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ORIGINAL ARTICLE

Pharmacognostic Specifications and Lawsone Content of *Lawsonia inermis* Leaves

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ABSTRACT

Background: *Lawsonia inermis* L. has been used as a traditional or folk medicine for the treatment of a wide range of skin infectious diseases. **Objective:** The objective of this study was to determine the pharmacognostic specifications and lawsone contents of *L. inermis* leaves. **Materials and Methods:** The pharmacognostic specifications of *L. inermis* leaves from 12 sources were evaluated according to the WHO guideline of quality control method for medicinal plant materials. The lawsone contents were analyzed by thin-layer chromatography (TLC) coupled with densitometry and image analysis. **Results:** Microscopic evaluation of *L. inermis* powders showed the fragment of mesophyll, fragment of parenchyma, epidermis layer with stomata, and the rosette crystal of calcium oxalate. Physicochemical parameters revealed that total ash, acid-insoluble ash, loss on drying, and water content should be not <6.98, 1.12, 8.08, and 9.86% of dried weight, respectively, whereas ethanol and water extractive values should be not < 19.67 and 23.06% of dried weight, respectively. The content of lawsone in *L. inermis* leaves by TLC-densitometry was found to be 0.76 ± 0.05 g/100 g of dried crude drug, whereas the lawsone content evaluation by TLC image analysis was found to be 0.87 ± 0.11 g/100 g of dried crude drug. The validation of the methods revealed that both TLC-densitometry and TLC image analysis showed a good sensitivity and accuracy for lawsone quantitation in *L. inermis*. **Conclusion:** The pharmacognostic specifications could be used as the standardization data of *L. inermis* leaves, and the development of TLC method could be applied to determine lawsone content in this plant material.

Key words: Lawsone, *Lawsonia inermis* L, pharmacognostic specification, thin-layer chromatography

SUMMARY

- The pharmacognostic specification of *Lawsonia inermis* leaves could be used as the standardization data of L. *inermis* leaves in Thailand.
- Both TLC-densitometry and TLC image analysis showed a good sensitivity and accuracy for lawsone quantitation.

Abbreviations Used: LOD: Limit of detection; LOQ: Limit of quantitation; RSD: Relative standard deviation; TLC: Thin layer chromatography; UV: Ultraviolet; Rf value: Relative to front value

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INTRODUCTION

Lawsonia inermis L., commonly referred to henna, belongs to the *Lythraceae* family and is the sole species in the genus. It has been used as a traditional or folk medicine for the treatment of a wide range of skin infectious diseases.[1] The extract of *L. inermis* showed positive results for antimicrobial and strong antioxidant activities.[2-5] Moreover, previous studies reported that various parts of *L. inermis* contained a number of secondary metabolites such as coumarins, flavonoids, and naphthoquinone, especially lawsone.[6,7] However, there has been no report on the pharmacognostic evaluation and lawsone content of *L. inermis* leaves in Thailand, which can greatly affect the quality, purity, identification, and consequently, the therapeutic value of the plant.[8-10]

The present study attempted to investigate the pharmacognostic specifications of *L. inermis* leaves from 12 different sources in Thailand and analyze lawsone content by thin-layer chromatography (TLC) coupled with densitrometry and with image analysis to validate suitable methodology to standardize and control the quality of raw materials. Moreover, to establish an herbal pharmacopoeia, the pharmacognostic parameters and standardization are also the information incorporated.

MATERIALS AND METHODS

Sample collection

Twelve samples of *L. inermis* leaves were collected from different geographical areas in Thailand. All sets of crude drugs were authenticated by N. Ruangrungsi and compared to the herbarium at the Department of National Parks, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand. Voucher specimens were deposited at the College of Public Health Sciences, Chulalongkorn University, Thailand.

Sample extraction

The ground samples of *L. inermis* leaves (5 g) were continuously extracted with hexane, dichloromethane, and 95% ethanol, respectively, until exhaustion using soxhlet apparatus. The extract was filtered and evaporated to dryness under vacuum. The ethanolic extracts of 12 samples were used for lawsone determination.

