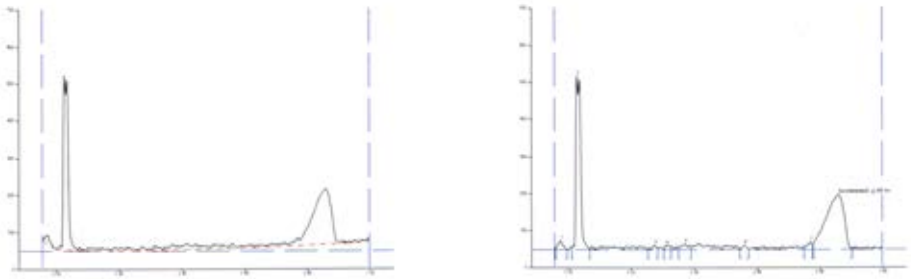

Figure 28 TLC-densitometry chromatogram of standard dioscorine (7.5 µg/spot)

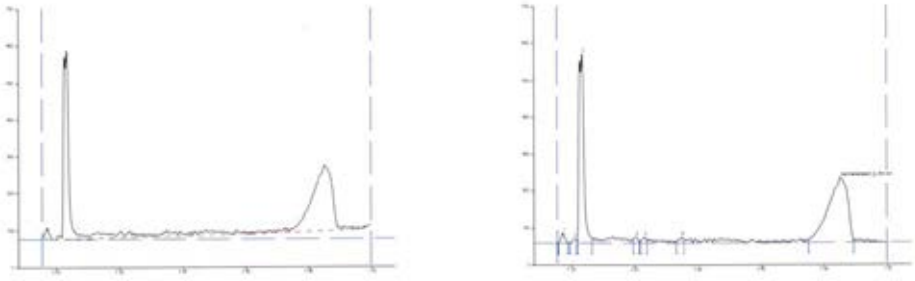

Figure 29 TLC-densitometry chromatogram of standard dioscorine (10.0 µg/spot)

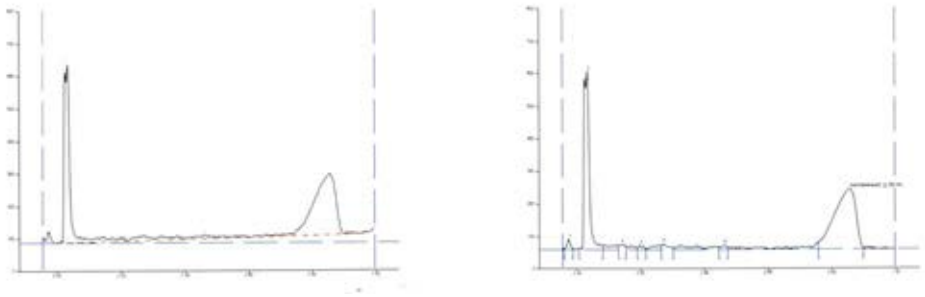

Figure 30 TLC-densitometry chromatogram of standard dioscorine (12.5 µg/spot)

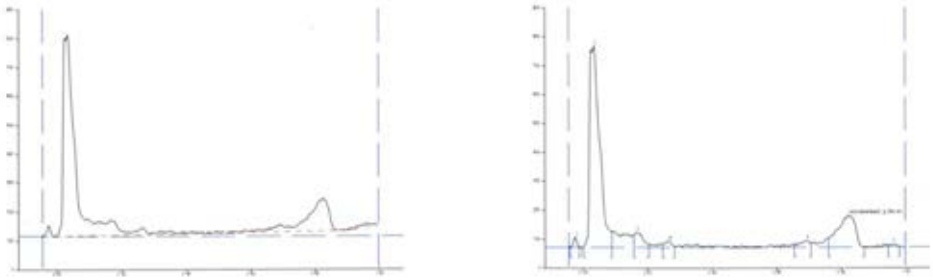

Figure 31 TLC-densitometry chromatogram of dioscorine content in *Dioscorea hispida* tuber from Bangkok 3

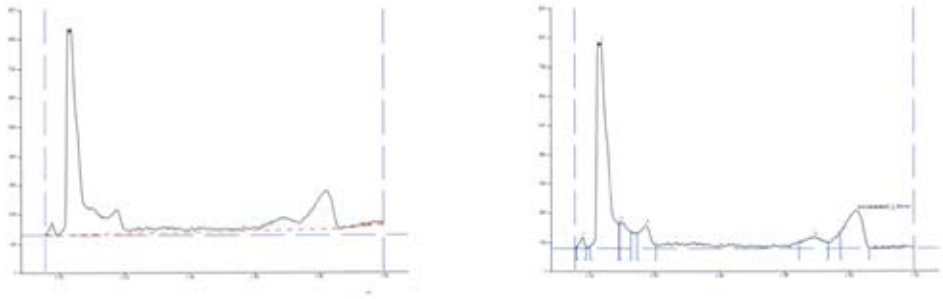

Figure 32 TLC-densitometry chromatogram of dioscorine content in *Dioscorea hispida* tuber from Nakhon Si Thammarat

