PHARMACOGNOSTIC SPECIFICATION AND VASICINE CONTENT OF *ADHATODA VASICA* LEAVES

Miss Paphitchaya Thetsana

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Public Health Sciences
College of Public Health Sciences
Chulalongkorn University
Academic Year 2014

ข้อกำหนดทางเภสัชเวทและปริมาณวิเคราะห์วาไซซีนของใบเสนียด

นางสาวปพิชญา เทศนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์สาธารณสุข
วิทยาลัยวิทยาศาสตร์สาธารณสุข จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

Thesis Title	PHARMACOGNOSTIC SPECIFICATION AND VASICINE CONTENT OF *ADHATODA VASICA* LEAVES
By	Miss Paphitchaya Thetsana
Field of Study	Public Health Sciences
Thesis Advisor	Assistant Professor Chanida Palanuvej, Ph.D.
Thesis Co-Advisor	Associate Professor Nijsiri Ruangrungsi, Ph.D.

Accepted by the Faculty of College of Public Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the College of Public Health Sciences
(Associate Professor Sathirakorn Pongpanich, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Naowarat Kanchanakhan, Ph.D.)
.....Thesis Advisor
(Assistant Professor Chanida Palanuvej, Ph.D.)
.....Thesis Co-Advisor
(Associate Professor Nijsiri Ruangrungsi, Ph.D.)
.....External Examiner
(Chaisak Chansriniyom, Ph.D.)

ปพิชญา เทศนา : ข้อกำหนดทางเภสัชเวทและปริมาณวิเคราะห์วาไซซีนของใบเสนียด (PHARMACOGNOSTIC SPECIFICATION AND VASICINE CONTENT OF *ADHATODA VASICA* LEAVES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ชนิดา พลานุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. นิจศิริ เรืองรังษี, 85 หน้า.

เสนียดเป็นสมุนไพรที่นิยมใช้กันอย่างแพร่หลาย ในตำรับยาไทยนิยมใช้ใบเสนียดในการรักษาไข้และอาการไอ ในการนำสมุนไพรมาใช้ ผู้ใช้จะต้องคำนึงถึงคุณภาพที่ได้มาตรฐานของตัวยา ดังนั้นงานวิจัยนี้ จึงได้ศึกษาข้อกำหนดทางเภสัชเวท และการวิเคราะห์หาปริมาณสารสำคัญในใบเสนียด โดยการศึกษาใบเสนียดจาก 12 แหล่งทั่วประเทศ ในการตรวจลักษณะทางจุลทรรศน์ของใบเสนียดทั้งในรูปแบบของผงยาและภาคตัดขวางของเส้นกลางใบ พบว่า ในการตรวจวัดท้องใบพบจำนวนของปากใบ ดัชนีปากใบ จำนวนของขน ดัชนีขุมขน จำนวนของผลึกแคลเซียมคาร์บอเนต มีค่าเท่ากับ 288.27 ± 3.70, 17.84 ± 0.83, 28.53 ± 7.63, 2.45 ± 0.53 และ 36.50 ± 9.20 ตามลำดับ และในการตรวจวัดหลังใบพบค่าอัตราส่วนเซลล์รั้ว มีค่าเท่ากับ 6.57 ± 0.56 ในการศึกษาเอกลักษณ์ทางเคมี-ฟิสิกส์ของใบเสนียด พบว่า มีปริมาณน้ำ น้ำหนักที่หายไปเมื่อทำให้แห้ง ปริมาณเถ้ารวม และเถ้าที่ไม่ละลายในกรด ไม่เกินร้อยละ 11, 9, 21 และ 6 โดยน้ำหนัก ตามลำดับ ปริมาณสิ่งสกัดด้วยเอทานอล และปริมาณสิ่งสกัดด้วยน้ำ ไม่น้อยกว่าร้อยละ 4 และ 22 โดยน้ำหนัก ตามลำดับ การศึกษาด้วยเทคนิคทินเลเยอร์โครมาโทกราฟี โดยใช้ตัวทำละลายคลอโรฟอร์ม และ เมทานอล (9 : 1) เป็นวัฏภาคเคลื่อนที่ ตรวจวัดภายใต้แสงอัลตราไวโอเลต (254 และ 365 นาโนเมตร) และใช้น้ำยาพ่นชนิดเฉพาะเจาะจง คือน้ำยาพ่นดราเจนดอฟ (Dragendorff's reagent) การวิเคราะห์เชิงปริมาณด้วยเทคนิคทางทินเลเยอร์โครมาโทกราฟีโดยใช้ตัวทำละลายโทลูอีน เอทิลอะซีเทต และ ไดเอทิลเอมีน (5 : 2 : 3) เป็นวัฏภาคเคลื่อนที่ วิเคราะห์ปริมาณวาไซซีนโดยวิธีทางทินเลเยอโครมาโทกราฟี-เด็นซิโทเมทรีโดยใช้เครื่อง CAMAG TLC scanner ร่วมกับโปรแกรม winCATS และวิธีการวิเคราะห์รูปภาพทางทินเลเยอร์โครมาโทกราฟีโดยใช้โปรแกรม ImageJ มีช่วงวิเคราะห์แบบโพลีโนเมียล (1 - 5 ไมโครกรัมต่อจุด) และมีค่าสัมประสิทธิ์สหสัมพันธ์ เท่ากับ 0.9998 และ 0.9988 ตามลำดับ ระดับความเที่ยงของวิธีวิเคราะห์ ประเมินจากค่าสัมประสิทธิ์ของการกระจาย มีค่าระหว่างร้อยละ 1.30 – 3.96 และ 6.29 – 10.45 ตามลำดับ ค่าเฉลี่ยการคืนกลับระหว่างร้อยละ 83.60 ± 1.09 และ 85.32 ± 5.42 ตามลำดับ ขีดจำกัดของการตรวจพบและขีดจำกัดของการหาปริมาณมีค่า 0.188, 0.067 และ 0.570, 0.202 ไมโครกรัม ตามลำดับ ค่าความคงทนของวิธี มีค่าสัมประสิทธิ์ของการกระจายร้อยละ 4.48 และ 11.12 ตามลำดับ วิเคราะห์ปริมาณวาไซซีนในใบเสนียด มีค่าเฉลี่ยที่ 0.130 ± 0.065 และ 0.134 ± 0.061 กรัมต่อ100กรัมของพืชแห้ง ตามลำดับ การเปรียบเทียบปริมาณวาไซซีนระหว่าง 2 วิธี ถูกทดสอบโดยใช้สถิติ pair *t*-test พบว่า ปริมาณวาไซซีนที่วิเคราะห์โดยวิธีทั้งสองวิธีไม่แตกต่างกัน (t = 1.796 , *P* = 0.249) ผลการศึกษาครั้งนี้สามารถจัดทำเป็นข้อกำหนดมาตรฐานของสมุนไพรใบเสนียดในประเทศไทย ซึ่งจะเป็นประโยชน์ต่อการควบคุมวัตถุดิบ และการวิจัย เพื่อพัฒนาตัวยานี้ต่อไป

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5678952353 : MAJOR PUBLIC HEALTH SCIENCES
KEYWORDS: ADHATODA VASICA LEAF / QUALITY PARAMETERS / LEAF MEASUREMENT / VASICINE ANALYSIS / TLC-DENSITOMETRY / TLC IMAGE ANALYSIS

PAPHITCHAYA THETSANA: PHARMACOGNOSTIC SPECIFICATION AND VASICINE CONTENT OF *ADHATODA VASICA* LEAVES. ADVISOR: ASST. PROF. CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 85 pp.

Adhatoda vasica Nees or Malabar nut tree is a shrub which in Acanthaceae family. *A. vasica* is a famous herb that used for a long time mostly in Thai's remedies. *A. vasica* leaves are used to treat fever and cough. For the good quality of herbal drugs, standardization is needed to be done. Leaf sample was collected from 12 different sources throughout Thailand. Whole plant of *A. vasica* was drawn by hand writing. In the microscopic method, *A. vasica* leaves powder and the cross section of mid rib was found calcium carbonate. Palisade ratio was 6.57 ± 0.56 whilst stomata number, stomata index, trichome number, trichome index and calcium carbonate number were 288.27 ± 3.70, 17.84 ± 0.83, 28.53 ± 7.63, 2.45 ± 0.53 and 36.50 ± 9.20, respectively. Physico-chemical method was shown that water content, loss on drying, total ash and acid insoluble ash were not more than 11, 9, 21 and 6 % by weight while ethanolic extract and water extract were not less than 4 and 22 % by weight. Thin layer chromatographic fingerprint of *A. vasica* leaf was performed using chloroform and methanol (9 : 1) as mobile phase and the developed TLC was photographed at 254 nm, 365 nm and strain with Dragendorff's reagent. TLC quantitative analyses of samples using toluene : ethylacetate : diethylamine (5 : 2 : 3) as a mobile phase and analyzed by TLC-densitometry (CAMAG TLC scanner and winCATS) and TLC image analysis (ImageJ software). The calibration range were polynomial (1 – 5 µg/spot) and had R^2 0.9998 and 0.9988, precision of method was in the range of 1.30 – 3.96 and 6.29 – 10.45, accuracy was 83.60 ± 1.09 and 85.32 ± 5.42, LOD and LOQ were 0.188, 0.067 and 0.570, 0.202 µg/spot and robustness of the method was 4.48 and 11.12 %, respectively. The total of vasicine in *A. vasica* leaves using TLC-densitometry and TLC image analysis was 0.130 ± 0.065 and 0.134 ± 0.061 g/100g of dried crude drug. The comparison of the total vasicine in both methods was not significant different (t = 1.796, *P* = 0.249). This study could be used for the standardization parameter of *A. vasica* leaves in Thailand and the development of this herbal drug species.

Field of Study: Public Health Sciences
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

The author would like to express her sincere thanks to her thesis advisor, Assistant Professor Dr. Chanida Palanuvej and her thesis co-advisor, Associate Professor Dr. Nijsiri Ruangrangsi for their advices, guidances, valuable suggestions, encouragements, kindness and supports throughout this study.

The author also wishes to thank Assistant Professor Dr. Naowarat Kanchanakhan as the chair of the thesis committee and Dr. Chaisak Chansriniyom as the thesis external examiner for their valuable suggestions and advices to improve thesis and the author wishes to thank Mr. Worathat Thitikornpong at Faculty of Pharmaceutical Sciences, Chulalongkorn University for his helping hand in densitometric instrumentation.

The author is grateful to College of Public Health Sciences, Chulalongkorn University for Teaching Assistant Scholarship and also thankful to all stuff members for their assistances kindness and friendships.

Most importantly, the author would like to express all her love and gratitude to her family for their supporting, understanding and encouragement to through her study.

CONTENTS

Page

Page

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABRREVIATIONS

%	=	Percent
°C	=	Degree Celsius
cm	=	Centimeter
EC_{50}	=	Fifty percent effective concentration
g	=	Gram
g/mol	=	Gram per mole
GC	=	Gas chromatography
HPLC	=	High performance liquid chromatography
ICH	=	The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
kg	=	Kilogram
LOD	=	Limit of detection
LOQ	=	Limit of quantification
m	=	Meter
mg	=	Milligram
mg/kg	=	Milligram per kilogram

mg/ml	=	Milligram per milliliter
min	=	Minute
ml	=	Milliliter
ml/kg	=	Milliliter per kilogram
mm	=	Millimeter
mm^2	=	Square Millimeter
nm	=	Nanometer
R_f	=	Retention factor
RSD	=	Relative standard deviation
TLC	=	Thin layer chromatography
UV	=	Ultraviolet
WHO	=	World Health Organization
µg/mL	=	Microgram per milliliter
µl	=	Microliter
σ	=	Sigma

CHAPTER I

INTRODUCTION

Background and rationale

Herbal medicines have been used to treat diseases for a long time. Traditional Thai medicine remedies are influential from Indian remedies. The important evidence is Ayurvedic scripture that used Sanskrit language to diagnostic disease. More than hundred thousand types of herbs were found in Thailand. The important things are the weather that comfortable for agriculture and the difference in geographic environment of each part of Thailand that makes the diversity of the plants. Herbs are used for treating diseases for a long time and many diseases can be treated by just only one herb for example, the root of turmeric for treating gastritis, clove buds used for toothache; or herb remedies which mixed of many kinds and parts of herbs for treatment of many diseases, including digestive diseases, inflammation diseases, skin diseases, fever and respiratory system [1]. Herbal drug can be divided into four types; traditional drugs, modified traditional drugs, herbal medicines and new drugs. Traditional drugs are the drugs that inherited the properties, dosage and instruction for a long time. Modified traditional drugs are the traditional drugs that used for a long time but packaged in the modern dosage form. Herbal medicines are the semi-purified drugs or extracts by the scientific methods and contain known amount

of active compound. New drugs are developed herbal drugs by scientific methods to get the purified substance that exactly known the chemical structures and these remedies can be registered as modern drugs [2]. The examinations of herb have many methods; for example, macroscopic method, microscopic method, chemical method, physical method and spectroscopic method. Macroscopic method is the method that used to examine the external crude drug by organoleptic. Microscopic method is the method that used to examine anatomical and histological characters of herbal crude drugs. Chemical method is the method that uses specific reagents to test for secondary metabolite specific color reaction or precipitation reaction. Physical method is the method that uses ultraviolet light or polarized light to examine the herbal drugs. Spectroscopic method is the method that used to examine chemical constituents in the herb [3].

Adhatoda vasica Nees is a medicinal plant native to Asia for treatment of respiratory system especially bronchodilatory activity. It is a green shrub and has a bitter taste. There are the studied reports for standardization, pharmacognostic evaluation, pharmacological and toxicological properties of *A. vasica* in India. From the pharmacological investigation, all parts of *A. vasica* have many activities such as bronchodilator activity, antitussive activity, wound healing activity, antimicrobial activity and antibacterial activity. The toxicological studies of *A. vasica* reported that the patient had allergy to the pollen grains of this plant during the month of

October and November [4-6]. The main chemical compound of *A. vasica* is vasicine, which in extracted, purified and mixed into the modern remedy to treat antitussive and bronchodilatory activity [7]. In Thailand, *A. vasica* has been a part of Ya-Keaw remedy which is one of Thai's remedies that used for a long time. The properties of this remedy are used to treat chickenpox, fever in both children and adults [8].

Nowadays, herbs become interesting. Herbal medicines need a great quality of ingredients, so the standardization of herbs should be taken. Standardization is the process of developing, implementing technical standards and quality control. The basic quality control consists of determination of foreign matter, macroscopic-microscopic examination, determination of water, volatile matters and solvent extractive value. Since the quality parameters as well as vasicine content of *A. vasica* crude drug in Thailand has never been established. The purpose of this study is to investigate the standardization parameters by qualitative and quantitative analyses of *A. vasica* leaves.

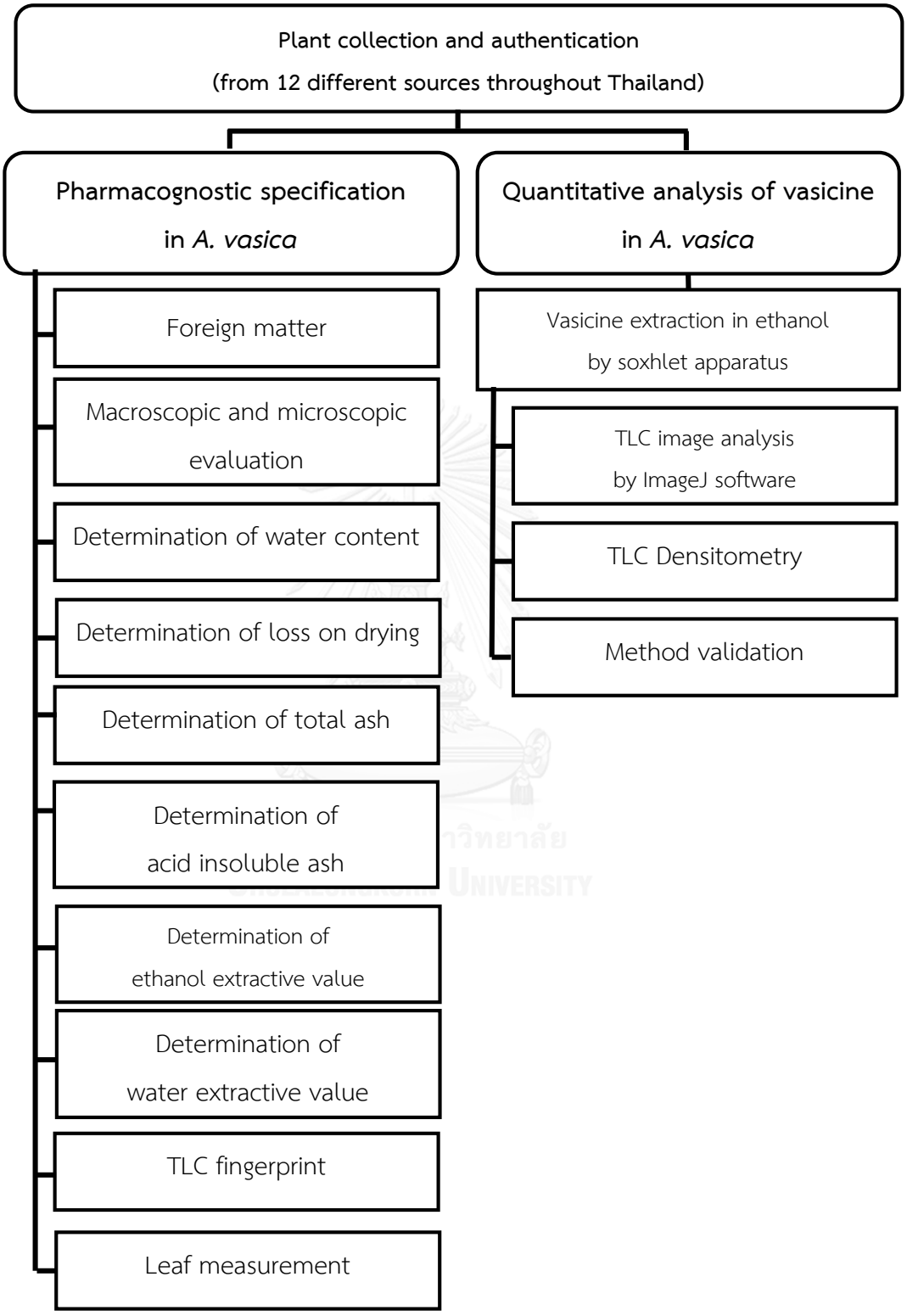
Research problems

The quality parameters as well as vasicine content of *A. vasica* crude drug in Thailand have never been established.

Objectives

1. To develop the standardization parameters of *A. vasica* crude drug.
2. To investigate the content of vasicine in *A. vasica* crude drug by TLC image analysis using ImageJ free software compared to TLC densitometry.
3. To examine leaf measurement value of stomatal number, stomatal index, trichome number, trichome index, lithocyst number and palisade ratio of *A. vasica* leaves.