Macroscopic and microscopic evaluations

The macroscopic character of *L. inermis* was illustrated as the drawing of the plant by the author. The anatomical and histological characters of the transverse section and powder of *L. inermis* leaf were examined

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under microscope to identify the structural features, cells, and ergastic substances of plant samples.[8]

Physicochemical evaluation

Total ash, acid-insoluble ash, loss on drying, moisture content, and extractive matter parameters of *L. inermis* leaves were analyzed according to the WHO guideline for quality control methods for medicinal plant materials.[11]

Thin-layer chromatographic fingerprint evaluation

The ethanolic extracts of *L. inermis* leaves were dissolved in ethanol (10 mg/ml). Five microliters of each sample was applied into silica gel 60 GF_{254} TLC plate. A mixture of chloroform, ethyl acetate, and formic acid (3:6:1) was used as the mobile phase. The spots were visualized under ultraviolet (UV) light at 254 nm and 366 nm, and then sprayed with detecting reagent (10% sulfuric acid in methanol) and heated at 110°C for 10 min.

Lawsone analysis

Preparation of lawsone standard solution

Lawsone (97%) was purchased from Sigma-Aldrich (St. Louis, USA). The stock solution of lawsone was prepared by dissolving 1 mg of lawsone in 1 ml of ethanol.

Preparation of ethanolic extracts of Lawsonia inermis leaves

Each *L. inermis* ethanolic extract was dissolved in ethanol to obtain a concentration of 10.0 mg/ml and then assayed by TLC.

Thin-layer chromatography-densitometry of lawsone

Five microliters of 12 ethanolic extracts and lawsone standard solutions with the concentration of 5, 20, 30, 40, and 60 μg/ml were applied onto the TLC plate coated with silica gel 60 GF_{254}. The plate was developed to a distance of 8.0 cm in a TLC chamber that contained a mixture of toluene: Ethyl acetate: Acetic acid (8:1:1) as the mobile phase. The developed TLC plate was scanned under the wavelength of maximum absorbance at 283 nm for quantitative analysis of lawsone at the Rf value of 0.41 by TLC scanner 3 and processed with winCATS software. Then, the analysis for the peak area of each spot was carried out. The content of lawsone was determined by comparing peak area to the calibration curve obtained from the same TLC plate. All analyses were performed in triplicate.

Thin-layer chromatography image analysis of lawsone by ImageJ software

The developed TLC plate from above was photographed under UV 365 nm by a digital camera and stored as JPEG files. The content of lawsone was determined using ImageJ software. The rectangular tool was used to crop the interested spot. The result from a rectangular tool showed peak area in each spot. The straight line was selected for drawing the line under the peak. The area under the peak was calculated by comparison with the calibration curve of lawsone obtained from the same TLC plate.

Method validation

The test of linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, and specificity were evaluated according to the ICH guidelines for the validation of analytical method.[12]

RESULTS

Pharmacognostic specifications

L. inermis is a small tree with 2–6 m tall. Leaves are opposite, entire, elliptic-to-broadly lanceolate, 1.5–5 cm × 0.5–2 cm, and acuminate. Flowers are numerous, large, pyramidal, calyx with 2 mm long tube, and 8 stamens inserted in pairs. Fruits are globose capsule with 4–8 in diameter.[13] Macroscopic and microscopic investigations were illustrated in Figures 1-4. Cytological and histological characterization proved to be a valuable tool for the identification of *L. inermis* leaves.

The physicochemical evaluation (% by weight) of *L. inermis* leaves was demonstrated in Table 1. The TLC fingerprint of the ethanolic extract of *L. inermis* leaves was illustrated in Figure 5.