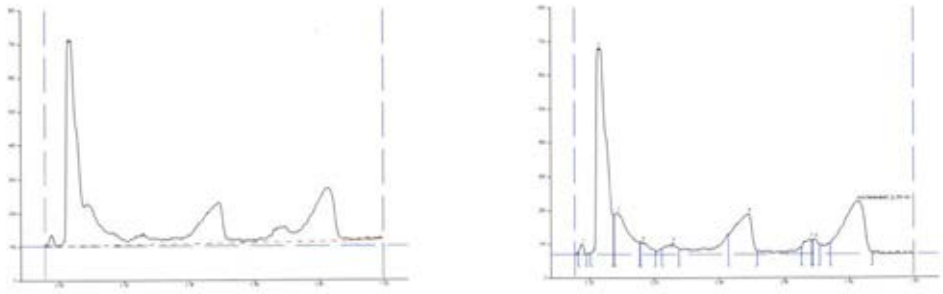

Figure 33 TLC-densitometry chromatogram of dioscorine content in *Dioscorea hispida* tuber from Kalasin

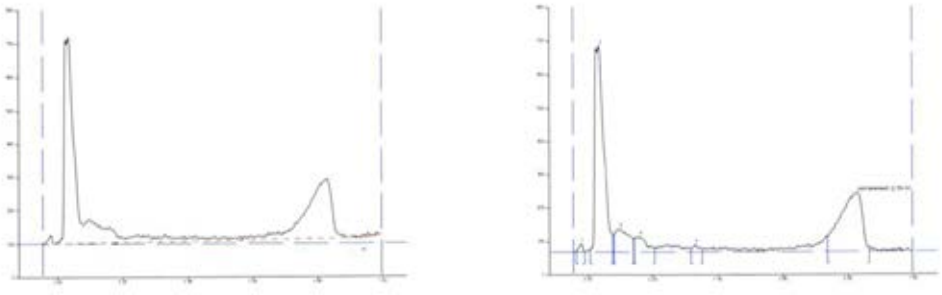

Figure 34 TLC-densitometry chromatogram of dioscorine content in *Dioscorea hispida* tuber from Bangkok 1

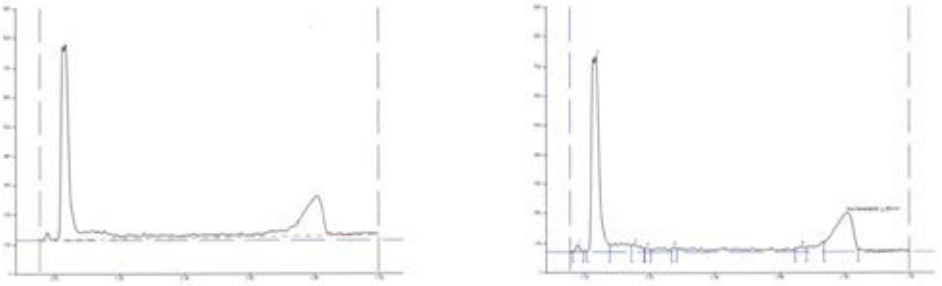

Figure 35 TLC-densitometry chromatogram of dioscorine content in *Dioscorea hispida* tuber from Nakhon Sawan

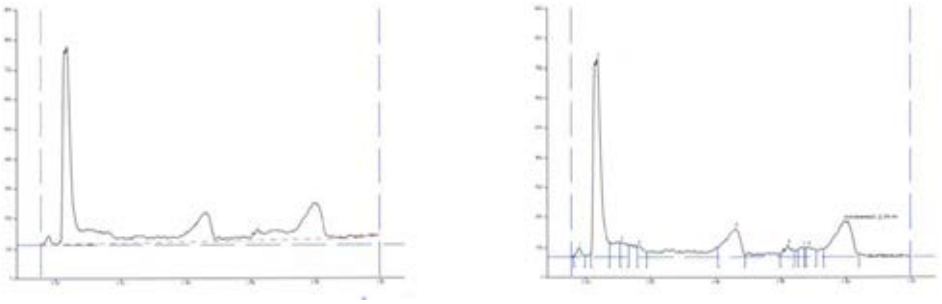

Figure 36 TLC-densitometry chromatogram of dioscorine content in *Dioscorea hispida* tuber from Ratchaburi