Conceptual framework



CHAPTER II

LITERTURE REVIEWS

Taxonomy

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Lamiales

Family: Acanthaceae

Genus: *Adhatoda*

Species: *vasica* [9]

Morphological characteristics

Description: *Adhatoda vasica* Nees (syn. *Justicia adhatoda* L.) or called in Thai Sa - Niat is a green perennial shrub, height 1 – 3 m with many opposite and ascending branches. Stem with yellowish bark. Leaves are broad and leathery, 12 – 20 cm in length and 3 – 6 cm in width, elliptic-lanceolate, acuminate, minutely puberulous when young, glabrous when mature, entire, dark green above paler beneath, base tapering, main nerves 10-12 pairs with reticulate venation between petioles 1 – 3 cm in length. Flowers are large, dense, terminal spikes with large, 2 – 7 cm in length, attractive white petals, streaked with purple on the lower lip. Filaments are

very hairy at the base, long stout, curved lower anther cells minutely apiculate at the base. Ovary pubescent, sub-acute shortly and bluntly pointed, pubescent, solid stalk flattened, 1 cm long seeds 6 mm long and 5 mm wide, orbicular oblong [9-11].

Distribution: *A. vasica* growing throughout in India, Sri Lanka, Myanmar, China, Laos, Malaysia and Thailand [12, 13].

Traditional uses of *Adhatoda vasica* Nees

A. vasica has been used in folk medicine as an herbal remedy for treating cold, cough, whooping cough and chronic bronchitis and asthma, as sedative expectorant, antispasmodic and anthelmintic. All parts of *A. vasica* were used to treat many diseases. Roots are used for diuretic, bronchitis, sore eyes and fever treatment. Flowers are used to improve blood circulation. Fruits are used to treat bronchitis and cold. Flowers and fruits are bitter taste, aromatic and used for anti-plasmodics. Leaves are used to treat bleeding, hemorrhage, headache, snake-bite, asthma and jaundice [10]. The remedy of the leaves mixed with roots of gingers is popularly used to treat cough and juice of fresh leaves mixed with honey is used to treat cough by liquefying the sputum. The extract of the leaves has been an ingredient in the Glycodin® used for the treatment of bronchitis. In conclusion, the main useful of all parts of *A. vasica* is to treat respiratory diseases. In the “Use of Traditional Medicine in Primary Health Care”, WHO recommended *A. vasica* for the treatment of cough, asthma and bleeding piles in both adults and children for a long time [12].

The main chemical constituents of *A. vasica* are alkaloids of which mostly found in the leaves is vasicine. Others are vasicol, vasicinone, vasicinol and deoxyvasicinone [7]. In previous studies, phytochemical screening of *A. vasica* leaves in many types of extractions: ethanol, methanol, aqueous and chloroform found that *A. vasica* leaves gave the positive results of alkaloids in the Dragendorff, Mayer, Hager and Wagner test; saponins in the foam test; tannins in 5% of $FeCl_3$ reagent; streols in Salkowaski test and Liebermann test and phenols in $FeCl_3$ and $Pb(NO_3)_2$ solution [14].

Vasicine

IUPAC: 1,2,3,9-tetrahydropyrrolo(2,1-b) quinozolin-3-ol

Molecular weight: 188.32 g/mol

Description: Yellow powder

Melting point: 210 °C

Solubility: soluble in organic solvents such as, acetone, chloroform, ethanol, methanol, and dichloromethane [15].

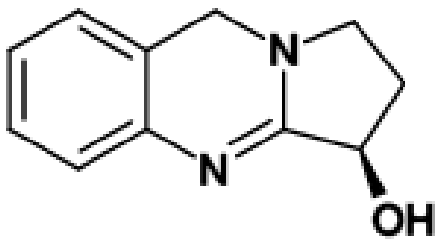


Figure 1 Structure of vasicine

Vasicine and distribution

Vasicine or peganine is a quinazoline alkaloids group that presents as a major alkaloid in all parts of *A. vasica*. Vasicine was first isolated as pure alkaloid by Hooper in 1888 [16].

Vasicine can be isolated from other sources for example, the root of *Sida cordifolia* (Malvaceae) and two species of Afrogalega i.e. whole plant and seed of *Galega battiscombei* Gillett and *Galega linblomi* Gillett [17, 18]. Nowadays, the most isolation of vasicine is from the leaves of *A. vasica* which widely done in India and on the month of September [19].

Medicinal use of vasicine in *Adhatoda vasica* Nees

Pharmacological properties of *A. vasica* are well known for a long time. The activities include: antioxidant, anti-inflammatory, bronchodilatory activities. The main activity of *A. vasica* is bronchodilatory activity which studied in both *in vitro* and *in vivo* experiments [20]. Other activities of *A. vasica* from previous studies have been reported for analgesics, antioxidant, antispasmodics, antimicrobials, abortifacient, antidiabetic agent, wound healing agent and hepatoprotective agent [21].

Biological activities of *Adhatoda vasica* Nees

Antioxidant and anti-inflammatory activities

Vasicine isolated from fresh leaves of *A. vasica* was treating for against lung disease in murine model. Albino rats were induced to have lung disease by injecting 1 ml of saline that contained 1 mg of ovalbumin and 20 mg of aluminum hydroxide and 1 ml of *Bordetella pertussis* vaccine as adjuvant twice a day for 21 days. They were divided into three groups; control, toxic and treated group. The result showed that 0.2 mg/kg of vasicine in treated group can reduce lipid peroxidation and oxidative stress [22].

In the carrageenan induced paw edema test and complete Freund's adjuvant (CFA) model in Wistar albino rats, vasicine in dose of 20 mg/kg was shown the most effective result in anti-inflammatory activity at 6 hours after injecting carrageenan [23].

Antitussive activity

A. vasica leaves and flowers extracts were used for study of antitussive activity. The animal models: guinea pig and rabbit were induced coughing by various irritants including irritant agent aerosol, mechanical and electrical trachea stimulation. The ethanolic extracts of *A. vasica* gave a good antitussive result compared to codeine [24].

In the other antitussive activity study, both male of female of albino mice were induced cough by sulphur dioxide (SO_2) exposure for 20 seconds. The result showed that ethanolic extract of *A. vasica* leaves in dose of 200 mg/kg had significant effect with 43.02 % inhibition on treatment with *A. vasica* within 60 min when compared to control group which treated with codeine sulphate [25].

The aqueous extract of *A. vasica* leaves was tested in animal model using guinea pigs. They were induced cough by citric acid and divided into 3 groups i.e. test group, positive control group (codeine dose 10 mg/kg) and negative control group (saline water dose 1 ml/kg). *A. vasica* extract in dose of 50 mg/kg was significantly effective [26].

Antibacterial activity

The dose of 125 µg/mL of vasicine acetate was a minimum inhibitory concentration which against bacteria therefor *Micrococcus luteus*, *Enterrobacter aerogenes*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* by using disc diffusion method [27].

Methanolic extract of *A. vasica* leaves was tested by agar well diffusion method for antibacterial activity against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria.

A. vasica leaves extract (15 mg/ml) was significantly effective against *P.aeruginosa* with the inhibition zone of 2.67 ± 0.06 mm [28].

Antibacterial activity against mastitis pathogens was done by disc diffusion method. The methanolic extract of fresh leaves of *A. vasica* in 150 mg/ml and 200 mg/ml showed significant antibacterial activity against *Staphylococcus aureus*, *Streptococcus agalactiae*, *Klebsiella pneumonia*, *Stretococcus dysgalactiae* and *Escherichia coli* [29].

Disc diffusion method was used for antibacterial activity. Various extracts of *A. vasica* leaves were tested. Hot aqueous of *A. vasica* leaves extraction, 500 mg/ml gave result of zone inhibition of 8.33±0.33 mm against *Staphylococcus* and hot methanolic *A. vasica* leaves extract 250 mg/ml gave result of zone inhibition of 12.33±0.88 mm against *Staphylococcus* and 500 mg/ml gave result of zone inhibition of 8.33±0.33 mm against *Klebsiella*, 14.00±0.57 mm against *Staphylococcus* and 8.67±0.88 mm against *Bacillus*, significantly [30].

Vasicine acetate was tested for antibacterial activity by DPPH radical scavenging assay. The minimum inhibitory concentration values was 125 µg/mL that against bacteria; *M. Luteus*, *E. aerogenes*, *S. epidermis* and *P. aeruginosa* [27].

Anti-ulcer activity

The extract of leaves of *A. vasica* (500 mg/kg in 0.2% agar), was tested in animal model which induced gastric ulcer by two methods (ethanol – induced ulcer and pylorus ligation plus aspirin – induced ulcer). The result showed that *A. vasica* had a high degree of protection (80%) in ethanol – induced ulcer model and 41% of degree protection in pylorus ligation plus aspirin – induced ulcer model when compared to control group [31].

The soxhlet extracts of *A. vasica* leaves with methanol, chloroform and diethyl ether were used for testing anti – ulcer activity in animal model. Wistar albino rats were induced gastric ulcer by three methods; aspirin, alcohol and pylorus – ligation induced gastric ulcer. The result showed that methanolic extract of *A. vasica* leaves in three methods significantly reduced the total volume of gastric acid secretion when compared to chloroform and diethyl ether extracts [32].

Anticestodal activity

The ethanolic extracts of *A. vasica* leaves in doses of 100, 200, 400, 800, 1600, and 3200 mg/kg were tested in animal model which inoculated with five cysticercoids (*Hymenolepis diminuta*) by feeding tube. The evaluation of immature worms (8 – 10 days after inoculation) and mature worms (21 – 25 after days after inoculation) were observed from faeces. The result showed that *A. vasica* in dose of

800 mg/kg significantly decreased worm recovery rate, the egg per gram (EPG) count from 100% to 20% in immature worms and from 100% to 16.60% in mature worms when compared to control group, respectively [33].

Antimutagenic activity

The methanolic and aqueous extracts of dried powder of *A. vasica* leaves were tested in *Allium cepa* chromosome assay to investigate mutagenic and antimutagenic activity. The EC_{50} values of aqueous extract and methanolic extract of *A. vasica* were 420 mg/kg and 460 mg/kg respectively. *A. vasica* in both extracts were shown none of mutagenicity [34].

Wound healing

Male Swiss albino mice being excision wound model were divided into three groups; treatment group, positive control group (povidine iodine ointment) and negative control group. Methanolic extract of *A. vasica* dried leaves gave result nearly positive control of wound healing comparing to other extracts [35].

Toxicology of vasicine

Acute toxic of vasicine

Vasicine was tested for acute toxicity in animal models: rats and dogs. The treatment groups of rats were given vasicine subcutaneously (10, 25 and 50 mg/kg body weight) and orally (20 and 100 mg/kg body weight) and the control group was given normal saline. The treatment groups of dogs were given vasicine subcutaneously (3.5 and 17.5 mg/kg body weight) and orally (35 mg/kg body weight). Vasicine did not have adverse effects by clinical observation, clinical chemistry and histopathological examination in this study [36].

Chronic toxic of vasicine

Vasicine was treated for chronic toxicity in animal models: rats and monkeys. The treatment groups of rats and monkeys were treated with vasicine orally (1, 2.5, 5 and 10 mg/kg body weight and 0, 5, 10 and 20 mg/kg body weight) for six months. The results were observed by clinical observations, clinical chemistry and histopathology of major organs compared to control group. The results of autopsy and histological investigation of the major organs showed no abnormality in the organs. [37].

Plant material quality control

Macroscopic and microscopic examination

Plant authentication has two major methods to identify herbal materials those are macroscopic and microscopic examination and chromatography. The advantage of the method is easily, used short time and inexpensive method [38].

Macroscopic examination is the identity of medicinal plant that based on shape, size, color, odor, taste, *etc.* of plant materials. This method can do with the naked eye or with a hand lens or stereomicroscope.

Microscopic examination is the observation method including the observation of the cellular structure and the content of plant material by using stereomicroscope. This method shows plant anatomical and histological characteristics [39, 40].

Determination of stomata type, stomatal number and stomatal index

The leaf crude drug can be specified, identified and characterized by the stomatal number and the stomatal index. The stoma is a pore that surrounded by two guard cells. The property of stomata is gas exchange. The stomata can mainly found in the epidermis of leaves, stems and the controlling gas organs. The changing shape of stomata makes opening and closing of pore. The characteristics of epidermal cells and stomata are firstly important in

the microscopic examination of leaves. The epidermal cells surrounding stomata called subsidiary cells which may be in different shapes.

The types of stomata can be identified into 4 types by the characters of the subsidiary cells.

Anomocytic type is the stomata type that surrounding by identical subsidiary cells.

Anisocytic type is the stomata type that has three or four subsidiary cells and one cell has smaller than others.

Diacytic type is the stomata type of which two subsidiary cells are right angled to the stoma.

Paracytic type is the stomata type of which two subsidiary cells are parallel to the stoma [41, 42].

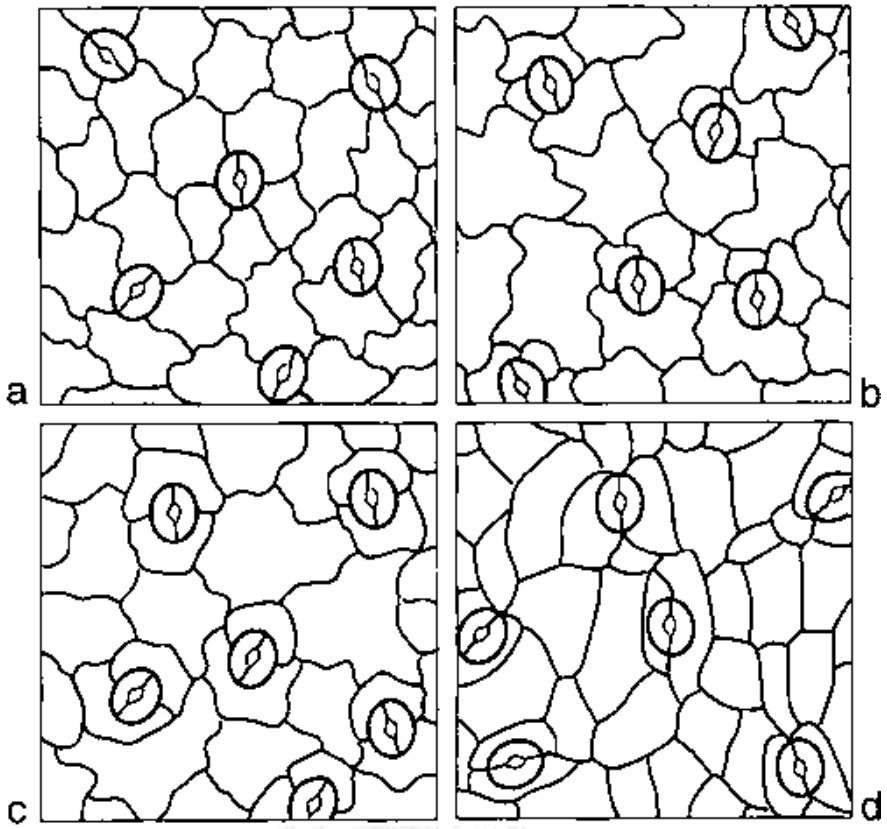


Figure 2 Types of stomata; a. Anomocytic type b. Anisocytic type c. Diacytic type d. Paracytic type

Stomatal number

Stomatal number is the average number of stomata in the area of 1 mm^2 of epidermis. Two guarding cells are counted into single unit of a stoma. It was designed by Timmerman in 1927 [43].

$$\text{Stomatal number} = \frac{\text{Number of stomata}}{\text{Area of epidermal cell } (mm^2)}$$

Stomatal index

Stomatal index is the percentage of stomata number per total number of epidermal cells [39]. The calculation of the stomatal index can be explained as;

$$\text{Stomatal index} = \frac{S \times 100}{E + S}$$

Where; S = the number of stomata per unit area

E = the number of ordinary epidermal cells in the same unit area

Palisade ratio

Palisade ratio is another identification and evaluation of leaf crude drug that defined as the average number of the palisade cells beneath one epidermal cell. T.E. Wallis and T. Dewar, introduced the term of “palisade ratio” in 1933 [44]. The determination was obtained by counting the total number of palisade cells beneath four upper epidermal cells and dividing the number by four [37]. The value of the palisade ratio in same species gives the same result. So, this value is very useful diagnostic feature for identification and characterization of the different plant species [40].

Thin layer chromatography

Thin layer chromatography (TLC) is a planar chromatographic method that used for separated mixture. For screening chemical constituents in herbal drugs, TLC is the first method to use because TLC is simply, needs a short time

and does not need expensive instruments [45-47]. For detection of the chemical constituents on TLC plate, many chemical compounds can absorb ultraviolet light or can emit fluorescence. In addition, universal reagents and specific reagents will be sprayed or dipped after developing TLC plates to produce chromophores. However, TLC analysis has limitations for low resolution, low sensitivity especially for detection of trace components [48].

Detecting reagents

The detecting reagents can be divided into two types: the general reagents and the specific reagents. The general reagents are commonly used for unknown compounds. The specific reagents are commonly used for known compounds. The most generally used staining reagents are shown in Table1.

Table 1 The general TLC detecting reagents for detection of the natural products [49-55]

Detecting reagent	Detection
Ferric (III) chloride	Phenols and phenolic acids
2-4, Dinitrophenol	Aldehydes and ketones
Calcium sulfate	Alkaloids
Morin hydrate	General reagent: Fluorescently actives
Potassium permanganate	Olefins and other readily oxidized groups
ρ-Anisaldehype/sulfuric acid	Phenols, sugars, steroids and terpenes
Vanillin/sulfuric acid	Terpenoids, steroids and saponins
Dragendorff's reagent	Alkaloids and quaternary nitrogen compounds
Ninhydrin	Amino acids, amines, amino sugar

Preparation of sample for TLC method

The concentration of the sample solution needs to be enough for detection after applied on TLC plate. The solvent that used for extraction needs to focus on the polarity of the solvent likely the mixture compound that use for separation and analyze. The solvent for dissolving the extract needs to be suitable and can dissolve all of chemical constituents in the sample extract. [56].

Silica gel, alumina, cellulose, gypsum and polyamines are the materials used for coating TLC plastic plate and also glass plate. Mobile phase is the mixture of two to five selected solvents that used to separate the chemical constituents in herbal extracts based on chemical properties such as polarity, ionic strength, affinity, *etc*. Spot of the sample after development can be detected under short wavelength UV (254 nm), long wavelength UV (366 nm) and visible light after derivatization [56, 57].

An important qualitative parameter, which characterizes the position of a spot on TLC plate, is the retardation factor (R_f) value. The calculation of the retardation factor (R_f) value is [57];

$$R_f = \frac{\text{Distance of the compound from original spot travelled to the developed spot}}{\text{Distance of the solvent from original line travelled to the developed line}}$$

Qualitative and quantitative analysis

Qualitative and semi-quantitative analyses by conventional TLC have commonly been used [57]. One of the analytical methods for the quality control of herbal materials is fingerprinting that has been acceptable method by WHO. This method is suitable for adulterations detection and plant species identification. Fingerprint can be obtained identify from chromatographic methods such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC). TLC is the first and popular method to perform chemical fingerprint for identification and authentication of herbal medicines. The advantages of TLC fingerprinting are simplicity, rapidity, specificity sensitivity and simple sample preparation [58].