Lawsone analysis

The contents of lawsone in *L. inermis* extract obtained from the chromatographic peak area integrated at the Rf value of 0.41 by densitometry and ImageJ software were found to be 0.76 ± 0.05 and 0.87 ± 0.11 g/100 g of dried crude drug, respectively [Figures 6 and 7]. Comparison of lawsone contents between TLC-densitometry and TLC image analysis was not statistically significantly different by paired *t*-test analysis ($P = 0.0525$) [Table 2].

Table 1: Physicochemical specification (% by weight) of *Lawsonia inermis* leaves

Content (% by weight)	Mean±SD*	Minimum-maximum
Loss on drying	8.08±0.15	5.86-12.79
Total ash content	6.98±0.12	4.56-8.96
Acid-insoluble ash content	1.12±0.09	0.56-2.07
Water content	9.86±0.65	7.78-14.11
Ethanol extractive values	19.67±1.34	6.21-29.22
Water extractive values	23.06±1.50	16.52-29.60

*Grand mean±pooled SD. The samples were from 12 different sources throughout Thailand, and each sample was done in triplicate. SD: Standard deviation

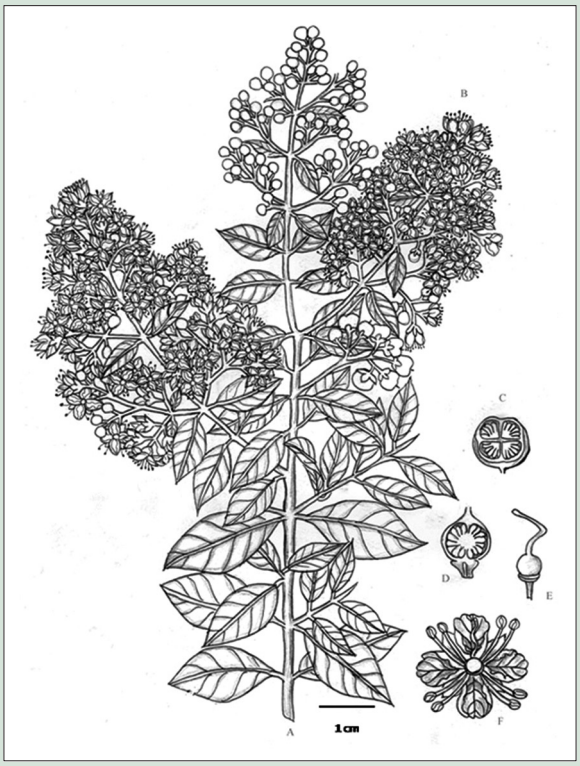

Figure 1: The flowering branch of *Lawsonia inermis* (L.) A: Flowering branch B: Inflorescence C: Transverse section of ovary D: Longitudinal section of ovary E: Pistil F: Flower

Method validation

The specificity evaluated by comparing the absorption spectrum pattern of *L. inermis* extract with the pattern of lawsone standard showed peak identity with the maximum wavelength at 283 nm. The calibration curves of lawsone by both methods were linear at the range of 5–60 μg/ml. The accuracy determined by recovery method which the extract was spiked with lawsone of low, medium, and high levels (10, 20, and 30 μg/ml, respectively) showed acceptable recovery (80–120%). The spiked samples were processed for precision as the repeatability (intra-assay) and intermediate precision (inter-assay). LOD and LOQ were estimated based on the residual standard deviation of the regression line (σ) and the slope of the calibration curve (S). The results were shown in Table 3.

DISCUSSION

The physicochemical evaluation of plant drugs is important for detecting adulteration and grading the quality of the drug. The procedures normally adopted to obtain the qualitative information about the purity and standard of a crude drug include several parameters.[14,15]

