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THE PHARMACOGNOSTIC SPECIFICATION OF *ARDISIA ELLIPTICA* FRUITS AND THEIR EMBELIN CONTENTS BY TLC IMAGE ANALYSIS COMPARED TO TLC DENSITOMETRY

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Abstract:

Ardisia elliptica Thunb. (Myrsinaceae) or Pilangkasa is one of the herbs in traditional Thai medicine. Its fruits are used for treatment of diarrhea, fever, liver disease, and leprosy disease. *A. elliptica* fruits contain a quinone derivative, embelin as a major constituent. TLC combination with densitometry or image analysis is easy and rapid method for quantitation of the active compound in plant extracts. The pharmacognostic specification of *A. elliptica* fruits collected from 15 different sources throughout Thailand was performed. Embelin content was analyzed by successively soxhlet extraction with hexane followed by TLC-densitometry and image analysis using n-butanol, n-propanol and 4 N ammonia (1:7:2) as mobile phase and UV254 visualization. Quantitative analysis of embelin in *A. elliptica* fruits by TLC densitometry and TLC image analysis were validated. The results demonstrated the contents of loss on drying, total ash, acid insoluble ash, ethanol-soluble extractive, water-soluble extractive and moisture as 10.54 ± 0.08, 5.53 ± 0.15, 0.33 ± 0.06, 4.92 ± 0.45, 9.42 ± 0.91 and 10.61 ± 0.69 % by weight respectively. Embelin contents were 1.64 ± 1.02 % by weight by TLC-densitometry and 1.54 ± 1.10 % by weight by TLC image analysis. The embelin contents by both methods were not statistically significant different.

Keywords: *Ardisia elliptica* Thunb., pharmacognostic specification, embelin, TLC densitometry, TLC image analysis

INTRODUCTION

In the last decades herbal medicines have been popular and developmental in many countries around the world since the World Health Organization urged its member countries to use folk healing practices and herbal medicines as part of the basic public health projects. The herbal medicines are used as remedies, over-the-counter drug products and raw materials for the pharmaceutical industry. In Thailand, herbal medicines have been used for a long time and the popularity of herbal medicines has been greatly increased in the last few years. There are increasing medicinal plants and herbal drugs published in the National List of Essential Medicines. However, there have been a lot of factors affected the usage of herbal medicines such as inadequate scientific researches, low confidence of physicians to use herbal medicine and the appropriate drug use (Satyapan *et al*., 2010). *Ardisia elliptica* Thunb. (Myrsinaceae) or Pilangkasa is one of the herbs in traditional Thai medicine. Its dried fruit is used for treatment of diarrhea, fever, liver disease, and leprosy disease (Kobayashi *et al*., 2005). *A. elliptica* fruit contains a quinone derivative, embelin as a major constituent. Myricetin, quercetin, berginin, norbergenin, kaempferol, quercetin 3-*O*-*β* - d -glucopyranoside and gallic

acid were reported as well (Sumino *et al*., 2002). Even though *A. elliptica* fruit is widely used in traditional Thai formularies, there are no standardization parameters to justify the quality of this crude drug. Thin layer chromatography is a method for screening plant extracts. This method is very easy, rapid and cheap methods for screening the active compound in plant extract. Quanlitative or semi-quantitative TLC analysis is usually done by visual comparison. Quantitative TLC analysis can be precisely performed by the technique of densitometry. It is based on measuring the absorbance or fluorescence of different zones on the plate exposed to monochromatic source of light. However, the densitometry equipment is also expensive (Hess, 2007). In recent study, there is an alternative way to quantitative evaluation using charged coupled device (CCD) camera. By coupling CCD detection with TLC, the entire chromatographic plate can be imaged in a single exposure yielding rapid quantification in shorter analysis time than of slit scanning densitometers. CCD detector has demonstrated extremely low dark current and read noise characteristics, high sensitivity and excellent linearity. These features have made the CCD an excellent detector for many imaging applications in chemical analysis, such as fluorescence detection (Hoeltz *et al.*, 2010). This study aimed to report the current information on the pharmacognostic specification of *A. elliptica* fruit with the special reference to embelin marker quantified by TLC image analysis compared to TLC densitometry.

MATERIALS AND METHODS

Materials and Chemicals

The dried *Ardisia elliptica* fruits were purchased from 15 traditional drug stores at different locations throughout Thailand during 2011 and authenticated by one of the authors (N.R.). Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University. The crude drug samples were examined and the other parts of plants, for example leaves, branches were removed. The crude drugs were kept in the closed container protected from light and heat. The standard embelin was purchased from ChromaDex, USA. The chemicals used were of analytical grade.