For the quantitative analysis by modern TLC method, the data from TLC chromatogram can be analyzed by coupling with TLC scanner or densitometer. Densitometry is the specific method that used for scanning TLC plate by specific or non-specific wavelength. The scanner quantitates the chemical constituents by measuring the intensity of absorbance or fluorescence signal between sample spot and background. Image analysis is an alternative method that can quantitate the chemical constituents by using software e.g. ImageJ, Scion Image or Photoshop to measure the intensity of pixel in digital imaging of TLC chromatogram.

ImageJ is the image analysis software that provided freely by National Institutes of Health, USA [45, 47, 58-61].

CHAPTER III

MATERIALS AND METHODOLOGY

Chemicals

Chloral hydrate	Ajax Finechem Pty. Ltd., New Zealand
Ethanol	RCI Labscan Limited, Bangkok, Thailand
Hydrochloric acid	RCI Labscan Limited, Bangkok, Thailand
Sodium hypochlorite	Haiter Bleach, Kao industrial, Thailand
Toluene	RCI Labscan Limited, Bangkok, Thailand
Vasicine	Altavista Phytochemicals Pvt. Ltd., India

The chemicals used were of analytical grade.

Materials

Cover glasses	Menzel-Glaser, Germany
Filter paper No.4	WhatmanTM Paper, UK
Filter paper No.40 ashless	WhatmanTM Paper, UK
Microscope Slide	Sail Brand, China
TLC aluminium sheet 20 x 20 cm	Merck, Darmstadt, Germany
silica gel 60 GF_{254}, 200 µm thickness	

Instrument and equipments

Aqua-shaker	Adolf Kühner AG, Switzerland
Balance readability 0.01 g	Ohaus Corp. Pine Brook, NJ, USA
(PioneerTM, PA2102)	
Balance readability 0.0001 g	SI-234, Denver Instrument, Germany
CAMAG Linomat 5	CAMAG, Switzerland
CAMAG TLC Chamber	CAMAG, Switzerland
CAMAG TLC Scanner 3	CAMAG, Switzerland
CAMAG TLC Visualizer	CAMAG, Switzerland
Digital camera	Canon Marketing (Thailand) Co., LTD,
(Canon PowerShot A650)	Bangkok
Hot air oven	WTC Binder tuttlingen, Germany
ImageJ software	National Institutes of Health, USA
(Version: 1.48)	
Incinerator	Carbolite, UK
Microscope	Zeiss Axioskop, Germany
Rotary vacuum evaporator	Büchi, Switzerland

TLC syringe	Hamilton Company, USA
Ultrasonic bath	Analytical Lab Science Co., LTD, Bangkok
Ultraviolet fluorescence analysis Cabinet (Model CC-80)	Spectronic corp., USA
Water bath	Brinkmann, USA
winCATS software (version: 1.4.6.2002)	CAMAG, Switzerland

Research methodology

Morphological identification and standardization parameters of *A. vasica* specimens from different sources in Thailand were examined according to World Health Organization (WHO) guidelines of "Quality Control Methods for Medicinal Plant Materials".

Plant collection

The leaves of *A. vasica* were collected from 12 provinces throughout Thailand and then authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi, Chulalongkorn University. The voucher specimen was deposited at the College of Public Health Sciences, Chulalongkorn University. After removal of any foreign matters, each sample was shade dried and crushed into powders.

Standardization of *Adhatoda vasica* Nees

Macroscopic evaluation

The shade dried leaves of *A. vasica* were evaluated by surface characteristics, texture, fracture characteristics, appearance of the cut surface, shape, size, color, odor, taste, and other characters.

Microscopic evaluation

The microscopic appearances of the *A. vasica* leaves were examined in cross section and in powdered form. The tissue section and powders were mounted onto a glass slide in water for microscopic observation under objective lens with 10X, 20X and 40X magnifications. Photographs were taken by a digital camera. The microscopic characters were illustrated in the proportion size related to the original.

Determination of water content (Azeotropic method)

The accurate 50 g of *A. vasica* dried leaf powders were transferred to round bottom flask, added with 200 ml of water – saturated toluene and boiled by using azeotropic distillation. After the water was completely distilled, allowed the receiving tube to cool in room temperature, read off the volume of water's content and calculated in percentage.

Determination of loss on drying

The accurate 3 g of *A. vasica* dried leaf powders were weighted in the pre-weighed crucible, dried the sample for 6 hours at 105 °C in an oven until constant weight, allowed the crucible to cool at room temperature, weighed and calculated the loss of weight in percentage.

Determination of total ash

The accurate 3 g of *A. vasica* dried leaf powders were weighed in the pre-weighed crucible, incinerated for 5 hours at 500 °C until its color turn to white, allowed the crucible to cool at room temperature, weighed and calculated the total ash in percentage.

Determination of acid insoluble ash

The aforementioned crucible total ash was added with 25.0 ml of hydrochloric acid (70 g/l), covered with a watch-glass and boiled gently for 5 minutes, filtered the insoluble matters with an ashless filter-paper No.40, transferred the filter-paper into the previous crucible, dried on a hot-plate and incinerated for 5 hours at 500 °C, allowed the crucible to cool at room temperature, weighed and calculated the acid insoluble ash in percentage.

Determination of ethanol soluble extractive value

The accurate 5 g of *A. vasica* dried leaf powders were macerated with 70.0 ml of 95% ethanol in a closed conical flask for 24 hours (6 hours under shaking and 18 hours standing) then were filtered rapidly. The marc was washed and adjusted the volume to 100.0 ml with ethanol and transferred 20.0 ml of the filtrate to a pre-weighed beaker and evaporated to dryness on a water-bath. Finally, the extract was dried at 105 °C to constant weight, allowed the beaker at room temperature, weighed and calculated the ethanolic extract in percentage.

Determination of water soluble extractive value

The accurate 5 g of *A. vasica* dried leaf powders were macerated with 70.0 ml of water in a closed conical flask for 24 hours (6 hours under shaking and 18 hours standing) then were filtered rapidly. The marc was washed and adjusted the volume to 100.0 ml with water and transferred 20.0 ml of the filtrate to a pre-weighed beaker and evaporated to dryness on a water-bath. Finally, the extract was dried at 105 °C to constant weight, allowed the beaker at room temperature, weighed and calculated the water extract in percentage.

Leaf measurement

The leaf samples were cut off from the middle of the fresh leaves and soaked in the mixture of water and sodium hypochlorite (1:1) about 1-3 days to remove chlorophyll, boiled in the mixture of chloral hydrate and water (4:1) until the leaf was transparent, rinsed with distilled water and then trichome number, trichome index, lithocyst number and palisade ratio were observed under digital microscope.

For the examination of stomatal number and stomatal index, fresh leaves were applied with nail polish in both sides, allowed the nail polish to dry, taped cellophane to the dried nail polish, peeled it slowly and observed under the microscope and calculated the average of thirty fields of upper and lower epidermis in area of 1 mm^2.

Thin layer chromatographic fingerprint

The 1 g of *A. vasica* dried leaf powders was macerated in 20 ml of 95% of ethanol for 6 hours and standing at room temperature for 18 hours then evaporated to dryness and re-dissolved in 1 ml of 95% of ethanol. The extract (3 µl) was applied on the 0.2 mm thickness of TLC silica gel 60 GF_{254} plate. Developed TLC plate in saturated TLC chamber with choroform : methanol (9:1) and observed the spots under short wavelength (254 nm) and long wavelength (365 nm) ultraviolet light then sprayed the plate with dragendorff's reagent.

Quantitative analysis of vasicine in *Adhatoda vasica* Nees

Preparation of standard solutions

One milligram of vasicine standard was dissolved in 1 ml of 95% of ethanol and diluted the concentrations to 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml to prepare the series of stock solution. These standard solutions were kept in refrigerator at 4ºC.

Preparation of ethanol extracts of *Adhatoda vasica* Nees

The accurate 3 g of *A. vasica* dried leaf powders were exhaustively extracted with 200 ml of 95% ethanol by soxhlet extraction. The ethanolic extract was filtered and evaporated to dryness by rotary evaporator. The extract was dissolved in 95% ethanol (25 or 50 mg/ml) for TLC-densitometry and TLC image analysis.

TLC image analysis by ImageJ software

The ethanolic extract of *A. vasica* and standard vasicine solution were accurately spotted (5.0 µl) on the 20 x 20 cm TLC silica gel 60 GF_{254} plate. TLC plate was developed in the saturated TLC chamber with toluene : ethyl acetate : diethylamine (5:2:3) and photographed under UV 254 nm using digital camera.

The image of TLC plate saved as TIFF file was analyzed by ImageJ software and the calibration curve was performed by plotting peak areas *versus* concentrations of vasicine in µg/spot.

TLC-densitometry

The developed TLC plate was scanned with CAMAG TLC densitometer at 290 nm and the calibration curve was done by plotting peak areas *versus* concentrations of vasicine in µg/spot.

Method validation

Method validation following the ICH guideline including specificity, calibration range, accuracy, precision, detection limit, quantitation limit and robustness were performed [62].

Specificity

Specificity was performed by the comparison of UV absorbance spectra at the peak apex among samples and standard (peak identity) and the comparison of UV absorbance spectra recorded at up-slope, apex and down-slope of the peak (peak purity).

Calibration range

The relationship between peak areas *versus* concentrations of standard vasicine per spot was constructed.

Accuracy

The spike method was done for accuracy. Different levels of vasicine standard (low, medium, high) were spiked into the sample for TLC analysis in triplicate. The % recovery was calculated from following formula.

$$\% \text{ recovery} = \left(\frac{C1}{C2 + C3}\right) \times 100$$

Where; C1 = the amount of vasicine found in spiked sample

C2 = the amount of vasicine found in un-spiked sample

C3 = the amount of standard vasicine added to the sample

Precision

Intra-day or repeatability and inter-day or intermediate precision were done for precision method. Three level concentrations of sample were analyzed in the same plate for repeatability and were analyzed in the different days for intermediate precision. The % relative standard deviation (% RSD) was calculated. Each precision was done in triplicate.

Limit of detection

The limit of detection (LOD) which is the lowest concentration that can be detected but not accurately quantitated was determined using following formula:

$$LOD = \frac{3.3 \times \sigma}{S}$$

Where, σ = the residual standard deviation of regression line

S = the slope of regression line

Limit of quantitation

The limit of quantitation (LOQ) which is the lowest concentration that can be accurately quantitated was determined using following formula:

$$LOD = \frac{10 \times \sigma}{S}$$

Where, σ = the residual standard deviation of regression line.

S = the slope of regression line

Robustness

The robustness of the method was done by small change of ratio of the mobile phase to ensure that the deliberation of small change will unaffected the result. The selected mobile phase composed of toluene : ethyl acetate : diethylamine at the ratio of 5:2:3 ; 4.8:2.1:3.1 ; 5.2:1.9:2.9 were performed and % RSD of peak area was calculated.

Data analysis

Paired student *t*-test was used for data comparison between TLC densitometry and TLC image analysis.

CHAPTER IV

RESULTS

Macroscopic evaluation

A shade-dried leaf of *A. vasica* was a dark green and brown color, 12 – 20 cm in length and 3 – 6 cm in width (Figure 3). The taste was bitter. The whole plant of *A. vasica* was shown in the Figure 4.

Figure 3 Shade-dried leaves of *Adhatoda vasica* Nees

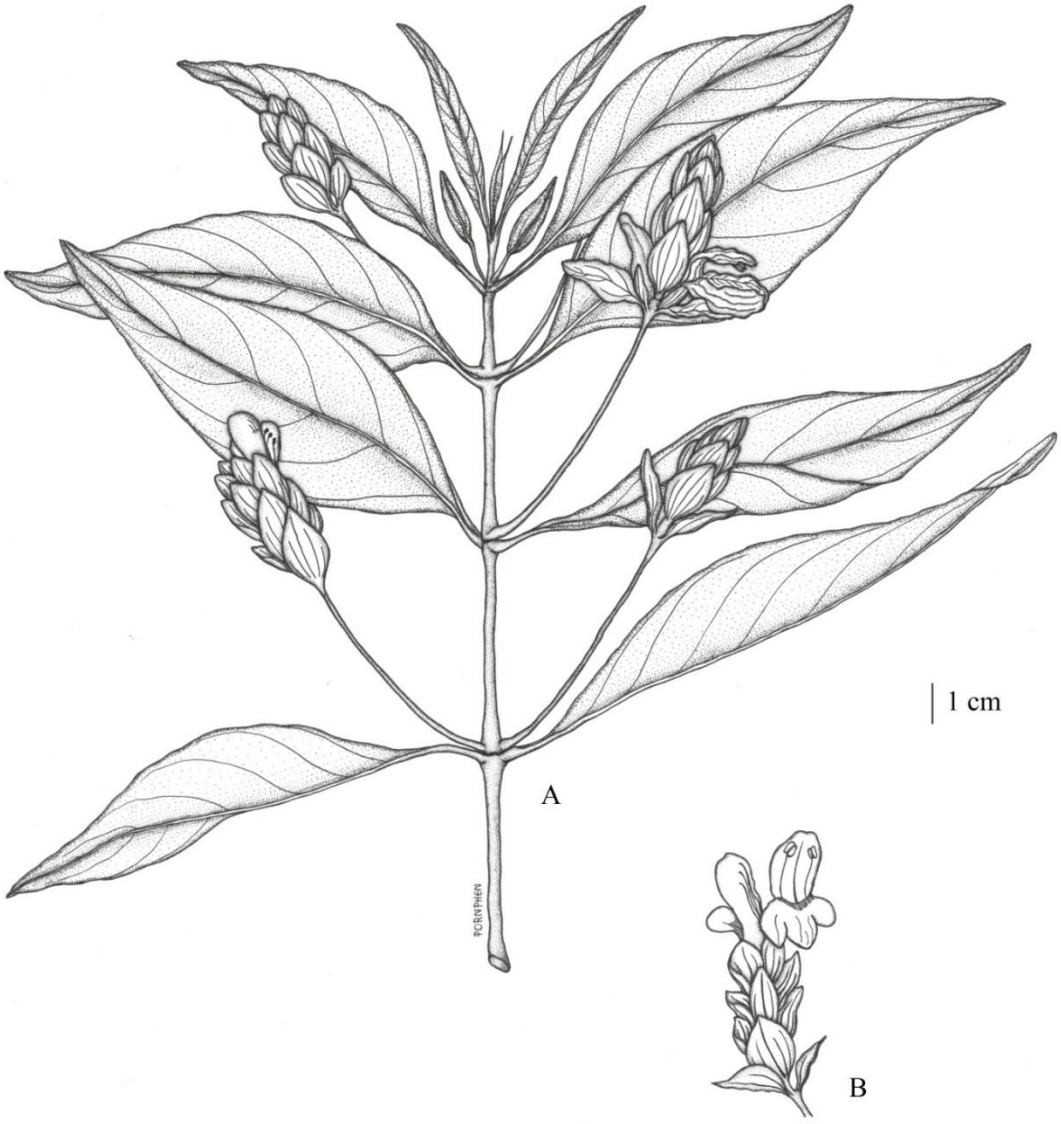


Figure 4 (A) Whole plant of *Adhatoda vasica* Nees (B) Flower of *Adhatoda vasica* Nees

Microscopic evaluation

The anatomical characters of *A. vasica* leaf, was shown in Figure 5. Palisade cell, multicellular uniseriate trichome, phloem, xylem, cortex, calcium carbonate crystal (cystolith), vessel, collenchyma and epidermis were illustrated. The histological characters of *A. vasica* leaf powder demonstrated epidermis, diacytic stomatal, glandular trichome and calcium oxalate prism (Figure 6).

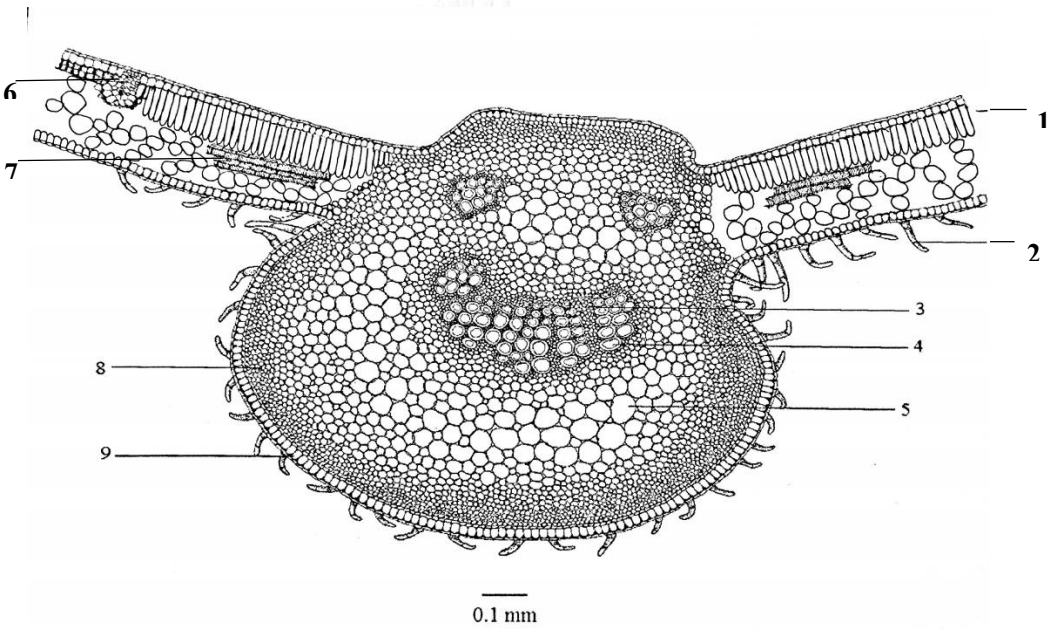


Figure 5 Cross section of *Adhatoda vasica* Nees leaf

1. Palisade cell
2. Multicellular uniseriate trichome
3. Phloem
4. Xylem
5. Cortex
6. Calcium carbonate crystal (cystolith)
7. Vessel
8. Collenchyma
9. Epidermis

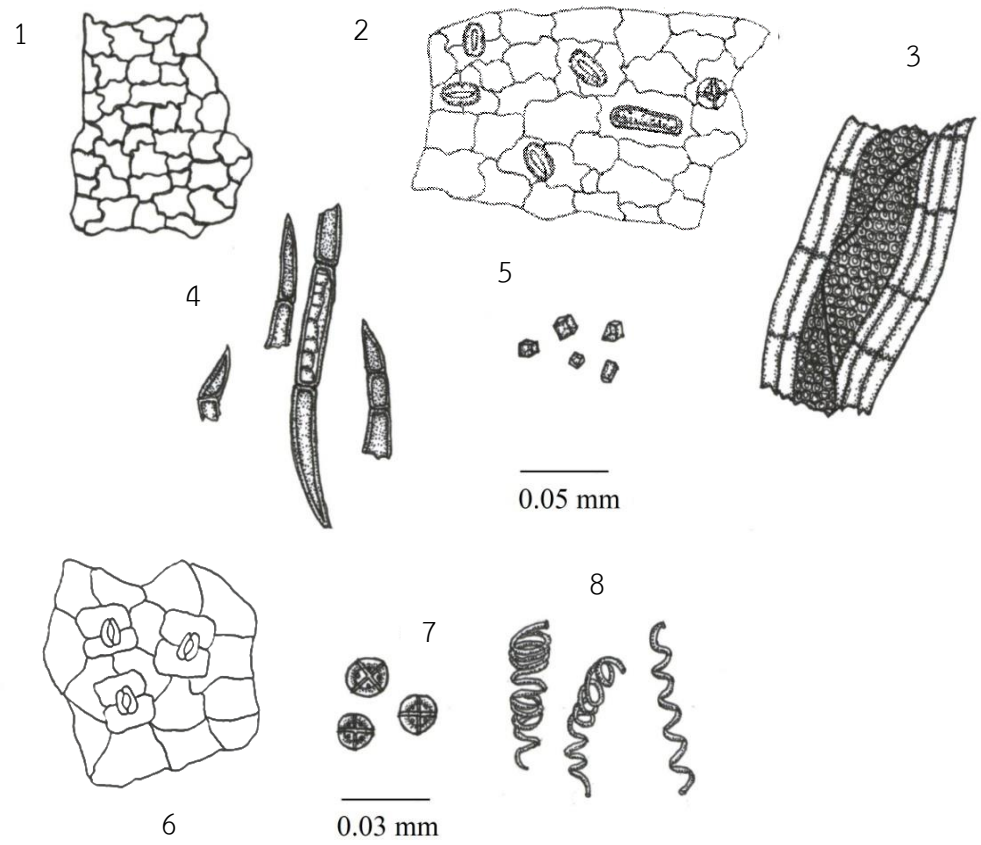


Figure 6 The histological evaluation of *Adhatoda vasica* Nees leaf powder

1. Epidermis
2. Lower epidermis with stoma, lithocyst cells and glandular trichome
3. Fragment of xylem ray
4. Multicellular trichome
5. Calcium oxalate prism
6. Stomata
7. Glandular trichome
8. Fragment of spiral vessels

Physico – chemical constants of *Adhatoda vasica* leaves

The results of standardization of *A. vasica* leaves were shown in Table 2. Determination of water content, loss on drying, total ash and acid insoluble ash should were not more than 11, 9, 21 and 6 % by weight but determination of ethanol soluble extractives and water soluble extractives should were not less than 4 and 22 % by dried weight, respectively.