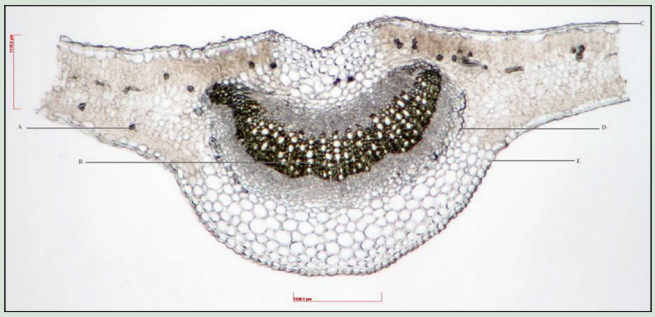

Figure 2: Transverse section of *Lawsonia inermis* leaf; A: Rosette crystal of calcium oxalate B: Xylem vessel C: Upper epidermis D: Lower epidermis E: Sclerenchyma cells

Therefore, this study proposed the upper limits for unwanted properties of *L. inermis* leaves' crude drugs such as loss on drying, total ash, acid-insoluble ash, and water contents, together with the lower limits for extractable matters such as the ethanol and water extractive values as shown in Table 1. However, the results were different with the previous studies that showed the physicochemical constant from the *L. inermis* leaves in India. The reason may be due to the difference in the nature and quantity of secondary metabolites, which are affected by temperature, rainfall, aspect, length of day, and altitude from different geographical areas.[16]

TLC-densitometry, a widely used quantitative analysis of major compounds in medicinal plants, is convenient and easy to perform with automated equipment. TLC-densitometry is instrumental TLC. Each step in the process is automated with a sample applicator, development chamber, and scanning densitometer which are controlled by a software.[17] Unfortunately,

Table 2: Lawsone contents of the ethanolic extract from *Lawsonia inermis* leaves determined by thin-layer chromatography-densitometry and thin-layer chromatography image analysis

Source	Lawsone content (g/100 g crude drug)	
	TLC-densitometry	TLC image analysis
1	1.04±0.05	1.13±0.09
2	0.01±0.00	0.04±0.02
3	0.28±0.02	0.18±0.01
4	0.89±0.02	0.92±0.12
5	0.56±0.08	0.51±0.07
6	1.08±0.07	0.99±0.15
7	1.09±0.08	1.58±0.08
8	1.30±0.04	1.40±0.08
9	1.47±0.04	1.55±0.10
10	0.10±0.07	0.10±0.09
11	0.81±0.04	1.27±0.14
12	0.53±0.04	0.84±0.22
Average**	0.76±0.05	0.88±0.11

n=3, **Grand mean±pooled SD. TLC: Thin-layer chromatography; SD: Standard deviation