Macroscopic Evaluation

For macroscopic evaluation, *A. elliptica* fruits were identified for their sensory or organoleptic characters such as color, odor, taste, size, shape and other characters.

Microscopic Evaluation

The microscopic appearances of *A. elliptica* fruits were determined in cross sectional view and in powder form.

Determination of Moisture Content (Azeotropic Method)

Weighed exactly 50 g of *A. elliptica* fruit powders and transferred to the flask. Added 600 ml of water-saturated toluene and boiled the flask until the water was completely distilled, removed heat, allowed the receiving tube to cool to room temperature and dislodged any droplets of water adhered to the walls of the receiving tube. Allowed the water and toluene layers to be separated and read off the volume of water, calculated the content of water as a percentage of air-dried material.

Determination of Loss on Drying

Placed exactly 3 g of *A. elliptica* fruit powders in a pre-weighed crucible, dried the sample at 105°C for 6 hour and weighed, calculated the loss of weight in a percentage of air-dried material.

Determination of Total Ash

Placed exactly 3 g of *A. elliptica* fruit powders in a pre-weighed crucible, ignited in an incinerator at 500°C until white, cooled in desiccator and weighed, calculated the content of total ash in a percentage of air-dried material.

Determination of Acid Insoluble Ash

To the crucible containing the total ash, added 25 ml of hydrochloric acid (70 g/l), covered with a watch-glass and boiled gently for 5 minutes. Rinsed the watch-glass with 5 ml of hot water and added this liquid to the crucible. Collected the insoluble matter on an ashless filter-paper No. 40 and washed with hot water until the filtrate was neutral, transferred the filter-paper containing the insoluble matter to the original crucible, dried on a hot-plate and ignited to constant weight. Allowed the residue to cool in desiccator then weighed without delay, calculated the content of acid-insoluble ash in a percentage of air-dried material.

Determination of Ethanol Soluble Extractive Value

Macerated exactly 4 g of *A. elliptica* fruit powders with 70.0 ml of 95% ethanol for 6 hours under shaking then allowed standing for 18 hours. Filtered, washed the marc with ethanol and adjusted the filtrate to exactly 100 ml with ethanol, transferred exactly 25 ml of the filtrate to a pre-weighed beaker and evaporated to dryness on a water-bath. Dried at 105°C for 6 hours, cooled in desiccator for 30 minutes and weighed without delay, calculated the content of extractable matter in a percentage of air-dried material.

Determination of Water Soluble Extractive Value

The method proceeded as above for determination of ethanol soluble extractive value, using water instead of ethanol.

Thin Layer Chromatographic Fingerprinting

From aforementioned ethanol soluble extractive procedure, transferred another 25 ml of the filtrate and evaporated to dryness. Re-dissolved in 1 ml of methanol and applied 3 µl of ethanolic extract of *A. elliptica* fruits on a 20 x 10 cm silica gel 60 F254 TLC plate. Developed the plate using a mixture of chloroform, ethylacetate, formic acid (5:4:1). After development, the plate was visualized under UV 254 nm, 365 nm and stained with 0.5% w/v phosphomolypbic acid in ethanol.

Quantitative Analysis of Embelin in A. elliptica Fruits

Hexane Extraction of A. elliptica Fruits

A. elliptica fruit powders (20.0 g) were exhaustively extracted with hexane by soxhlet apparatus. The extract was filtered and evaporated to dryness under reduced pressure at 50ºC.

Standard Embelin Solutions

The standard solutions of embelin were prepared in methanol to the concentration of 0.2, 0.4, 0.6, 0.8, 1.0 mg/ml.

TLC densitometry of Embelin

Applied 3.0 µl of standard embelin solutions and 3.0 µl of hexane extract of *A. elliptica* fruits on a 20 x 10 cm silica gel 60 F254 TLC plate, developed using a mixture of n-butanol, n-propanol, 4 N ammonia (1:7:2). After development, the plate was scanned using densitometer (Camag TLC Scanner 3, USA). The calibration curve of embelin was prepared by plotting peak areas *vs.* concentrations of embelin applied.