Table 2 Physico - chemical constants of *A. vasica* leaves

Content (% by weight)	**Mean**	**SD**	**Range (Mean±3SD)***
Water content	10.511	0.388	9.348 – 11.674
Loss on drying	9.298	0.067	9.098 – 9.498
Total ash	20.770	0.095	20.484 – 21.55
Acid – insoluble ash	6.190	0.127	5.809 – 6.572
Ethanol soluble extractive	3.789	0.234	3.085 – 4.492
Water soluble extractive	22.155	0.504	20.643 – 23.668
Volatile oil	0	0	0

*The samples were from 12 different sources throughout Thailand and each sample was done in triplicate.

Leaf measurement

The fresh mature leaf was observed for palisade, stomata, trichome and lithocyst cells in both sides. Palisade was determined in the upper side. Diacytic stomata and glandular trichome were found in the lower side. The quantitative analyses of palisade ratio, stomatal number, stomatal index, lithocyst number, trichome number and trichome index were done in thirty fields and averaged. The results were shown in Figure 7 – 9 and Table 3.

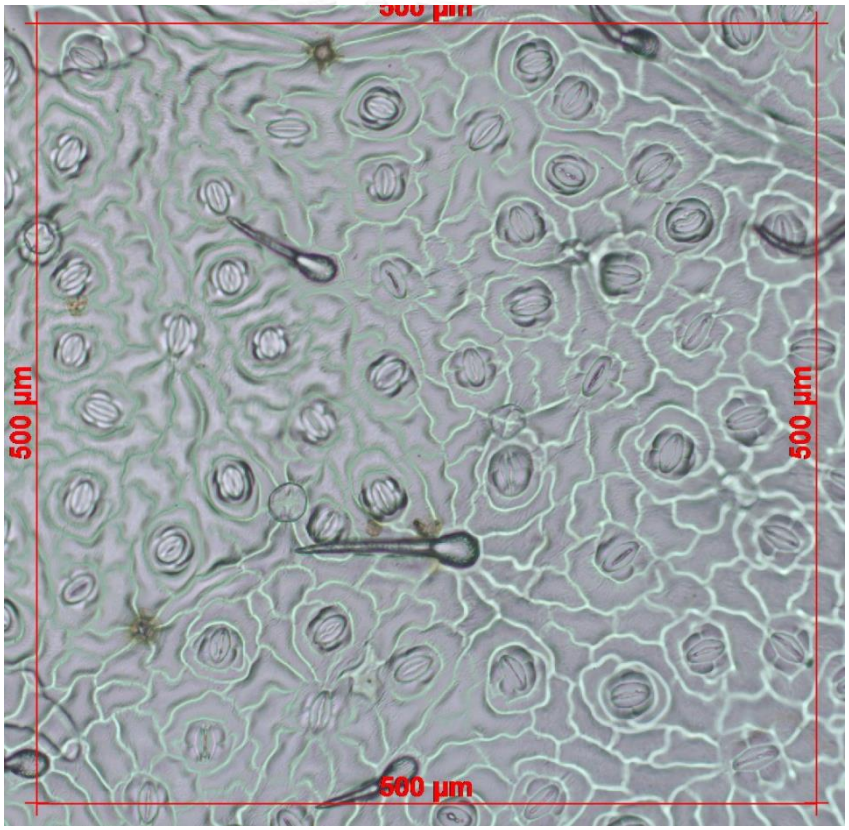


Figure 7 Stomata of *Adhatoda vasica* Nees leaf (diacytic type)

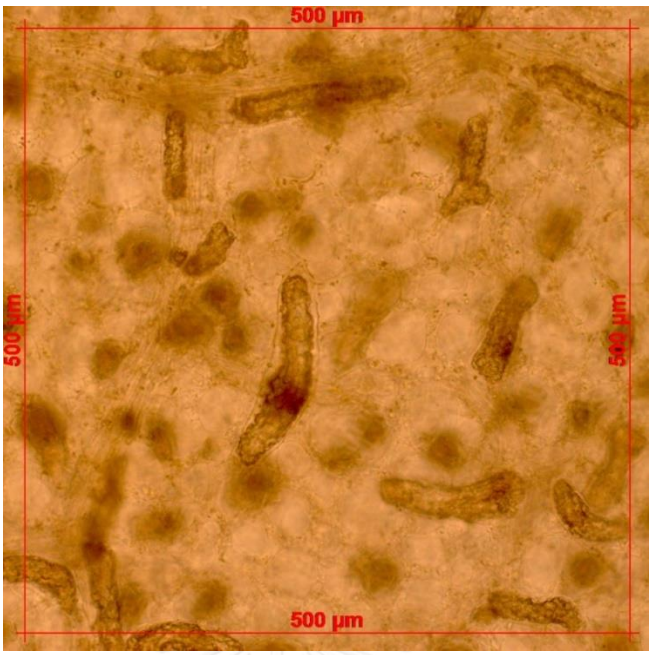


Figure 8 Cystolith in the upper epidermis of *Adhatoda vasica* Nees leaf

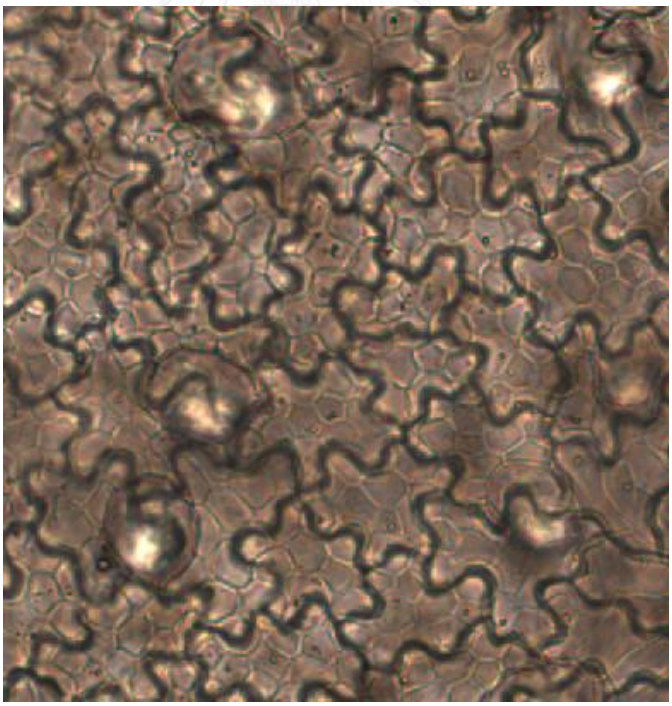


Figure 9 Palisade in the upper epidermis of *Adhatoda vasica* Nees leaf

Table 3 Stomatal number, stomatal index, palisade ratio, lithocyst number, trichome number and trichome index of *A. vasica* leaves

No.	Epidermal cell number in 1 mm^2	Epidermal cell area (μm^2)	Stomatal number in 1 mm^2	Stomatal index	Palisade ratio	Trichome number	Trichome index	Lithocyst number
1	1264	791.139	300	18.797	6.00	32	2.469	36
2	1368	730.994	292	17.381	7.25	20	1.441	28
3	1236	809.061	272	17.708	6.50	28	2.215	28
4	1228	814.332	276	18.110	7.50	20	1.603	28
5	1184	844.595	256	17.534	5.50	20	1.661	32
6	1228	814.332	272	17.801	6.50	28	2.229	40
7	1268	788.644	288	18.321	7.00	16	1.246	40
8	1372	728.863	324	18.794	6.25	28	2.000	36
9	1288	776.398	276	17.337	7.25	28	2.128	56
10	1344	744.048	296	17.661	6.50	36	2.609	24
11	1344	744.048	268	16.341	6.00	28	2.041	28
12	1224	816.993	252	16.890	7.00	16	1.290	40
13	1232	811.688	268	17.585	6.25	24	1.911	40
14	1224	816.993	260	17.241	6.25	24	1.923	28
15	1264	791.139	276	17.692	7.25	20	1.558	36
16	1312	762.195	308	18.644	6.50	32	2.381	40
17	1436	696.379	332	18.486	7.00	28	1.913	24
18	1332	750.751	252	15.672	6.75	24	1.770	48
19	1144	874.126	248	17.514	6.50	24	2.055	52
20	1376	726.744	308	17.991	7.25	28	1.994	36
21	1212	825.083	240	16.216	6.50	28	2.258	52
22	1340	746.269	312	18.483	6.75	36	2.616	44
23	1348	741.840	292	17.422	5.75	36	2.601	56
24	1456	686.813	348	18.913	6.75	36	2.413	28
25	1256	796.178	296	18.640	7.25	36	2.786	28
26	1312	762.195	284	17.488	7.00	28	2.090	32
27	1380	724.638	332	19.080	6.75	28	1.989	28
28	1284	778.816	296	18.362	5.75	32	2.432	40
29	1292	773.994	304	18.447	6.00	52	3.869	36
30	1360	735.294	320	18.605	5.50	40	2.857	32
MIN	**1144.000**	**686.813**	**240.000**	**15.672**	5.500	**16.000**	**1.246**	**24.000**
MAX	**1456.000**	**874.126**	**348.000**	**19.080**	7.500	**52.000**	**3.869**	**56.000**
MEAN	**1296.933**	**773.486**	**288.267**	**17.839**	6.567	**28.533**	**2.145**	**36.500**
SD	**74.142**	**44.190**	**27.451**	**0.833**	0.557	**7.628**	**0.531**	**9.200**

Thin layer chromatographic fingerprint

The extract of *A. vasica* was spotted on 0.2 mm thickness of TLC silica gel 60 GF_{254} plate and observed under short wavelength (254 nm), long wavelength (365 nm) ultraviolet light and sprayed with Dragendorff's reagent after developed in suitable mobile phase (chloroform : methanol (9 : 1)) (Figure 10).

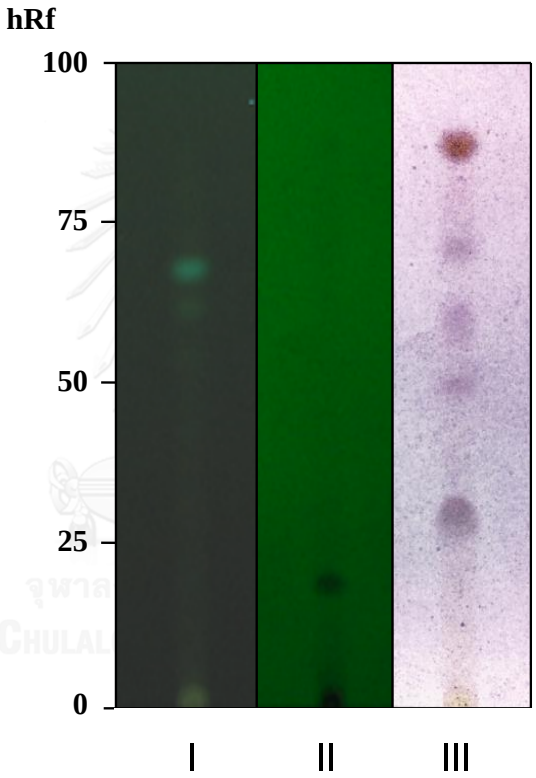


Figure 10 TLC fingerprint of the ethanolic extract of *Adhatoda vasica* Nees leaves

I = under UV light 365 nm

II = under UV light 254 nm

III = staining with Dragendorff's reagent

Ethanolic extraction of *A. vasica* leaves

The average percent yield of the ethanolic extract of *A. vasica* leaves by soxhlet extraction was 10.539 ± 2.781 % by weight (Table 4).

Table 4 The percent yield of ethanolic extract of *A. vasica* leaves from 12 different sources in Thailand

Source	Weight of sample	Weight of extractive matter	% yield
1	5.000	0.452	9.044
2	5.000	0.372	7.446
3	5.000	0.555	11.098
4	5.000	0.439	8.778
5	5.000	0.328	6.566
6	5.000	0.622	12.438
7	5.000	0.579	11.588
8	5.000	0.627	12.530
9	5.000	0.615	12.308
10	5.000	0.815	16.290
11	5.000	0.376	7.520
12	5.000	0.543	10.856
		Average	**10.539 ± 2.781**

Specificity

Peak identity

The absorbance spectra of vasicine in all samples were identical to standard vasicine. The maximum absorbance was at 290 nm (Figure 11).

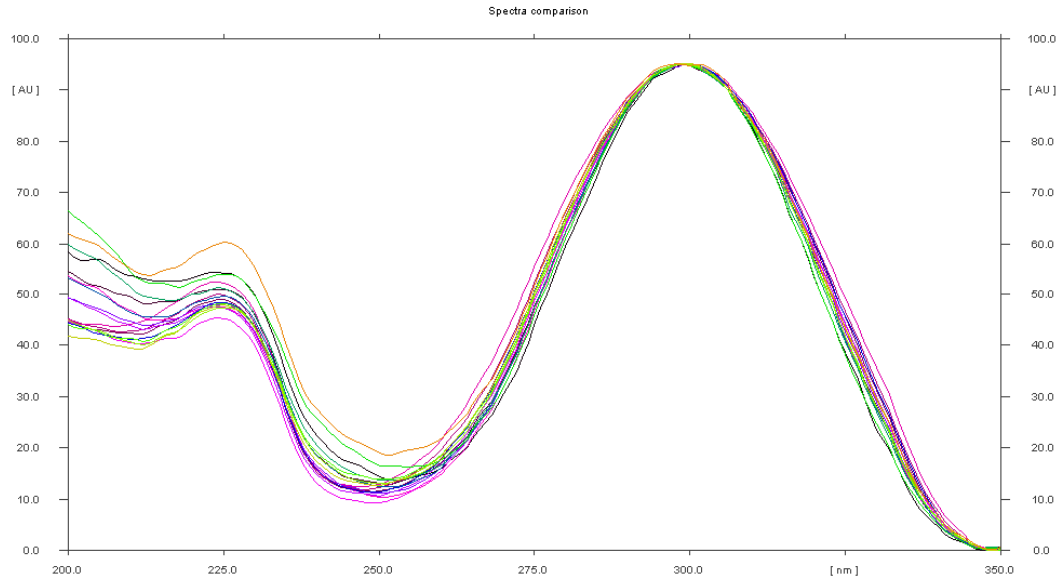


Figure 11 UV absorbance spectra of vasicine in *A. vasica* leaves and standard vasicine

Peak purity

Peak purity of vasicine determined by using up-slope, apex and down-slope of the peak showed identical UV absorbance spectra (Figure 12).

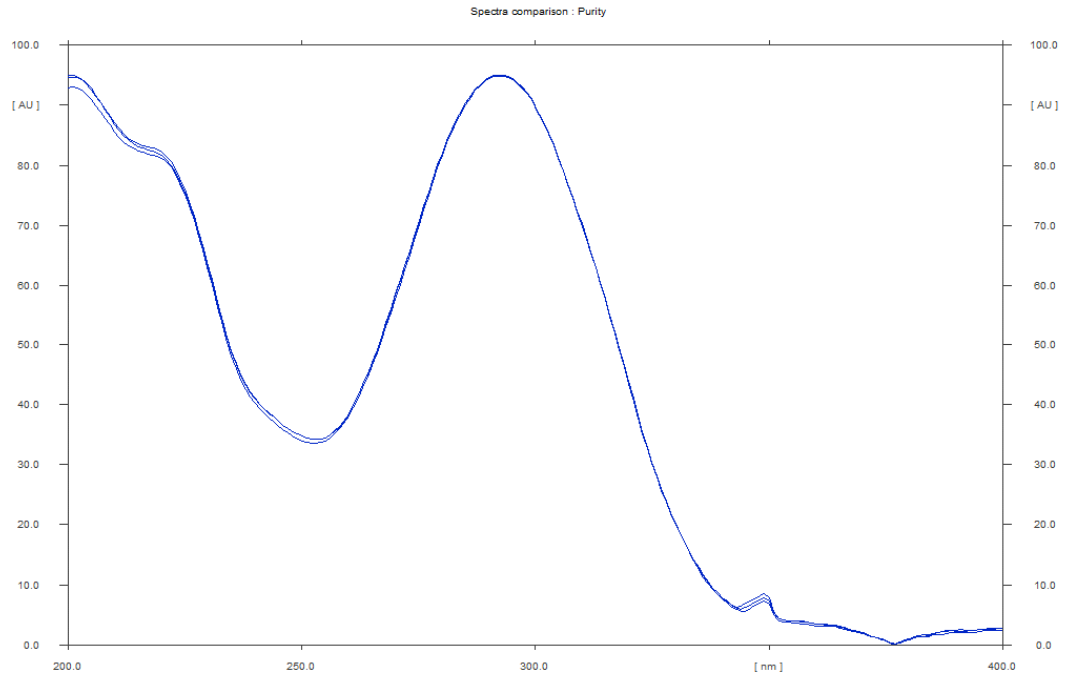


Figure 12 Peak purity determination using up-slope, apex and down-slope of the peak

Method validation (TLC image analysis)

Calibration range

The calibration of vasicine by ImageJ software was polynomial with the regression equation of $y = -254.24x^2 + 6511x - 2628.6$ and its coefficient of determination (R^2) of 0.9988. The calibration range of vasicine was 1 – 5 µg/spot.