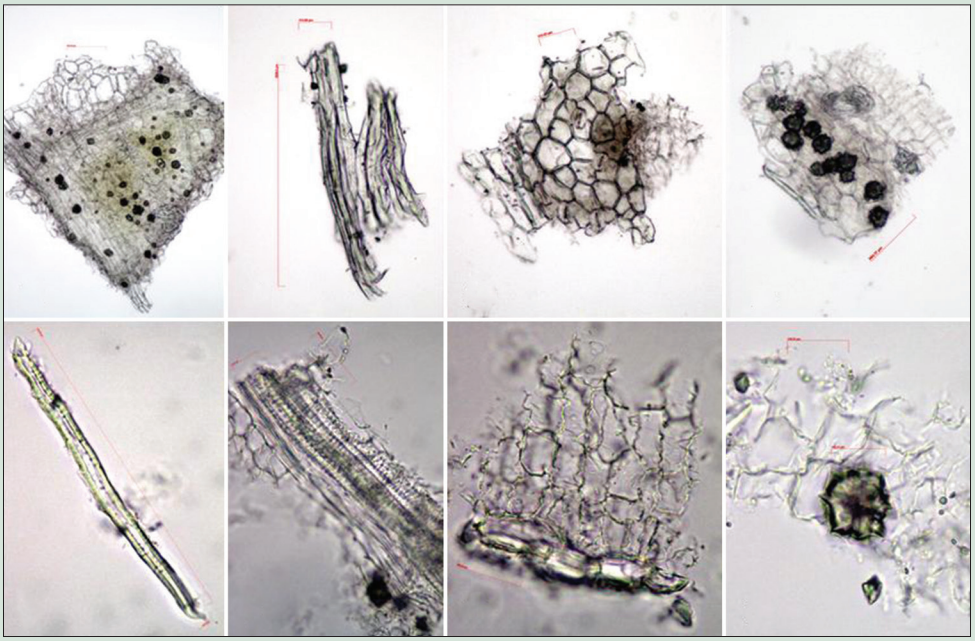

Figure 3: Powder of *Lawsonia inermis* leaf; (a) fragment of mesophyll associated with vascular tissue (b) group of fiber (c) epidermal layer with stomata (d) fragment of parenchyma containing rosette crystal (e) parts of single fiber showing thickening wall (f) fragment of parenchyma associated with vessel (g) fragment of parenchymatous cell (h) rosette crystal of calcium oxalate

Figure 4: *Lawsonia inermis* leaves

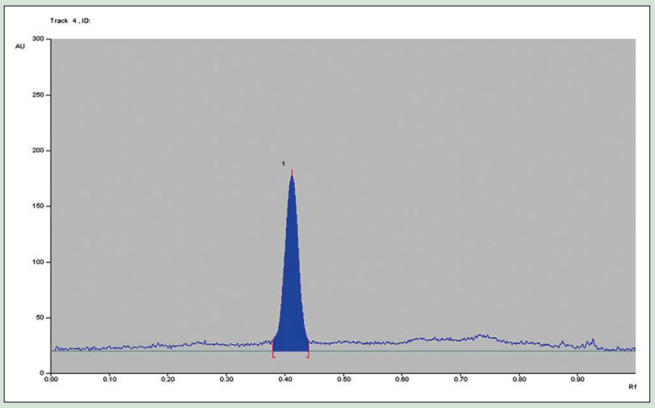

Figure 6: Chromatogram of standard lawsone

Table 3: Method validity of thin-layer chromatography-densitometry and thin-layer chromatography image analysis of lawsone content in *Lawsonia inermis* extract

Parameter	TLC-densitometry	TLC image analysis
Range (µg/ml)	5.00-60.00	5.00-60.00
Regression equation (µg/ml)	y=151.59x+609.15	y=449.26x+216
Coefficient of determination (R^2)	0.9945	0.9962
Recovery (%)		
Low	80.03	117.19
Medium	80.98	96.39
High	90.94	96.16
Repeatability (% RSD)		
Low	1.0	1.66
Medium	3.3	7.37
High	2.5	6.96
Intermediate precision (%RSD)		
Low	9.8	5.29
Medium	11.9	8.23
High	6.0	4.51
LOD (3.3 σ/S) (µg/ml)	2.94	2.44
LOQ (10 σ/S) (µg/ml)	8.90	7.39

RSD: Relative standard deviation; LOD: Limits of detection; LOQ: Limits of quantification; TLC: Thin-layer chromatography

most of the equipment are expensive which some laboratories cannot afford. To develop an alternative to TLC method for analyzing lawsone

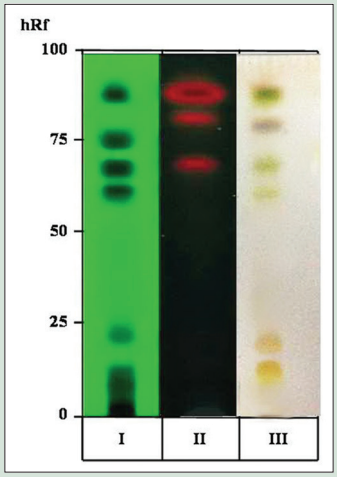

Figure 5: Thin-layer chromatography fingerprint of the *Lawsonia inermis* leaves' ethanolic extract with chloroform:ethyl acetate:formic acid (3:6:1) as the mobile phase (I: Detection under ultraviolet light 254 nm, II: Detection under ultraviolet light 366 nm, III: detection with 10% sulfuric acid)