TLC Image Analysis of Embelin

The developed TLC plate was examined under UV 254 nm by UV viewing cabinet (Spectroline, USA). The photos were taken using Canon, PowerShot A650 IS camera and stored as JPEG files with C mode ISO 80, fluorescent, largest and superfine image. The ImageJ software was used to analyze and quantitate the embelin spot on TLC plate. The calibration curve of embelin was prepared by plotting peak areas *vs.* concentrations of embelin applied.

Method validation

Both methods were validated in terms of its linearity, limits of detection (LOD), limits of quantification (LOQ), accuracy and precision according to ICH guideline.

Data analysis

The pharmacognostic specification was calculated as grand mean and pooled standard deviation. The embelin contents between TLC densitometry and TLC image analysis were compared by Wilcoxon signed ranks test statistical analysis.

RESULTS AND DISCUSSION

The pharmacognostic parameters of *A. elliptica* dried fruits were shown to represent the characteristics as well as the quality of crude drug (table 1). The moisture content should be not more than 13% for safe storage of plant materials (Müller *et al*., 2006). The average moisture content of *A. elliptica* dried fruits revealed in this study was around 10%. The anatomical characteristics represented by transverse section of *A. elliptica* fruit and the histological characters of the fruit powder as well as TLC fingerprint were shown in figure 1. These data could be used to authenticate the crude drug.

Table 1. The Pharmacognostic parameter of *Ardisia elliptica* fruits[a]

Content (% by weight)	Mean	SD	Range (Mean ± 3SD)
Acid-insoluble ash	0.335	0.059	0.158 - 0.512
Total ash	5.526	0.152	5.071 - 5.980
Ethanol-soluble extractive	4.916	0.446	3.579 - 6.252
Water-soluble extractive	9.418	0.910	6.688 - 12.148
Volatile oil	0	0	0
Loss on drying	10.538	0.079	10.302 - 10.775
Moisture	10.613	0.689	8.546 - 12.681

[a] The parameters were shown as grand mean ± pooled SD.
Samples were from 15 different sources throughout Thailand.
Each sample was performed in triplicate.