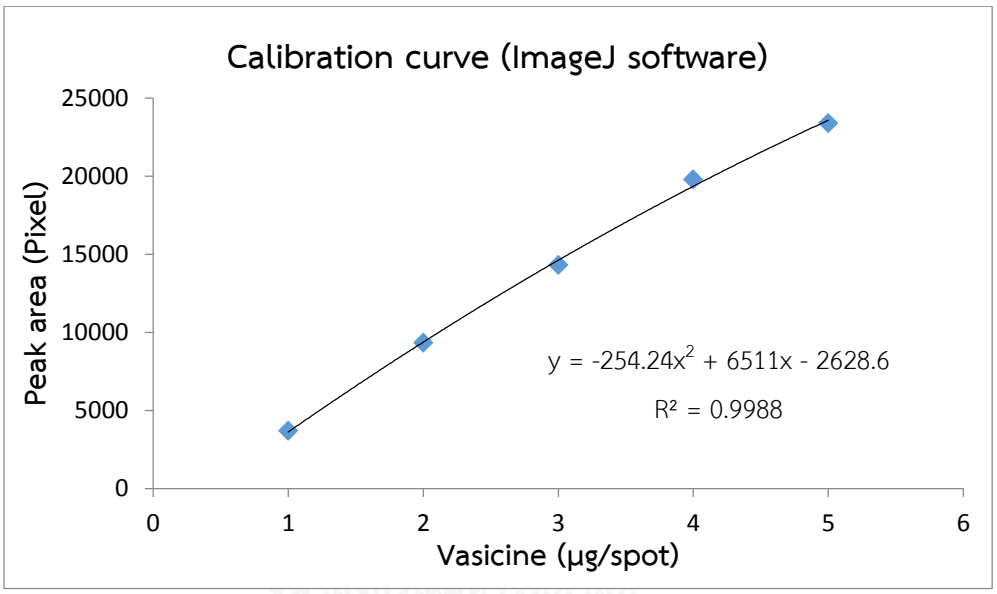


Figure 13 The calibration curve of vasicine in *A. vasica* leaves by TLC image analysis

Accuracy

The accuracy of the method was performed by recovery method. The samples were spiked with 3 concentration of vasicine standard (low, middle and high). The percent recovery was 85.32 ± 5.42 (Table 5).

Table 5 Accuracy of quantitation of vasicine in *A. vasica* leaves by TLC image analysis

Vasicine added (µg/spot)	**Vasicine found (µg/spot)**	**% Recovery**
0.00	1.94 ± 0.29	-
1.00	2.60 ± 0.42	88.28 ± 9.08
2.00	3.30 ± 0.35	83.77 ± 4.17
3.00	4.14 ± 0.33	83.91 ± 3.01
	Average	**85.32 ± 5.42**

Precision

Three different concentrations of vasicine in *A. vasica* leaves were analyzed in the same day for repeatability and different days for intermediate precision. The repeatability and intermediate precision were shown in Table 6.

Table 6 Repeatability and Intermediate precision of quantitation of vasicine in *A. vasica* leaves by TLC image analysis

Repeatability		**Intermediate precision**	
Amount (µg/spot)	**%RSD**	**Amount (µg/spot)**	**%RSD**
2.60 ± 0.42	10.29	2.39 ± 0.22	11.70
3.30 ± 0.35	4.97	3.18 ± 0.43	12.37
4.14 ± 0.33	3.59	3.92 ± 0.21	5.33
Average	**6.29 ± 3.54**		**10.45 ± 4.48**

Detection limit and quantitation limit

The evaluation of detection limit and quantitation limit were based on the residual standard deviation of the regression line and the slope of the calibration curve. The detection limit and quantitation limit were 0.188 and 0.570 µg/spot.

Robustness

The differences in peak area of vasicine by changes of the ratio of mobile phase were shown in Table 7. The robustness of the method was 11.12 %RSD.

Table 7 Robustness of vasicine in *A. vasica* leaves by image analysis

Mobile phase composition (toluene : ethyl acetate : diethylamine)	**Peak area**
4.9 : 2.1 : 3.1	7398.88
5 : 2 : 3	8318.30
5.2 : 1.9 : 2.9	6663.14
Mean ± SD	**7460.11 ± 829.28**

The content of vasicine in *A. vasica* dried leaves

All samples were determined for the vasicine content in ethanolic extracts of *A. vasica* leaves in triplicate by TLC image analysis using ImageJ software and calculated as grams per 100 grams of the crude drug (Table 8).

Table 8 The amount of vasicine in *A. vasica* leaves in % by weight (TLC image analysis)

Source	Vasicine in the ethanolic extract (mg/mg)	Yield of the ethanolic extract (g/100 g of dried crude drug)	Vasicine in *A. vasica* leaves (g/ 100g of dried crude drug)
1	0.010	9.044	0.095
2	0.020	7.446	0.149
3	0.022	11.098	0.248
4	0.015	8.778	0.128
5	0.028	6.566	0.185
6	0.015	12.438	0.184
7	0.006	11.588	0.065
8	0.004	12.530	0.045
9	0.006	12.308	0.074
10	0.006	16.290	0.100
11	0.020	7.520	0.150
12	0.015	12.500	0.190
	Average		**0.134 ± 0.061**

Method validation (TLC-densitometry)

Calibration range

The calibration of vasicine by TLC-densitometry was polynomial with the regression equation of $y = -531.04x^2 + 8851.9x + 8212.7$ and its correlation coefficient (R^2) of 0.9998. The calibration range of vasicine was 1 - 5 µg/spot.

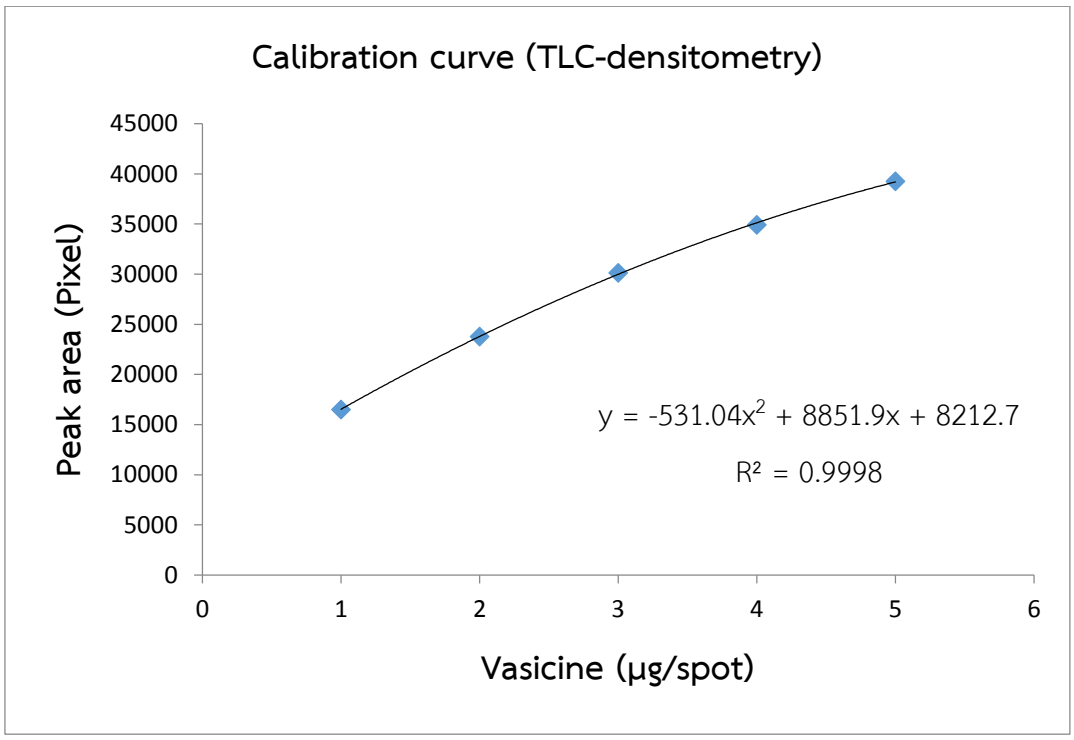


Figure 14 The calibration curve of vasicine in *A. vasica* leaves by TLC-densitometry

Accuracy

The accuracy of the method was performed by recovery method. The samples were spiked with 3 concentration of vasicine standard (low, middle and high). The percent recoveries were in the Table 9.

Table 9 Accuracy of quantitation of vasicine in *A. vasica* leaves by TLC-densitometry

Vasicine added (µg/spot)	**Vasicine found (µg/spot)**	**% Recovery**
0.00	1.73 ± 0.12	-
1.00	2.31 ± 0.09	84.78 ± 1.85
2.00	3.03 ± 0.12	81.27 ± 0.74
3.00	4.01 ± 0.08	84.79 ± 0.68
Average		**83.61 ± 1.09**

Precision

Three different concentrations of vasicine in *A. vasica* leaves were analyzed in the same day for repeatability and different days for intermediate precision. The repeatability and intermediate precision were shown in the Table 10.

Table 10 Repeatability and Intermediate precision of quantitation of vasicine in *A. vasica* leaves by TLC-densitometry

Repeatability		**Intermediate precision**	
Amount (µg/spot)	**%RSD**	**Amount (µg/spot)**	**%RSD**
2.31 ± 0.09	2.18	2.23 ± 0.09	4.22
3.03 ± 0.12	0.92	2.96 ± 0.12	4.14
4.01 ± 0.08	0.80	3.76 ± 0.13	3.52
Average	**1.30 ± 0.76**		**3.96 ± 0.39**

Detection limit and quantitation limit

The evaluation of detection limit and quantitation limit were based on the residual standard deviation of the regression line and the slope of the calibration curve. The detection limit and quantitation limit were 0.067 and 0.202 µg/spot.

Robustness

The differences in peak area of vasicine by changes of the ratio of suitable mobile phase were shown in Table 11. The robustness of the method was 4.489 %RSD.

Table 11 Robustness of vasicine in *A. vasica* leaves by TLC-densitometry

Mobile phase composition (Toluene : ethyl acetate : diethylamine)	**Peak area**
4.9 : 2.1 : 3.1	15095.37
5 : 2 : 3	16188.53
5.2 : 1.9 : 2.9	14909.13
Mean ± SD	**15397.68 ± 4.489**

The content of vasicine in *A. vasica* dried leaves

All samples were determined for the vasicine content in ethanolic extracts of *A. vasica* leaves in triplicate by TLC-densitometry and calculated as grams per 100 grams of the crude drug (Table 12).

Table 12 The amount of vasicine in *A. vasica* leaves in % by weight (TLC-densitometry)

Source	Vasicine in the ethanolic extract (mg/mg)	Yield of the ethanolic extract (g/100 g of dried crude drug)	Vasicine in *Adhatoda vasica* leaves (g/ 100g of dried crude drug)
1	0.010	9.044	0.089
2	0.020	7.446	0.146
3	0.022	11.098	0.242
4	0.015	8.778	0.130
5	0.028	6.566	0.181
6	0.013	12.438	0.166
7	0.006	11.588	0.064
8	0.000	12.530	0.001
9	0.007	12.308	0.084
10	0.006	16.290	0.104
11	0.023	7.520	0.170
12	0.015	12.500	0.189
	Average		**0.130 ± 0.065**

Comparison of vasicine contents between TLC image analysis and TLC-densitometry

Comparison of vasicine contents between TLC image analysis and TLC-densitometry were tested by using paired *t*-test statistical analysis. The result was shown that vasicine contents in both methods were not significantly different (P = 0.249) (Table 13).

Table 13 Comparison of vasicine contents in *A. vasica* leaves between TLC image analysis and TLC-densitometry

Source	Vasicine content (g/g)	
	TLC image analysis	TLC-densitometry
1	0.0105	0.0098
2	0.0200	0.0197
3	0.0224	0.0218
4	0.0145	0.0149
5	0.0282	0.0275
6	0.0148	0.0133
7	0.0056	0.0055
8	0.0036	0.0001
9	0.0060	0.0068
10	0.0061	0.0064
11	0.0200	0.0226
12	0.0152	0.0151
Average	0.0139 ±0.0078	0.0136 ± 0.0082

CHAPTER V

DISCUSSION AND CONCLUSION

The quality of plant materials affects safety and efficacy of the herbal medicines [63]. Standardization is important process for the quality control of herbal medicines [64]. The pharmacognostic specifications are set by macroscopic, microscopic examinations, physiochemical parameters, leaf measurement, chemical fingerprint profile and quantification of active chemical compound.

This study presented the pharmacognostic evaluation, leaf measurement and vasicine content of *A. vasica* leaves. Cross sectioning of the midrib of mature leaf demonstrated the anatomical structures of palisade cell, phloem, xylem, lithocyst cell and multicellular uniseriate trichome. Histological characteristics of *A. vasica* leaf powder showed diacytic stomatal type of which a pair of subsidiary cells stand right angle with the guard cells. Glandular trichomes and calcium oxalate prisms were found. This microscopic method was used as primary screening test for identification and authentication of plant materials. Furthermore, leaf constants could be applicable. The stomatal number of *A. vasica* mature leaf was found to be 288.3 ± 27.5 and the stomatal index was 15.7 – 19.1 per 1 mm^2. Two previous studies reported stomata index of 10.8 – 18.1 per 1 mm^2 and 11.5 – 13.5 per 1 mm^2 [65]. The variation in stomatal characters might be caused by genetic factors and

environmental factors especially atmospheric CO_2 [66]. The palisade ratio of 5 – 8 in this study was similar to the result of 5 – 8.5 from previous study in India [67]. This study revealed other leaf constants including lithocyst number, trichome number and trichome index of *A. vasica* leaf. The results showed that lithocyst number was 36.5 ± 9.2 per mm^2, trichome number was 28.5 ± 7.6 per mm^2 and trichome index was 2.1 ± 0.5 per mm^2.

In this study, loss on drying content of dried leaf powder of *A. vasica* was 9.3% that nearly same amount of the previous study in India (10.2%). Total ash was 20.8 % similar to the result of previous study in India which collected from Hindu University, Varanasi (20%) but the result of total ash in another study which *A. vasica* was collected from RKDF University, Madhya Pradesh was 13.5%. The result of acid insoluble ash in this study was 6.2% but from previous studies in India, they got 1.0% and 0.82% [21, 68]. The ash value meant the inorganic compounds containing in the part of herbal materials. Total ash is the measurement of total amount of materials remaining after incineration that including physiological ash and non-physiological ash. Acid insoluble ash is measurement of the silica presents after boiling in dilute hydrochloric acid. So, the result meant that *A. vasica* in Thailand has silica present more than in India that may be due to the difference in type of soil, water and weather. From the previous study, the water and ethanol extractive values of *A. vasica* dried leaf powders crude drug like this study, was 18.5% and 6.8%

respectively [68]. In this study, water extracts was 22.2% and ethanol extracts was 3.8%. The results of previous study and in this study can conclude that chemical compounds of *A. vasica* were mainly hydrophilic. Vasicine content of *A. vasica* leaf which analyzed by TLC image analysis by ImageJ software and TLC-densitometric method were found to be 0.130 and 0.134 % in dried crude drug. In India, vasicine content which analyzed by HPTLC-densitometry was 0.65% dry crude drug [69]. Muralidhar *et al.* analyzed vasicine by HPLC and demonstrated the yield of 0.59% - 0.74 % [70]. TLC image analysis is cheaper method which can be used as an alternative to TLC-densitometric method for quantitation of chemical constituents in crude drug.

In this study, TLC image analysis and TLC-densitometry were validated following ICH guideline which including accuracy, precision, specificity, detection limit (LOD), quantitation limit (LOQ) and robustness. For the robustness, this study designed the method by varying the small amounts of mobile phase ratio. In addition, other parameters could be adjusted for example, TLC chamber saturated time. Uncertainty, of these parameters should be checked to ensure method robustness [71]. Specificity of the methods was validated through UV absorbance spectra under the range of 200 – 350 nm among standard vasicine and vasicine in the extracts. The result revealed identical spectra representing chromatographic peak purity of vasicine. In this study, maximum absorption of vasicine

was 290 nm in agreement with the previous study of 292 nm [72]. The calibration curves were polynomial in both TLC image analysis and TLC-densitometry with the range of 1 – 5 µg/spot. Accuracy of TLC-densitometry and TLC image analysis were 84% and 85% that in the acceptable range (80 – 120 %). Repeatability and intermediate precisions of TLC-densitometry was in the acceptable value (< 5 %RSD). However, image analysis showed lower precisions (6 – 11 %RSD). LOD and LOQ of TLC image analysis represented lesser sensitivity than TLC-densitometry. These were due to the fact that densitometry detected UV_{290} absorption of vasicine while TLC image analysis detects picture elements of the image under UV_{254}. However, the comparison of vasicine content by TLC-densitometry and TLC image analysis showed no significant difference. So, TLC image analysis could be used instead of TLC-densitometry.

Benefits and application

1. This research provides the standardization parameters of *A. vasica* leaves.
2. This research provides the contents of vasicine in *A. vasica* leaves.
3. This research provides the simple, less expensive and valid method of TLC image analysis for vasicine quantitation in *A. vasica* leaves.