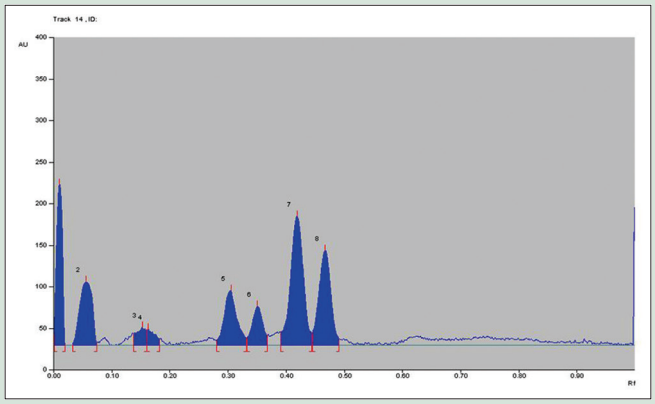

Figure 7: Chromatogram of lawsone in *Lawsonia inermis* extract

content in *L. inermis* leaves, TLC-densitometry and TLC image analysis using ImageJ software were performed on the specimen from 12 different sources in Thailand. Results from TLC-densitometry were compared with the results from TLC-image analysis.

The lawsone contents of *L. inermis* leaves extract analyzed by TLC-densitometry and TLC image were found to be 0.76 ± 0.05 and 0.87 ± 0.11 g/100 g of dried crude drug, respectively. This finding was related with the previous study that showed considerable variation in lawsone concentration ranged from 0.004% to 0.608%.[18] This is the first study for the quantitative analysis of lawsone in *L. inermis* leaves with both methods.

According to the ICH guidelines, the test of linearity, LOD, LOQ, precision, accuracy, and specificity should be performed for validation of an analytical method. In this study, TLC-densitometry and TLC image analysis for quantitative analysis were validated in terms of specificity, linearity, accuracy, precision, LOD, and LOQ to confirm that the analytical procedure employed reliable and accurate information. Lawsone at five concentration levels was investigated for linearity of the TLC methods. The calibration curves of lawsone obtained from both TLC-densitometry and TLC image methods were linear in the range of 5–60 µg/ml. A good correlation coefficient (R^2) was obtained in this study ($R^2 > 99$). The precision of *L. inermis* extract was analyzed as % relative standard

deviation (RSD) of nine determinations covering the specific range. The accuracy was determined by recovery test. The %RSD of repeatability and intermediate precision revealed that the method was precise.[12] The recoveries of lawsone ranged from 80.03% to 117.19%. Good agreement of recovery was ranged from 80% to 120% with the requirement for complex matrices.[12] Hence, the results indicated that these two methods were accurate for lawsone determination in *L. inermis* extract. Thus, the result from method validation and paired *t*-test statistical analysis indicated that TLC image analysis is an efficient, reliable, and suitable technique for using in the quantitative analysis of lawsone in *L. inermis* leaves. Moreover, this study suggests that TLC image analysis method can be used as an alternative method in any laboratory due to its advantages, which is easy to perform, fast, and inexpensive. Because the digital camera with charge-coupled device image sensor becomes more widely used as it is much faster and efficient than scanning densitometer,[19] image analysis using ImageJ software was not required as it is a sophisticated instrument and easily applicable. The software is easy to operate as well.[20] In addition, it has enabled the simple and cost-effective use of TLC as a quantitative analysis. The combined technique, digitally enhanced-TLC, applies to determine the accurate amount of compound in the sample solution.[21]

CONCLUSION

This present study reported for the first time the pharmacognostic specification parameters as well as the lawsone content of *L. inermis* leaves in Thailand. The pharmacognostic specifications of *L. inermis* leaves could be used as the standardization data to authenticate and evaluate the quality of plant materials before used as therapeutic drugs. Moreover, the TLC methods in this study can be applied to determine lawsone content in other traditional medicine.

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Conflicts of interest

There are no conflicts of interest.

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