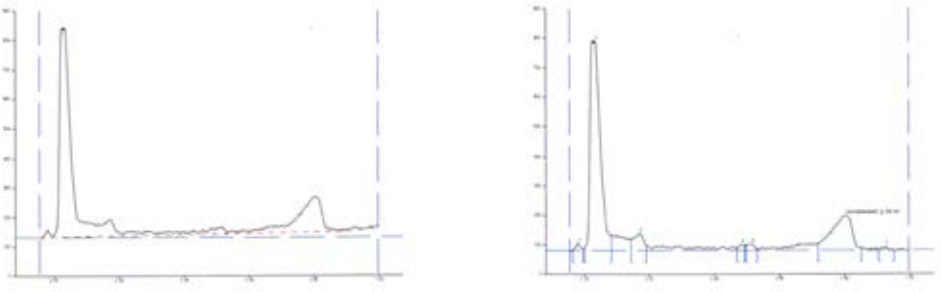

Figure 37 TLC-densitometry chromatogram of dioscorine content in *Dioscorea hispida* tuber from Chiang Mai

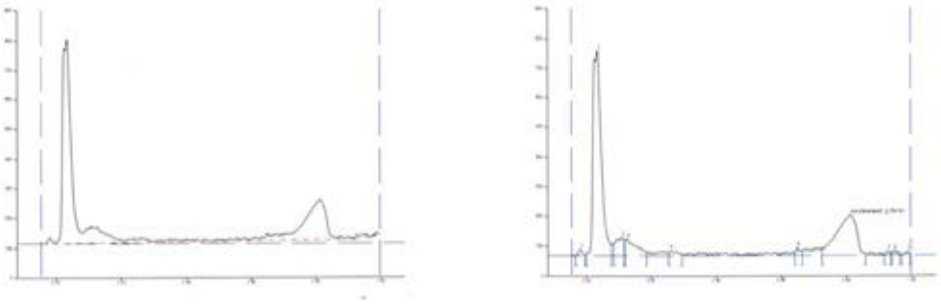

Figure 38 TLC-densitometry chromatogram of dioscorine content in *Dioscorea hispida* tuber from Uthai Thani

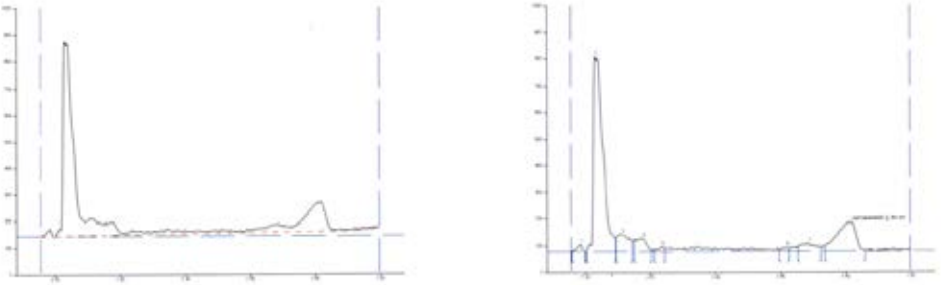

Figure 39 TLC-densitometry chromatogram of dioscorine content in *Dioscorea hispida* tuber from Rayong

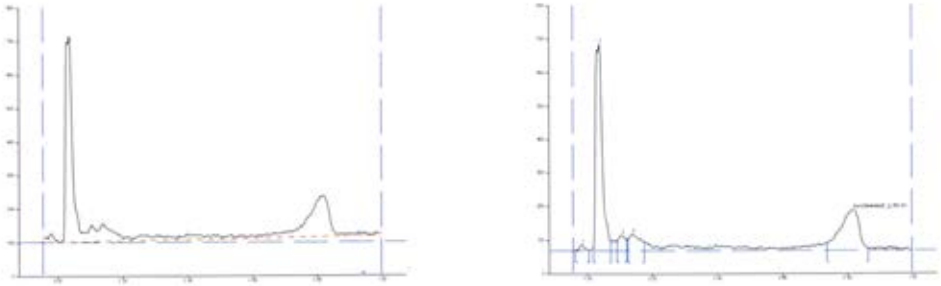

Figure 40 TLC-densitometry chromatogram of dioscorine content in *Dioscorea hispida* tuber from Lop Buri