Table 14 Determination of water content of *A. vasica* dried crude drug

Source	No.	Amount (% by weight)	Mean	SD
1	1	10.00	10.000	0.000
	2	10.00		
	3	10.00		
2	1	7.00	6.999	0.499
	2	6.50		
	3	7.50		
3	1	10.40	10.133	0.231
	2	10.00		
	3	10.00		
4	1	15.50	14.500	0.866
	2	14.00		
	3	14.00		
5	1	12.00	12.000	0.000
	2	12.00		
	3	12.00		
6	1	11.00	10.667	0.577
	2	11.00		
	3	10.00		
7	1	10.00	10.000	0.000
	2	10.00		
	3	10.00		
8	1	11.00	10.667	0.577
	2	11.00		
	3	10.00		
9	1	8.00	7.666	0.289
	2	7.50		
	3	7.50		
10	1	13.00	12.667	0.577
	2	13.00		
	3	12.00		
11	1	13.00	13.000	0.000
	2	13.00		
	3	13.00		
12	1	8.00	7.832	0.288
	2	7.50		
	3	8.00		
Grand mean			**10.511**	
Pooled SD			**2.282**	

Table 15 Determination of loss on drying of *A. vasica* dried crude drug

Source	No.	Amount (% by weight)	Mean	SD
1	1	6.615	6.573	0.110
	2	6.657		
	3	6.448		
2	1	10.597	10.595	0.035
	2	10.630		
	3	10.560		
3	1	9.790	9.801	0.017
	2	9.821		
	3	9.793		
4	1	11.598	11.541	0.050
	2	11.506		
	3	11.520		
5	1	7.993	7.954	0.111
	2	7.829		
	3	8.039		
6	1	9.965	9.927	0.046
	2	9.875		
	3	9.941		
7	1	7.305	7.305	0.084
	2	7.390		
	3	7.221		
8	1	8.495	8.492	0.066
	2	8.556		
	3	8.424		
9	1	11.550	11.485	0.089
	2	11.521		
	3	11.383		
10	1	10.681	10.622	0.059
	2	10.623		
	3	10.563		
11	1	7.935	8.028	0.088
	2	8.110		
	3	8.039		
12	1	9.207	9.249	0.054
	2	9.310		
	3	9.231		
Grand mean			**9.298**	
Pooled SD			**1.588**	

Table 16 Determination of total ash of *A. vasica* dried crude drug

Source	No.	Amount (% by weight)	Mean	SD
1	1	18.882	18.909	0.080
	2	18.847		
	3	19.000		
2	1	21.570	21.565	0.054
	2	21.616		
	3	21.509		
3	1	20.302	20.486	0.165
	2	20.623		
	3	20.532		
4	1	18.871	19.079	0.180
	2	19.183		
	3	19.183		
5	1	30.305	30.436	0.134
	2	30.431		
	3	30.572		
6	1	20.437	20.515	0.071
	2	20.536		
	3	20.573		
7	1	20.509	20.482	0.047
	2	20.509		
	3	20.428		
8	1	18.892	18.903	0.021
	2	18.888		
	3	18.927		
9	1	18.894	18.979	0.093
	2	19.078		
	3	18.963		
10	1	20.759	20.848	0.078
	2	20.900		
	3	20.885		
11	1	18.649	18.720	0.077
	2	18.803		
	3	18.708		
12	1	20.388	20.313	0.067
	2	20.261		
	3	20.290		
Grand mean			**20.770**	
Pooled SD			**3.096**	

Table 17 Determination of acid insoluble ash of *A. vasica* dried crude drug

Source	No.	Amount (% by weight)	Mean	SD
1	1	3.587	3.668	0.089
	2	3.763		
	3	3.654		
2	1	12.543	12.447	0.240
	2	12.625		
	3	12.174		
3	1	4.384	4.495	0.112
	2	4.494		
	3	4.607		
4	1	4.475	4.496	0.143
	2	4.365		
	3	4.648		
5	1	16.388	16.460	0.064
	2	16.480		
	3	16.511		
6	1	5.273	5.117	0.219
	2	4.866		
	3	5.212		
7	1	6.479	6.575	0.097
	2	6.674		
	3	6.572		
8	1	4.634	4.586	0.124
	2	4.680		
	3	4.445		
9	1	2.907	2.916	0.081
	2	3.001		
	3	2.840		
10	1	4.938	5.044	0.108
	2	5.154		
	3	5.039		
11	1	4.287	4.331	0.065
	2	4.406		
	3	4.301		
12	1	4.303	4.147	0.149
	2	4.130		
	3	4.007		
Grand mean			**6.190**	
Pooled SD			**3.932**	

Table 18 Determination of ethanol soluble extractive of *A. vasica* dried crude drug

Source	No.	Amount (% by weight)	Mean	SD
1	1	6.136	6.648	0.591
	2	6.515		
	3	7.294		
2	1	1.797	1.795	0.025
	2	1.769		
	3	1.819		
3	1	3.154	3.238	0.086
	2	3.326		
	3	3.235		
4	1	3.898	4.027	0.140
	2	4.007		
	3	4.176		
5	1	3.585	3.357	0.263
	2	3.417		
	3	3.069		
6	1	4.018	3.923	0.171
	2	3.725		
	3	4.026		
7	1	3.447	3.570	0.106
	2	3.629		
	3	3.634		
8	1	4.342	4.696	0.318
	2	4.788		
	3	4.958		
9	1	2.157	2.172	0.042
	2	2.220		
	3	2.139		
10	1	3.636	3.799	0.149
	2	3.834		
	3	3.928		
11	1	4.258	4.309	0.092
	2	4.253		
	3	4.415		
12	1	3.703	3.928	0.198
	2	4.006		
	3	4.075		
Grand mean			**3.789**	
Pooled SD			**1.205**	

Table 19 Determination of water soluble extractive of *A. vasica* dried crude drug

Source	No.	Amount (% by weight)	Mean	SD
1	1	26.829	26.477	0.361
	2	26.107		
	3	26.496		
2	1	14.811	14.882	0.075
	2	14.961		
	3	14.874		
3	1	19.438	19.963	0.469
	2	20.113		
	3	20.340		
4	1	20.279	20.067	0.185
	2	19.940		
	3	19.982		
5	1	20.452	19.560	1.125
	2	18.296		
	3	19.932		
6	1	22.620	22.186	0.442
	2	21.737		
	3	22.201		
7	1	18.956	19.423	0.419
	2	19.766		
	3	19.546		
8	1	22.310	22.940	0.684
	2	23.667		
	3	22.842		
9	1	19.857	19.475	0.425
	2	19.551		
	3	19.016		
10	1	19.874	19.969	0.339
	2	20.345		
	3	19.687		
11	1	35.407	34.857	0.484
	2	34.499		
	3	34.664		
12	1	26.179	26.068	0.159
	2	26.138		
	3	25.886		
Grand mean			**22.155**	
Pooled SD			**4.953**	

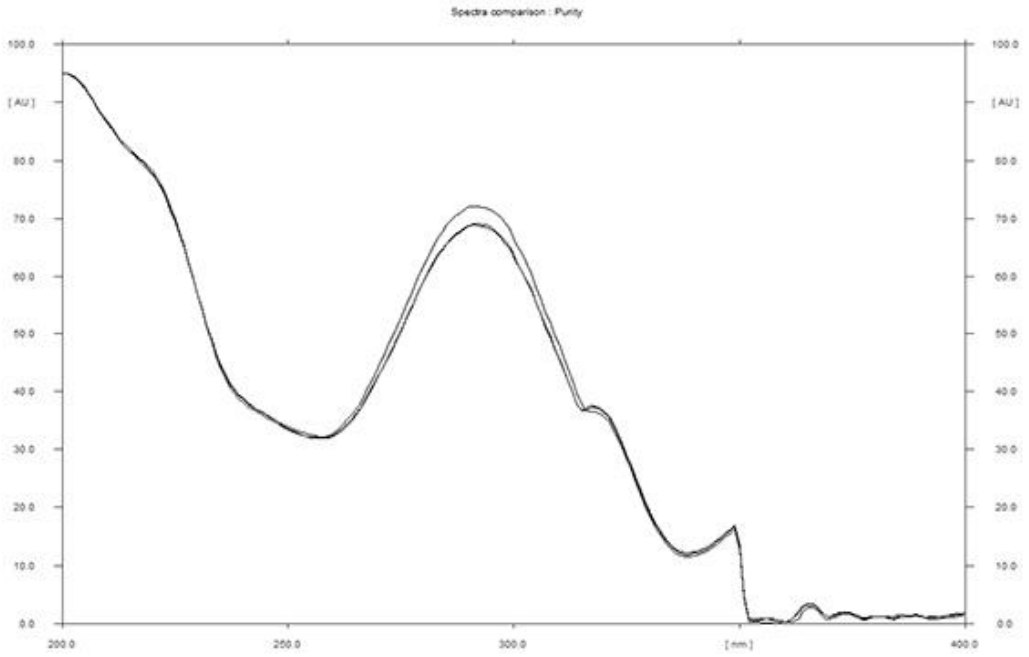


Figure 15 Peak purity determination of standard 1 using up-slope, apex and down-slope of the peak

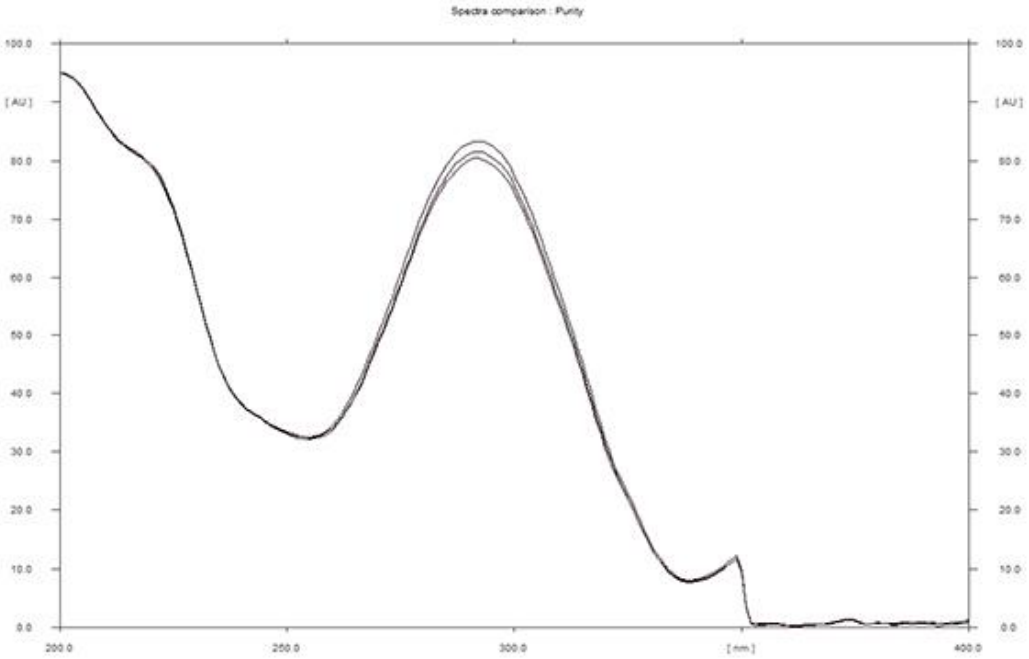


Figure 16 Peak purity determination of standard 2 using up-slope, apex and down-slope of the peak

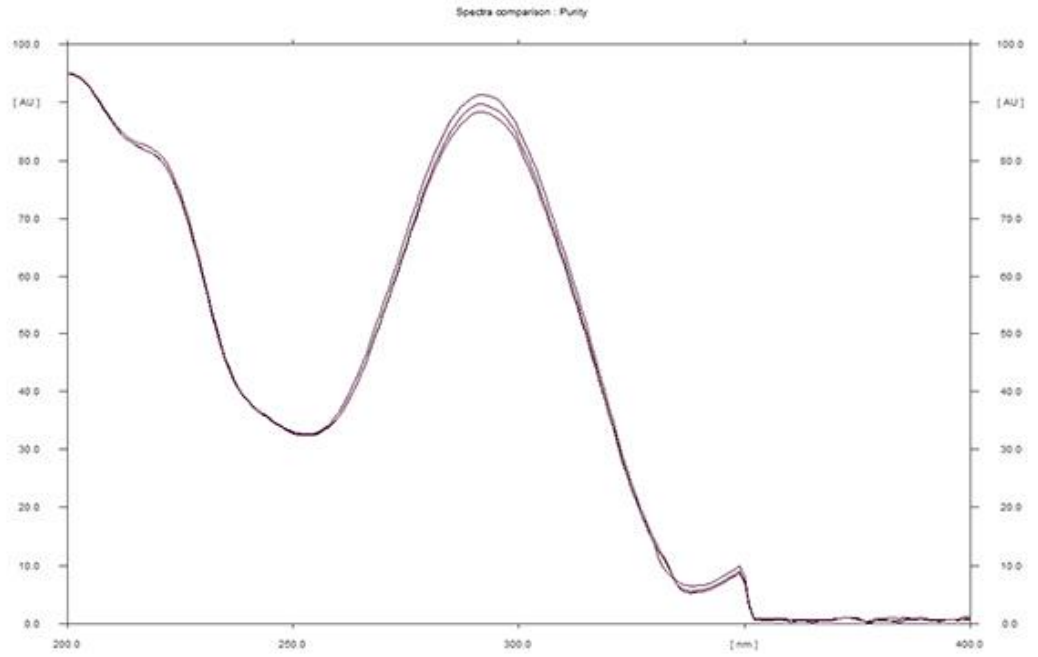


Figure 17 Peak purity determination of standard 3 using up-slope, apex and down-slope of the peak

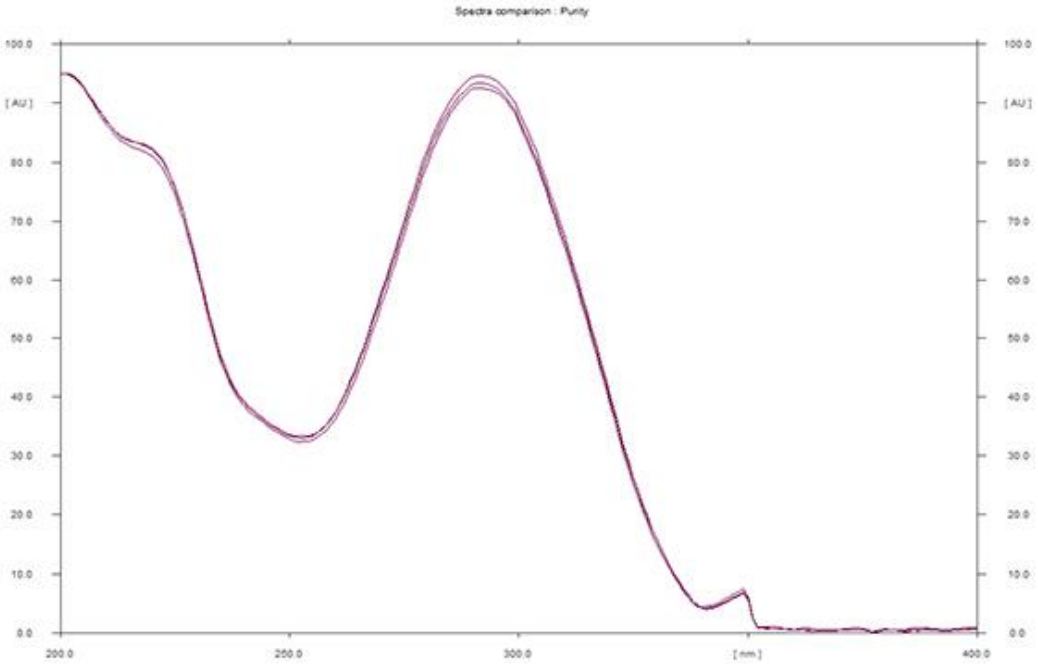


Figure 18 Peak purity determination of standard 4 using up-slope, apex and down-slope of the peak

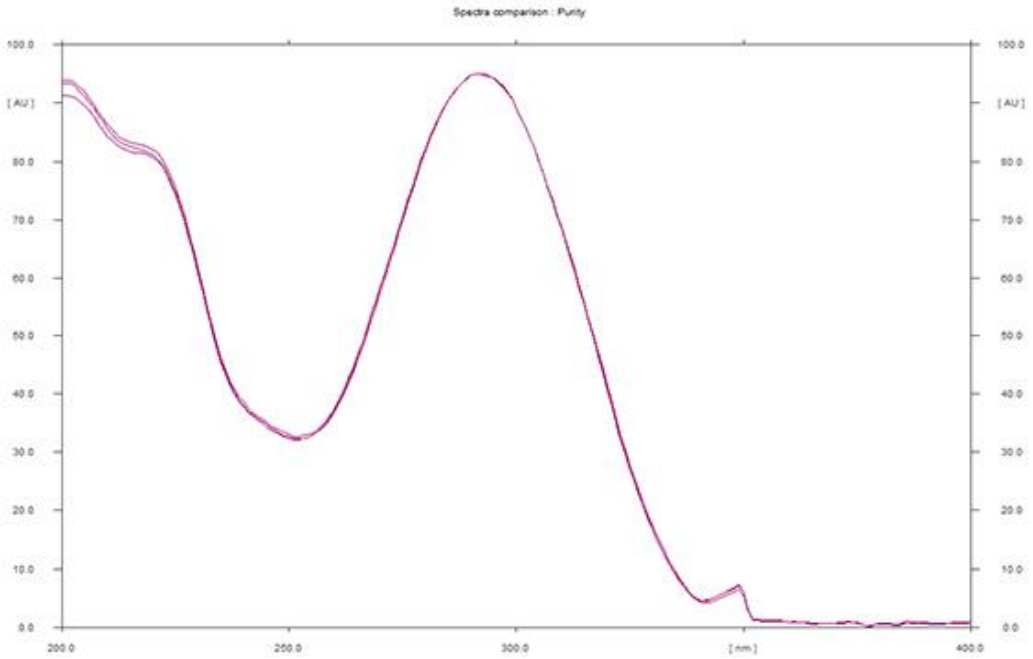


Figure 19 Peak purity determination of standard 5 using up-slope, apex and down-slope of the peak

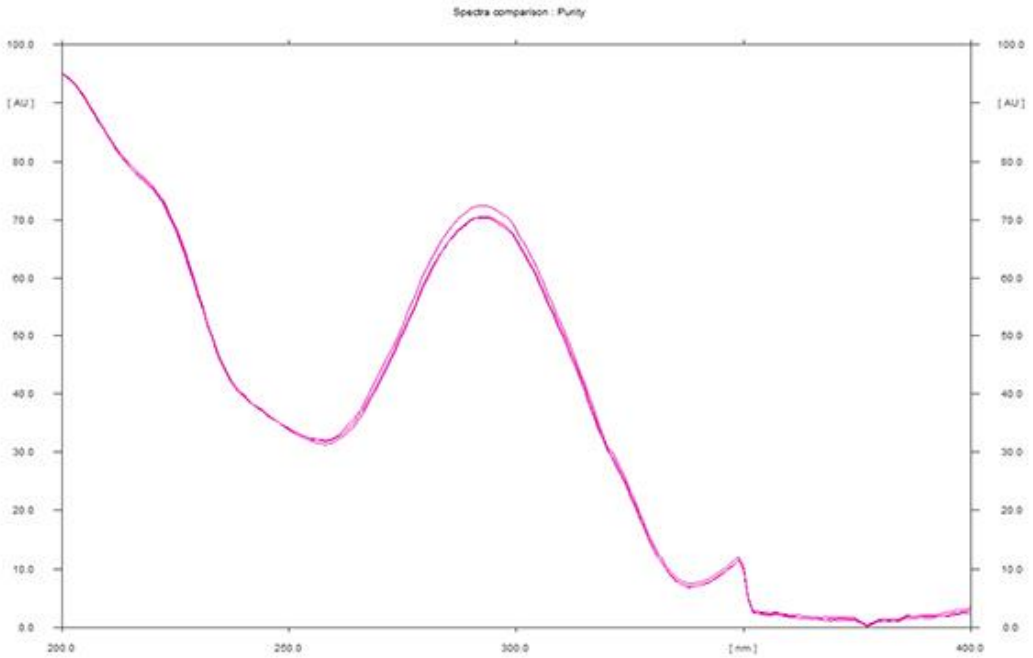


Figure 20 Peak purity determination of sample 1 using up-slope, apex and down-slope of the peak

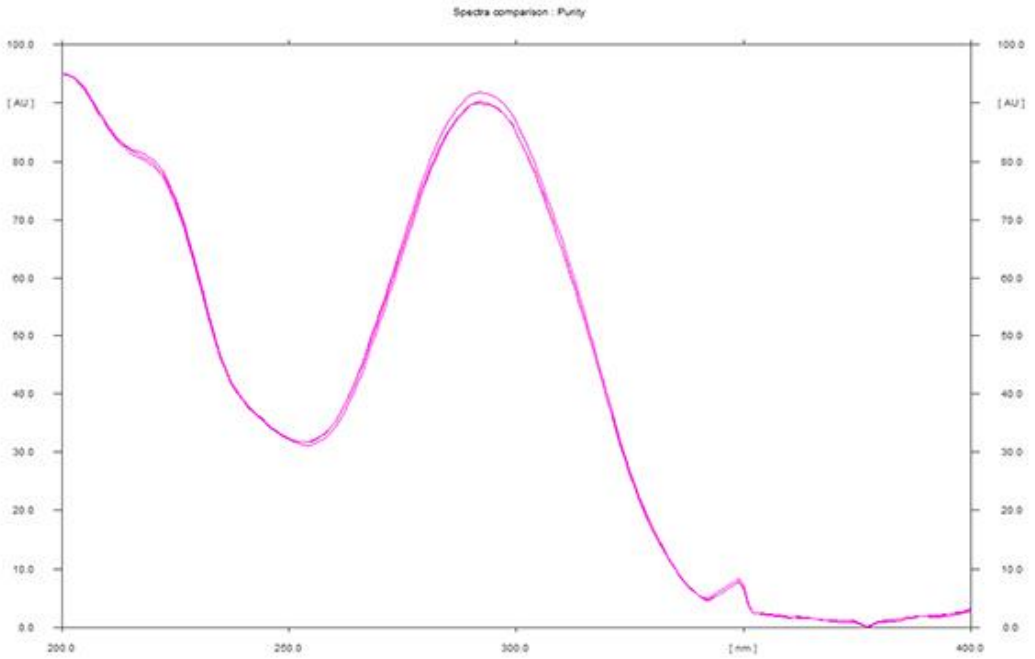


Figure 21 Peak purity determination of sample 2 using up-slope, apex and down-slope of the peak

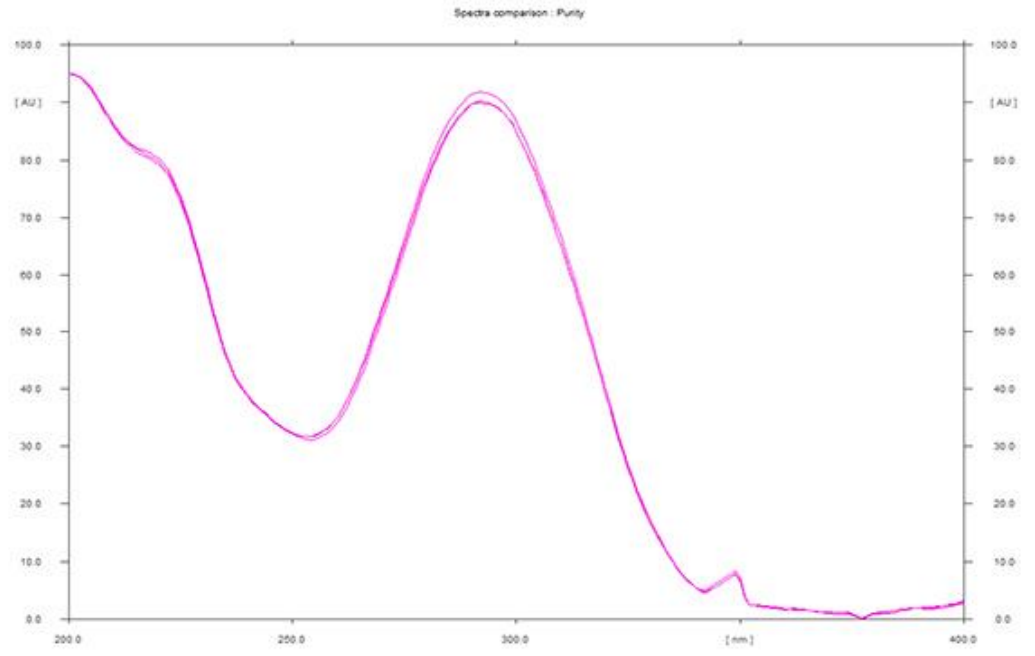


Figure 22 Peak purity determination of sample 3 using up-slope, apex and down-slope of the peak

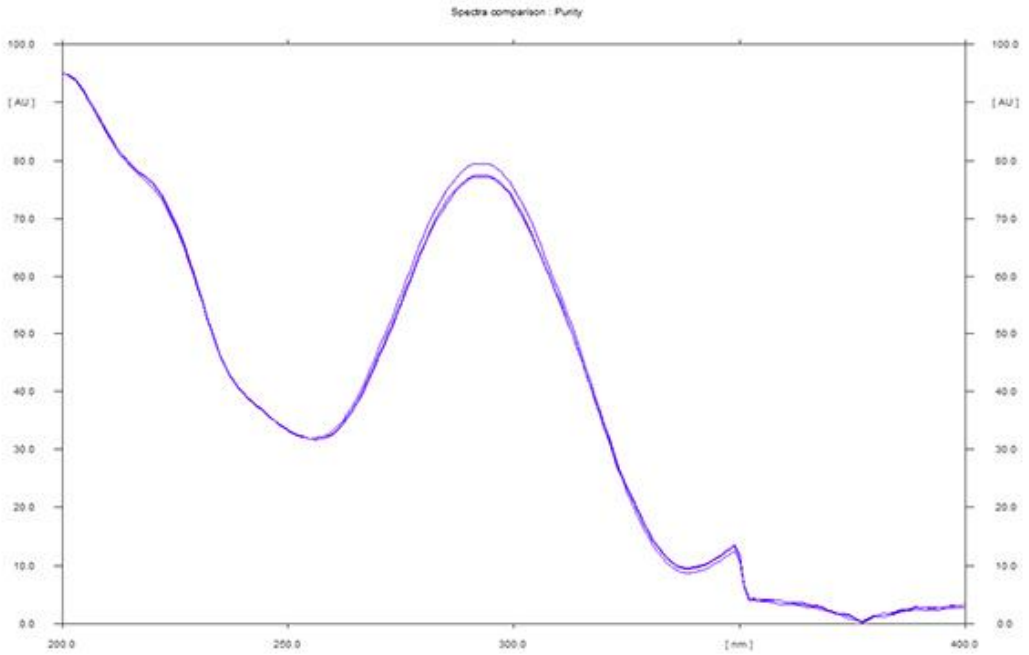


Figure 23 Peak purity determination of sample 4 using up-slope, apex and down-slope of the peak

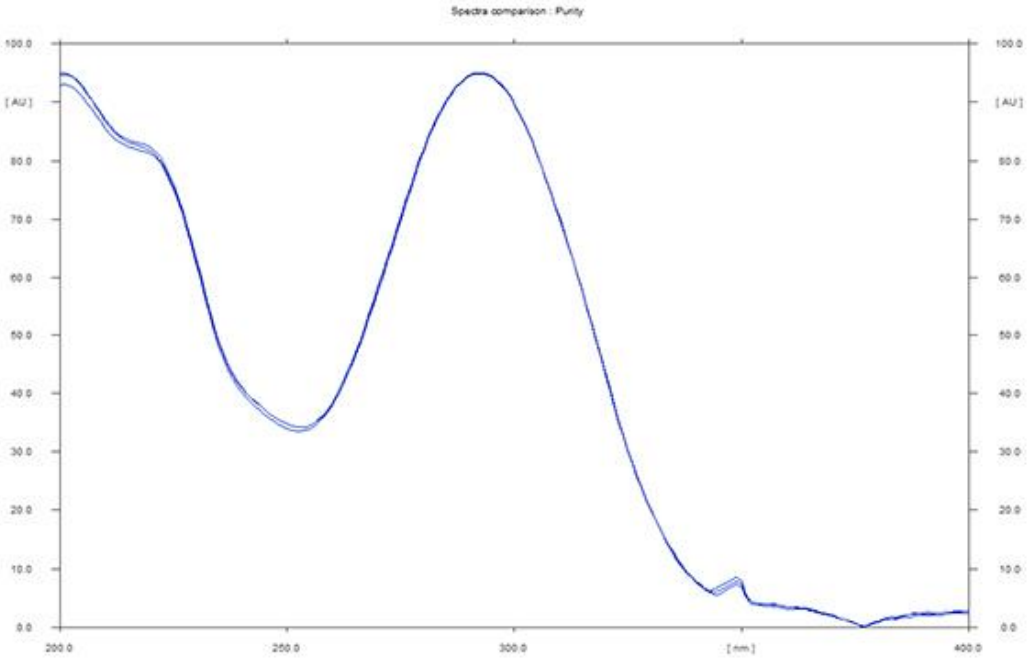


Figure 24 Peak purity determination of sample 5 using up-slope, apex and down-slope of the peak

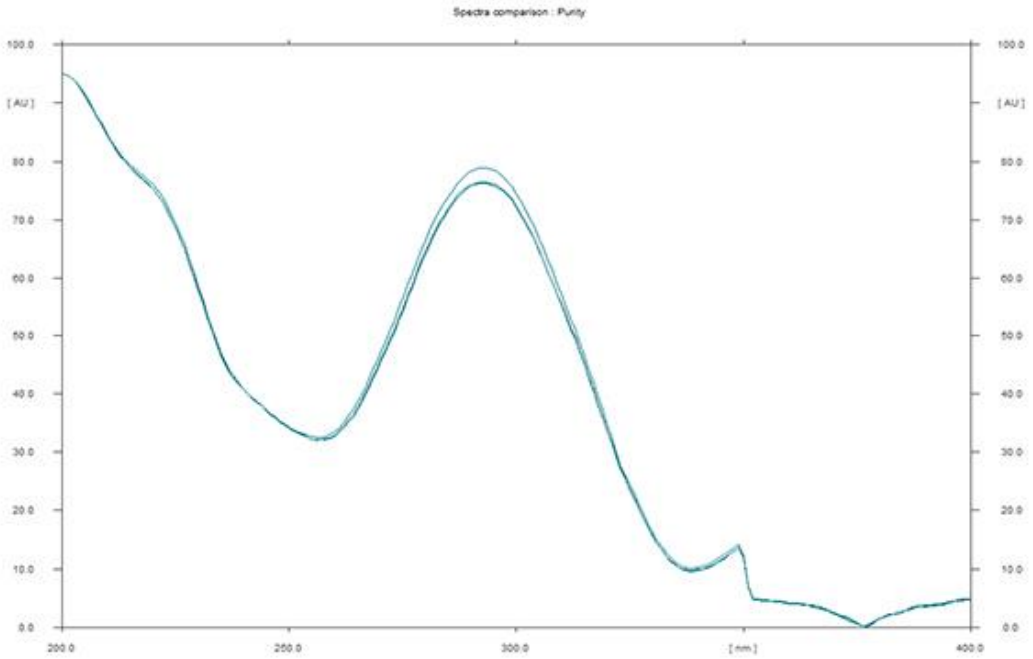


Figure 25 Peak purity determination of sample 6 using up-slope, apex and down-slope of the peak

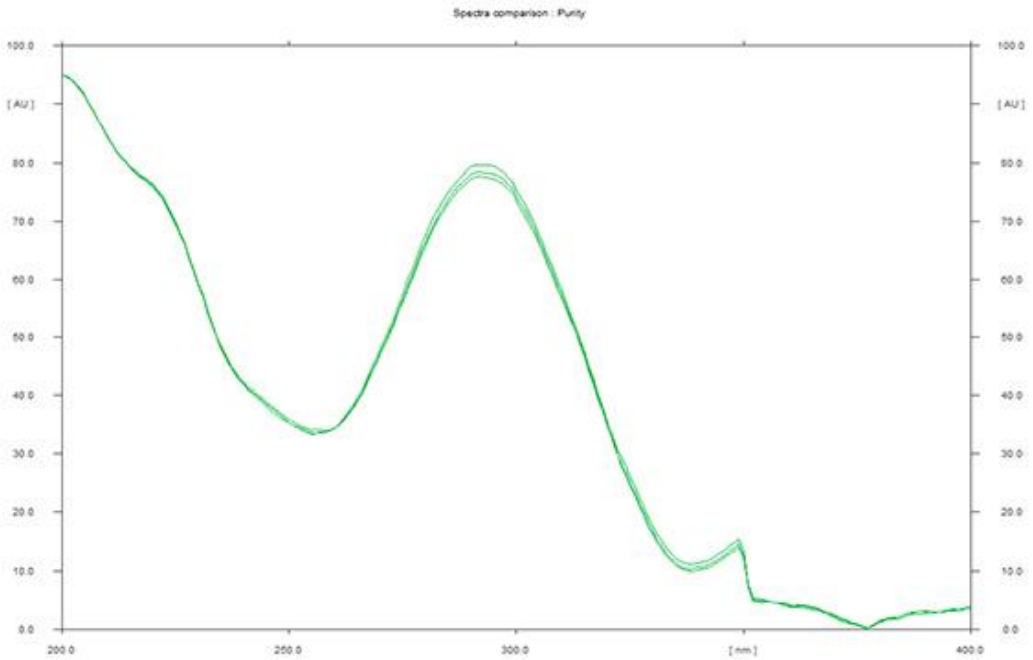


Figure 26 Peak purity determination of sample 7 using up-slope, apex and down-slope of the peak

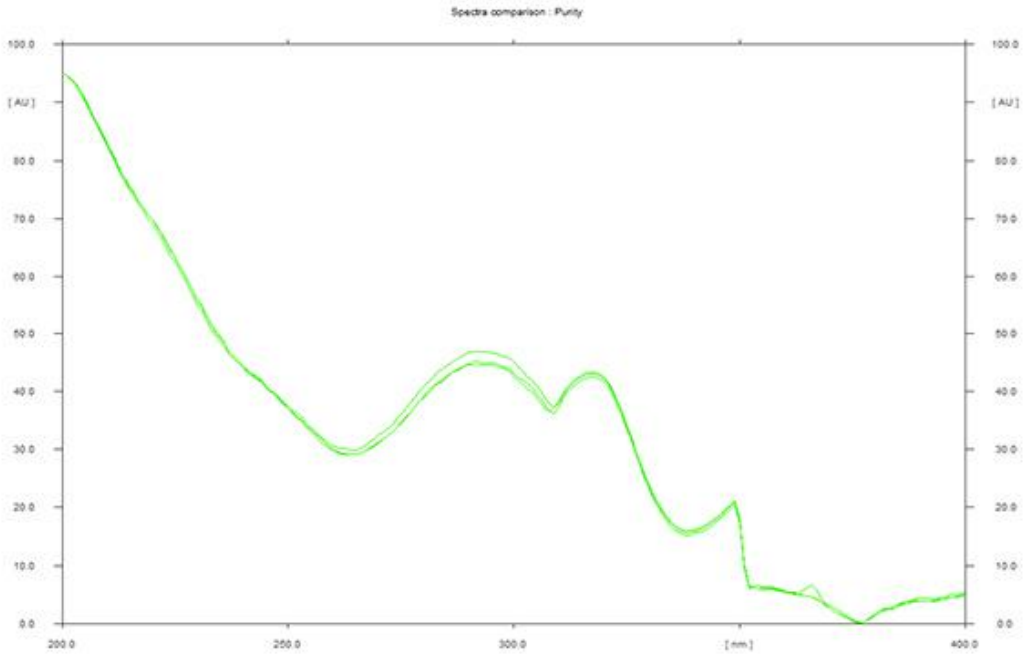


Figure 27 Peak purity determination of sample 8 using up-slope, apex and down-slope of the peak

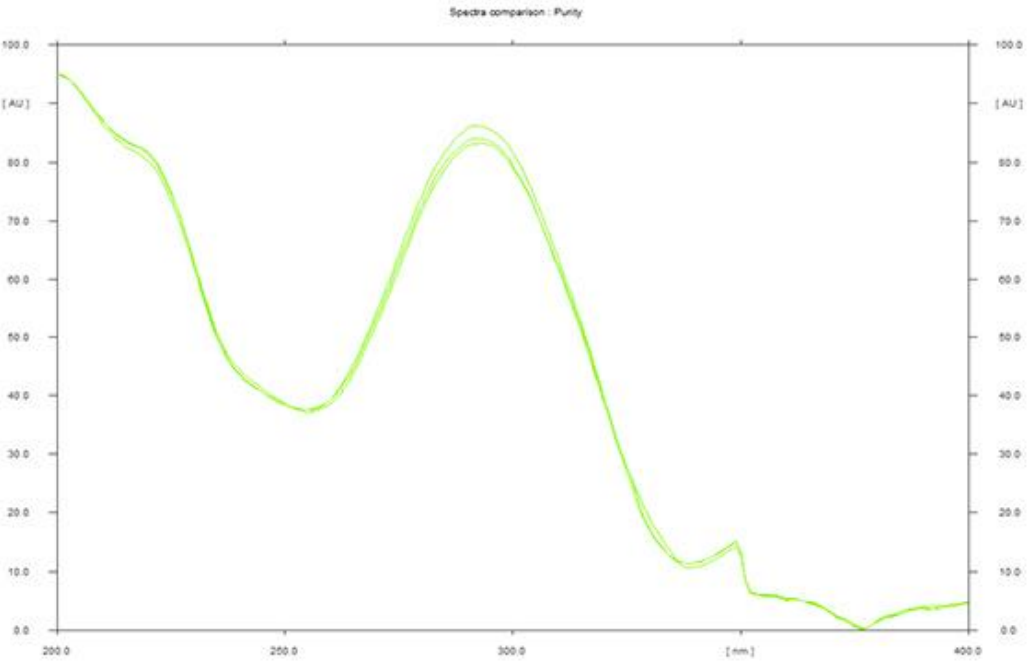


Figure 28 Peak purity determination of sample 9 using up-slope, apex and down-slope of the peak

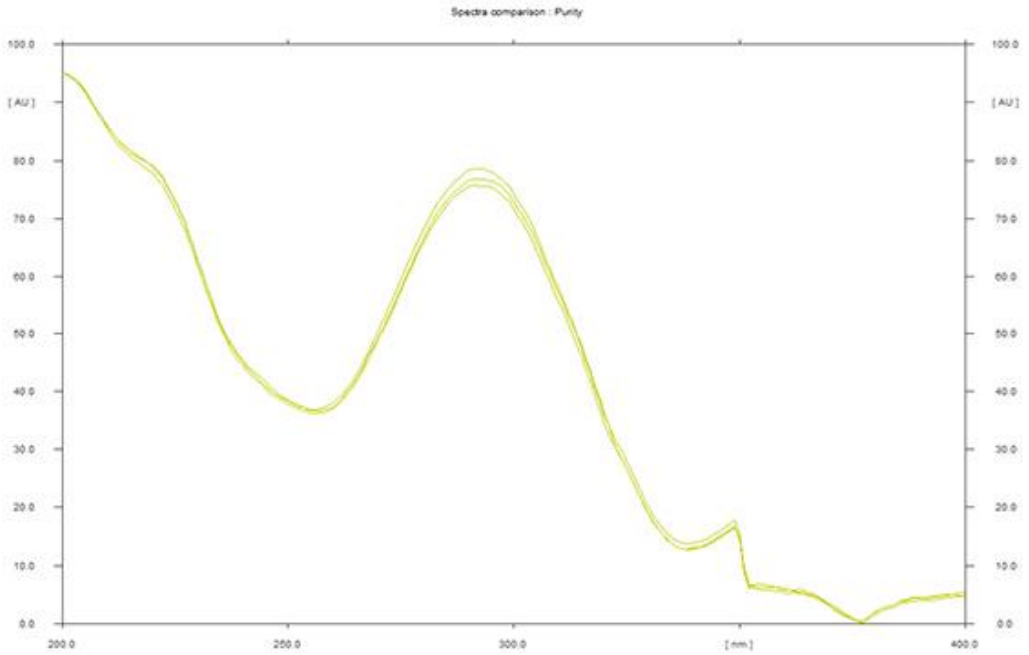


Figure 29 Peak purity determination of sample 10 using up-slope, apex and down-slope of the peak