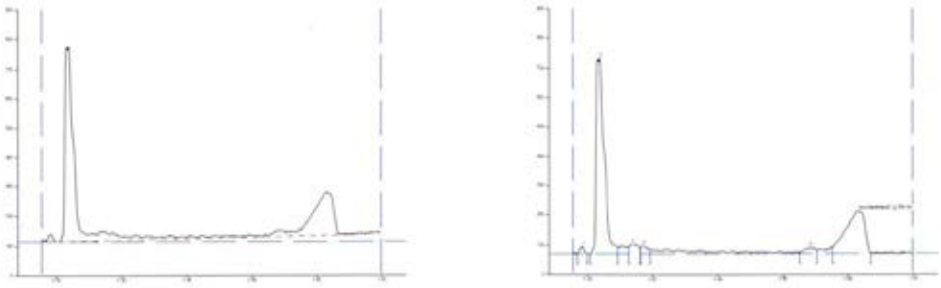

Figure 41 TLC-densitometry chromatogram of dioscorine content in *Dioscorea hispida* tuber from Nong Khai

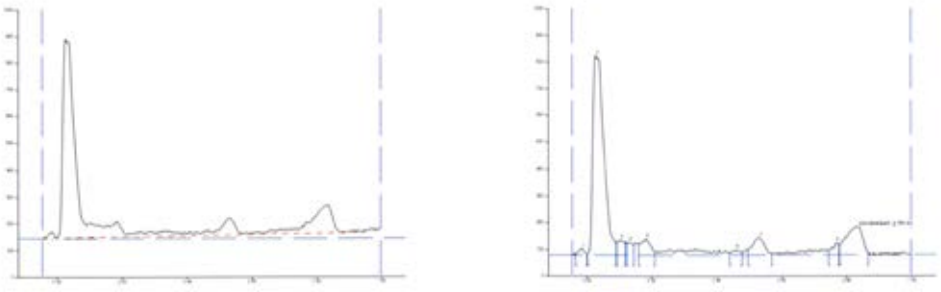

Figure 42 TLC-densitometry chromatogram of dioscorine content in *Dioscorea hispida* tuber from Bangkok 2

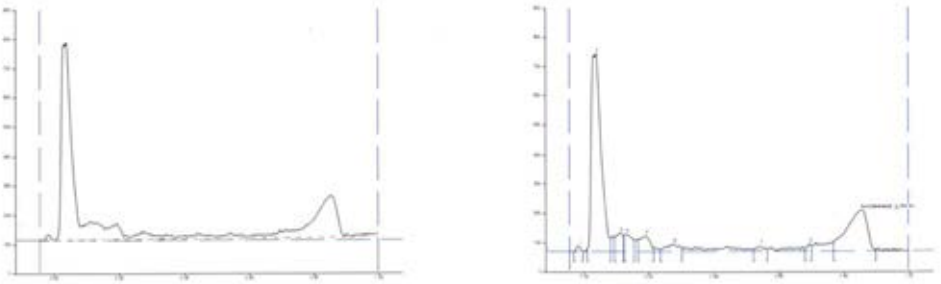

Figure 43 TLC-densitometry chromatogram of dioscorine content in *Dioscorea hispida* tuber from Nakhon Pathom

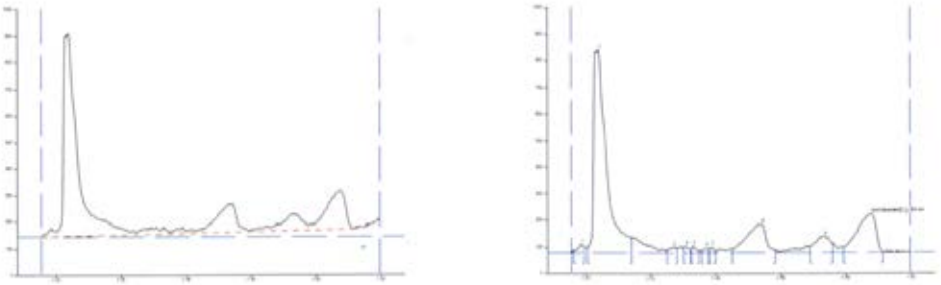

Figure 44 TLC-densitometry chromatogram of dioscorine content in *Dioscorea hispida* tuber from Surat Thani

VITAE

Miss Nonglapat Sasiwatpaisit was born on September 7, 1985 in Nan, Thailand. She received her Bachelor's degree of Applied Thai Traditional Medicine from School of Health Sciences, Mae Fah Luang University, Thailand in 2008. She worked at Strategic Information Center, Department of Development for Traditional and Alternative Medicine, Ministry of Public Health, until 2010.

Publication

Sasiwatpaisit, N., Palanuvej, C., and Ruangrungsi, N. Pharmacognostic specification and dioscorine contents of *Dioscorea hispida* tubers. Proceedings of the 7th Indochina Conference on Pharmaceutical Sciences, pp. 284 - 287. Bangkok, 2011.

Scholarships

1. Research Fund; the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund).
2. The Herbal Remedies and Alternative Medicine Task Force of STAR: Special Task Force for Activating Research under 100 Years Chulalongkorn University Fund.