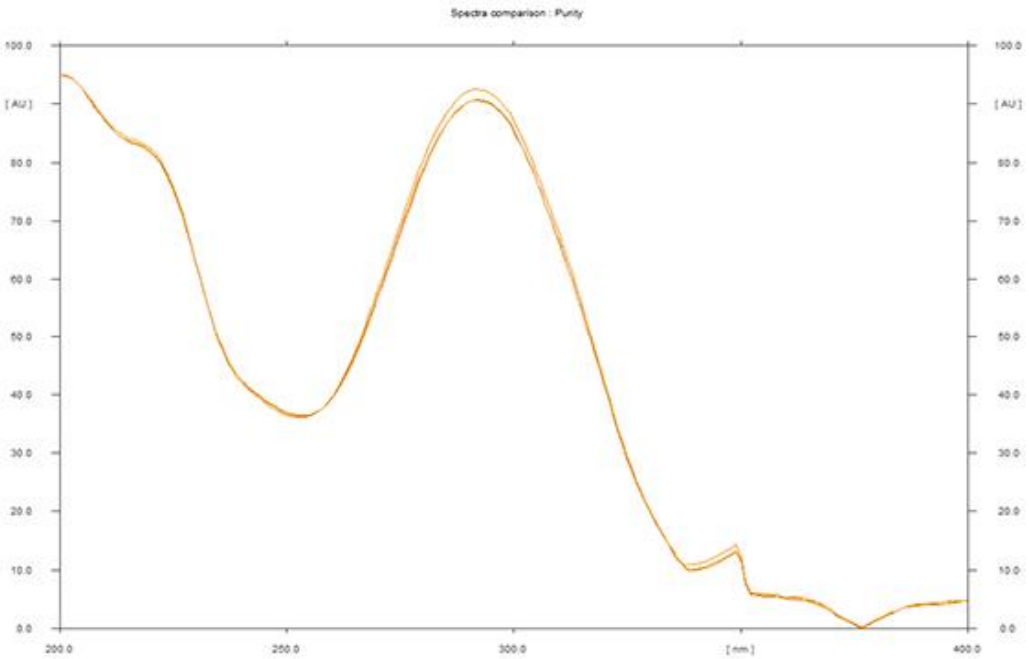


Figure 30 Peak purity determination of sample 11 using up-slope, apex and down-slope of the peak

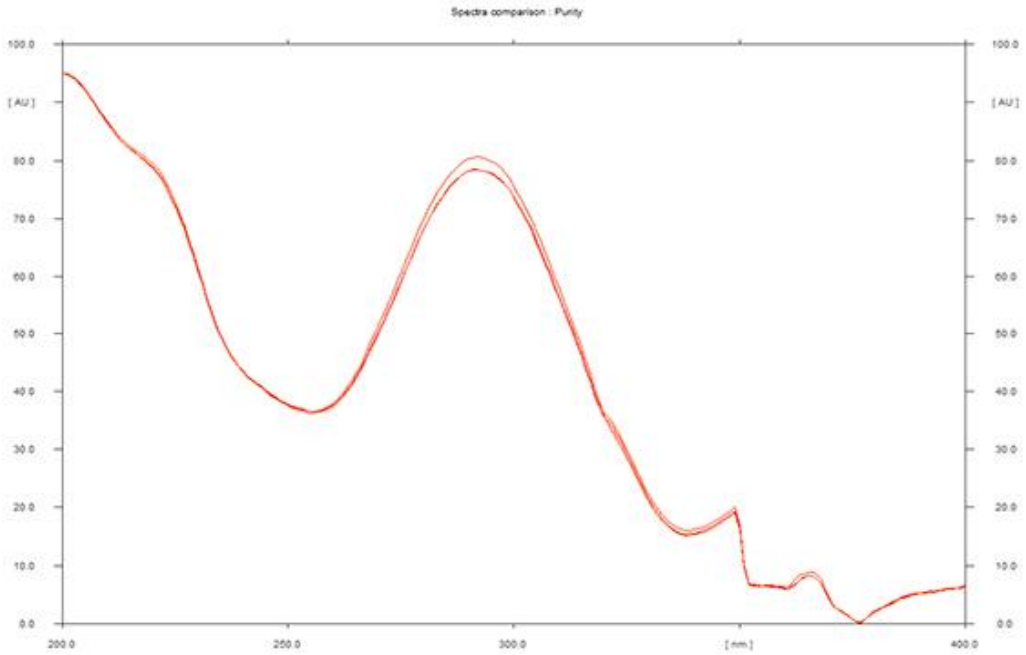


Figure 31 Peak purity determination of sample 12 using up-slope, apex and down-slope of the peak

REFERENCES

1. Plant Genetic Conservation Project Office. 1996 16/3/58; Available from: http://www.rspg.or.th/plants_data/herbs/herbs_200.htm.
2. National Drug Committee, *List of Herbal Medicine Products A.D.2006*. 2006.
3. Supavita, T., *Pharmacognostic identification: Plant histology by microscopy*. 2010, Songkhla, Thailand: Neo Point Co.,Ltd. 80.
4. Saha, J. and S. Kalyanasundaram, *Studies on pollen allergy in Pondicherry.* Indian Journal of Medical Research, 1965. **50**: p. 193-198.
5. Chaubal, P.D. and S.B. Gadve, *Study of pollen allergy in Kolhapur during monsoon.* Indian Journal of Chest Diseases and Allied Sciences, 1984. **26**: p. 38-40.
6. Arbat, A. and G.V. Patil, *Common allergenic pollen and spores causing respiratory allergy from capital.* J. Soc. Pure Appl. Nat. Sci., 1985. **1**(5-7).
7. Rachana, et al., *Review & Future Perspectives of Using Vasicine, and Related Compounds.* Indo-Global Journal of Pharmaceutical Sciences, 2011. **1**(1): p. 85-98.
8. Temsiririrkkul, R. ยาเขียว ยาไทยใช้ได้ทั้งผู้ใหญ่และเด็ก. 2013; Available from: http://www.pharmacy.mahidol.ac.th/th/knowledge/article/156.
9. Alam, K., D. Pathak, and A. SH, *Phytochemical and Pharmacological Investigations on Adhatoda zeylanica (Medic.): A Review.* PHCOG J, 2010. **2**(12): p. 513-519.
10. Dhankhar, S., et al., *A review on Justicia adhatoda: A potential source of natural medicine.* African Journal of Plant Science, 2011. **5**(11): p. 620-627.
11. Raja, S.S., et al., *Variation in Vasicine Content and Pharmacognostic Characters of Morphotypes of Adhatoda zeylanica Medic.* Journal of Plant Sciences 3, 2008. **1**: p. 61-68.
12. Singh, T.P., O.M. Singh, and H.B. Singh, *Adhatoda vasica Nees: Phytochemical and Pharmacological Profile.* The Natural Products Journal, 2011. **1**: p. 29-39.

13. Maurya, S. and D. Singh, *Quantitative Analysis of Total Phenolic Content in Adhatoda vasica Nees Extracts.* International Journal of PharmTech Research, 2010. **2**(4): p. 2403-2406.
14. Singh, S., A. Hussain, and D. Singh, *Phytochemical Screening and Determination of Quinazoline Alkaloid in Adhatoda vasica.* International Journal of Pharmaceutical Sciences Review and Research, 2012. **14**(2): p. 115-118.
15. SIGMA-ALDRICH. *vasicine*. 2014 [cited 2014 14.03]; Available from: www.sigma-aldrich.com.
16. Nepali, K., S. Sharma, and R. Ojha, *Vasicine and structurally related quinazolines.* Medicinal Chemistry Research, 2013. **22**: p. 1-15.
17. Ghosal, S., R.B.P.S. Chauhan, and R. Mehta, *Alkaloids of Sida Cordifolia.* Phytochemistry, 1974. **14**: p. 830-832.
18. Susag, L., S. Mathenge, and M. Benn, *The alkaloids of two species of Afrogalega.* Biochemical Systematics and Ecology 2002. **31**: p. 645-647.
19. Rajani, M. and K. Pundarikakshudu, *A note on the seasonal variation of alkaloids in Adhatoda vasica Nees.* International Journal of Pharmacognosy, 1996. **34**(4): p. 308-309.
20. Gangwar, A.K. and A.K. Ghosh, *Medicinal uses and pharmacological activity of Adhatoda vasica.* International Journal of Herbal Medicine, 2014. **2**(1): p. 88-91.
21. KumarSingh, S., et al., *Pharmacognostic study and phytochemical screening of leaf of Adhatoda vasica (Acanthaceae).* Journal of Medicinal Plants Studies, 2014. **2**(4): p. 29-31.
22. Srinivasarao, D., et al., *A study on Antioxidant and Anti-inflammatory activity of Vasicine against lung damage in rats.* Indian J Allergy Asthma Immunol, 2006. **20**(1): p. 1-7.
23. Singh, B. and R.A. Sharma, *Anti-inflammatory and antimicrobial properties of pyrroloquinazoline alkaloids from Adhatoda vasica Nees.* Phytomedicine, 2013. **20**: p. 441-445.

24. Dhuley, J.N., *Antitussive effect of Adhatoda vasica extract on mechanical or chemical stimulation-induced coughing in animals.* Journal of Ethnopharmacology, 1999. **67**: p. 361-365.
25. Jahan, Y. and H.H. Siddiqui, *Study of antitussive potential of Glycyrrhiza Glabra and Adhatoda vasica using a cough model induced by sulphur dioxide gas in mice.* International Journal of Pharmaceutical Sciences and Research, 2012. **3**(06): p. 1668-1674.
26. Chattopadhyay, N., et al., *Structural features and antitussive activity of water extracted polysaccharide from Adhatoda vasica.* Carbohydrate Polymers, 2011. **83**: p. 1970-1974.
27. Duraipandiyan, V., et al., *Antimicrobial, Antioxidant, and Cytotoxic Properties of Vasicine Acetate Synthesized from Vasicine Isolated from Adhatoda vasica L.* Hindawi, 2014. **2015**: p. 1-7.
28. Walter, C., et al., *Antibacterial activity in herbal products used in Pakistan.* Pakistan Journal of Botany, 2011. **43**(Special): p. 155-162.
29. S., M., et al., *Evaluation of antibacterial activity of methanol extract of leaves of Adhatoda vasica on mastitis pathogens.* Hygeia :: journal for drugs and medicines, 2013. **5**(1): p. 1-4.
30. Prakash, K.C., et al., *Studies on chromatographic finger print analysis and antibacterial activity of Adhatoda vasica leaves extracts.* Pharmacologyonline, 2011. **3**: p. 1322-1329.
31. Shrivastava, N., et al., *Anti-Ulcer Activity of Adhatoda vasica Nees.* Journal of Herbal Pharmacotherapy, 2006. **6**(2): p. 43-49.
32. G., V. and S. K., *Anti-ulcer activity of Adhatoda vasica leaves against gastric ulcer in rats.* Journal of Global Pharma Technology, 2011. **3**(2): p. 7-13.
33. Yadav, A.K. and V. Tangpu, *Anticestodal activity of Adhatoda vasica extract against Hymenolepis diminuta infections in rats.* Journal of Ethnopharmacology, 2008. **119**: p. 322-324.
34. Rathnasamy, S., et al., *Evaluation of cytotoxic, mutagenic and antimutagenic potential of leaf extracts of three medicinal plants using Allium cepa*

chromosome assay. International Current Phamaceutical Journal, 2013. **2**(8): p. 131-140.

35. S., S. and K.D. Arunachalam, *Investigations on the phytochemical activities and wound healing properties of Adhatoda vasica leaves in Swiss albino mice.* African Journal of Plant Science, 2011. **5**(2): p. 133-145.
36. Atal, C.K. and *et al*., *Cultivation and utilization of medicine plants.* Council of Scientific and Industrial Research, 1982: p. 41.
37. Pahwa, G.S. and *et al*., *Chronic toxicity studies with vasicine from Adhatoda vasica Nees in rats and monkeys.* Indian Journal Experimental Biology, 1987. **25**: p. 467-470.
38. Zhao, Z. and *et al.*, *Authentication is fundamental for standardization of Chinese medicines.* Planta Medica, 2006: p. 864-874.
39. Trease, G.E. and W. C. Evans, *Pharmacognosy*, ed. 16. 2009, London: W & B Saunders Press.
40. Mukherjee, P.K., *Quality Control of Herbal Drugs*. 2 ed., New Delhi: Business Horizons Pharmaceutical.
41. Stahl, E., *Drug Analysis by Chromatography and Microscopy : A Practical Supplement to Pharmacopoeias*. Michigan: Ann Arbor.
42. World Health Organization, *WHO Guidelines, Quality control methods for medicinal plant materials*. 2011, Geneva: World Health Organization.
43. Youngken, H.W., *Textbook of Pharmacognosy*. 6 ed., New York: McGrawHill.
44. Wallis, T.E., *Textbook of Pharmacognosy*. 14 ed., London: J & A Churchill Press.
45. Rafi, M., et al., *Differentiation of Curcuma longa, Curcuma xanthorrhiza and Zingiber cassumunar by thin laver chromatography.* Indonesian Journal of Chemistry, 2011. **11**: p. 71-74.
46. Shewiyo, D.H., *HPTLC methods to assay active ingredients in pharmaceutical formulation : A review of the method development and validation steps.* Journal of Pharmaceutical and Biomedical Analysis, 2012. **66**: p. 11-23.

47. Zhang, L. and X. Lin, *Quantitative evaluation of thin-layer chromatography with image background estimation based on charge-coupled device imaging.* Journal of Chromatography, 2006. **1109**: p. 273-278.
48. Hoeltz, M., J. Noll, and H.A. Dottori, *Photometric procedure for quantitative analysis of Aflatoxin B1 in peanuts by thin-layer chromatography using charge coupled device detector.* Quimica Nove, 2010. **33**: p. 43-47.
49. Sherma, J. and B. Fried, *Handbook of thin-layer chromatography*. 2 ed. 1996, New York: Marcel Dekker.
50. Wagner, H. and S. Bladt, *Plant drug analysis a thin layer chromatography atlas*. 2001, Germany: Springer.
51. Delloyd's Lab Tech resources reagents and solutions, *Preparation of chromatography spray reagents*. 2013: http://delloyd.50megs.com/spray_reagents.html.
52. Spangenberg, B., D.F. Poole, and C. Weins, *Quantitative thin-layer chromatography*. 2011, Germany: Springer - Verlag Berlin Heidelberg.
53. Kaalea, E., P. Rishaa, and T. Layloff, *TLC for pharmaceutical analysis in resource limited countries.* Journal of Chromatography 2011. **A 1218**: p. 2732-2736.
54. Liu, W.J.H., *Traditional herbal medicine research methods: identification, analysis, bioassay and pharmaceutical and clinical studies*. 2011, Singapore: A John Wiley & Sons.
55. University of Michigan. *TLC Stains*. Available from: http://www.umich.edu/~mssgroup/docs/TLCStains.pdf.
56. Yongyu, Z., et al., *Quality Control Method for Herbal Medicine-Chemical Fingerprint Analysis, Quality Control of Herbal Medicines and Related Areas*. 2011.
57. Schneider, C.A., W.S. Rasband, and K.W. Eliceiri, *NIH Image to ImageJ 25 years of image analysis.* Nature Methods, 2012. **9**: p. 671-675.
58. Sherma, J., *Encyclopedia of chromatography*. 2010, New York: CRC Press.
59. Mohammad, A., *Analysis of herbal products by thin-layer chromatography : a review.* International Journal of Pharma and Bio Sciences, 2010: p. 1-50.

60. Ciesla, L., *Biological Fingerprinting of Herbal Samples by Means of Liquid Chromatography.* Chromatography Research International, 2012: p. 1-9.
61. Hajimehdipoor, H. and *et al.*, *Fingerprint Study of Thymus spp. by TLC.* Journal of Medicinal Plant, 2009. **8**: p. 19-24.
62. ICH Harmonized Tripartide Guideline, V.o.A.P., Text and Methodology, Q2(R1)," *The international conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*. 2005, Geneva.
63. Kadam, P.H.N.J.K.A.J.V., *Future trends in standardization of herbal drugs.* Journal of Applied Pharmaceutical Science, 2012. **02**(06): p. 38-44.
64. Dey, N.S.P.M.S., *Herbal drugs: Standards and regulation.* Fitoterapia, 2010. **81**: p. 462-471.
65. Unnati, S., et al., *Pharmacognostical and phytochemical evaluation of Adhatoda vasica leaf.* International Journal of Research Studies in Biosciences, 2014. **2**(11): p. 144-148.
66. Hetherington, A.M. and F.I. Woodword, *The role of stomata in sensing and driving environmental change.* NATURE, 2003. **424**: p. 901-908.
67. Shah, U.R., et al., *Comparative pharmacognostic study of leaves of Adhatoda vasica and Ailanthus excelsa.* International Journal of Pharmacognosy, 2014. **1**(2): p. 95-98.
68. VK, G.A.J.A.J., *Pharmacognostical study of Justicia adhatoda Linn. leaf.* International Journal of Herbal Medicine, 2014. **1**(6): p. 01-04.
69. Chowdhury, C.D.R.P.A., *HPTLC determination of vasicine and vasicinone in Adhatoda vasica.* PHYTOCHEMICAL ANALYSIS, 2005. **16**: p. 90-92.
70. E., S.M.S.S.R.P.E.H.N.R.C., *HPLC analysis of Adhatoda vasica obtained from differentgeographic sources.* International Journal of Drug Development & Research, 2010. **2**(4): p. 676-680.
71. Suthar, A.C., et al., *Quantitative estimation of vasicine and vasicinone in Adhatoda vasica by HPTLC.* Journal of Pharmacy Research, 2009. **2**(12): p. 1893-1899.

72. Biradar, Y.S., *TLC densitometric quantification of vasicine vasicinone and embelin from Adhatoda zeylanica leaves and Embelia ribes fruits.* 2010: p. 132-144.

APPENDIX

VITA

Name: Paphitchaya Thetsana

Born: September 4, 1988, Bangkok, Thailand

Education: Bachelor of Applied Thai Traditional Medicine (Applied Thai Traditional Medicine), School of Health Science, Mae Fah Luang University, Thailand in 2011

Poster presentation with proceedings

Thetsana, P., Palanuvej, C. and Ruangrangsi, N. Pharmacognostic specification of Adhatoda vasica and quantitative analysis of vasicine by Thin layer chromatography. Proceedings of The 7th Thailand-Japan international academic conference 2014 (TJIA2014), pp. 256 – 259. University of Tokyo, Kongo Campus, Japan, 2014

Oral presentation

Thetsana, P., Palanuvej, C. and Ruangrangsi, N. Pharmacognostic specification and microscopic analysis of Adhatoda vasica leaves. The 2nd International Conference on Advanced Pharmaceutical Research, March 12, 2015, Rangsit University, Phatumthani, Thailand