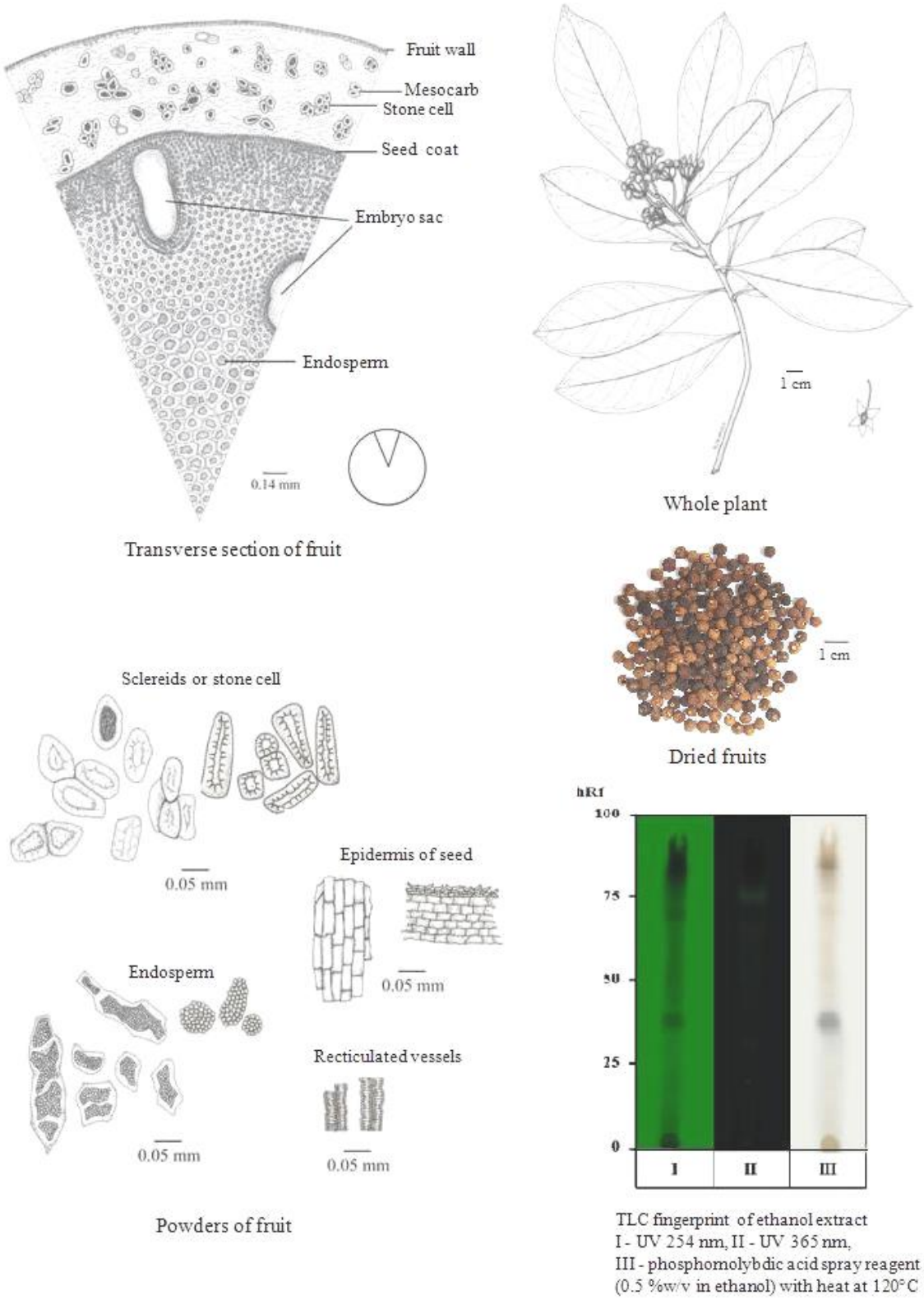
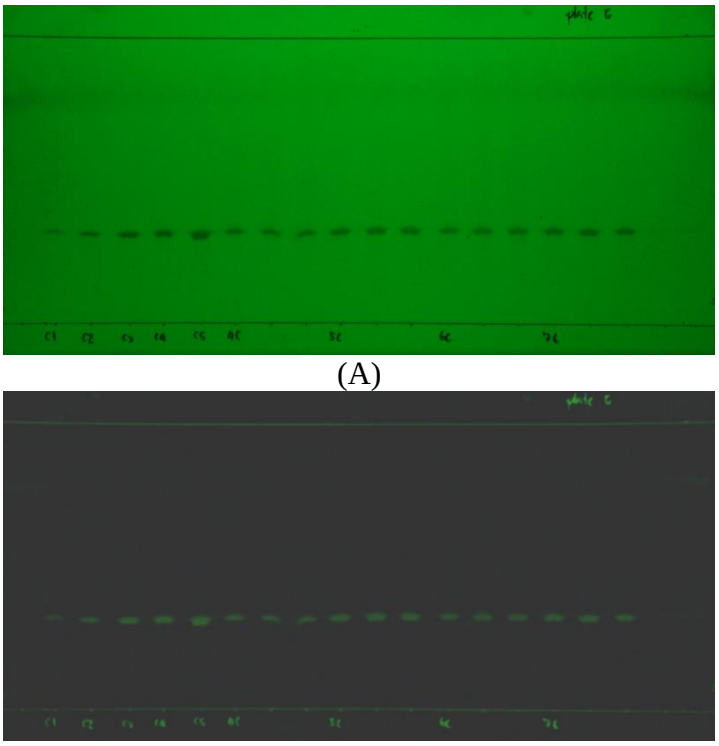


Figure 1. Macroscopic, microscopic characteristics and TLC fingerprints of dried fruits of *Ardisia elliptica* Thunb.

In addition to the pharmacognostic parameters that should be used for herbal material standardization, active chemical constituents are markers for quality control as well. TLC is easy, rapid and cheap to use and also has promising analytical potential if combined with TLC scanner or TLC image analysis to quantify the chemical constituents in herbal materials. ImageJ is a public domain, Java-based image processing program developed by the National Institutes of Health in Bethesda, Maryland, USA. This image analysis software is freely available program and can be used by any computers (Burger and Burge, 2008). Moreover, it is an open source that each developer or user has freedom to fix the problem and develop the program to ultimate processing e.g. removal noise and baseline drift correction to fix the error (Schneider *et al*., 2012).

TLC chromatograms of embelin in *A. elliptica* dried fruits were shown in figure 2. Several trials for the optimal mobile phase were done and the mixture of n-butanol, n-propanol, 4 N ammonia (1:7:2) was efficacious as previously reported (Madhavan *et al*., 2011). The quenching spot of embelin was obviously inspected and the background was clear. The retention factor (hRf) of embelin was around 35.



(A)

(B)

Figure 2. The TLC plates developed with n-butanol, n-propanol, 4 N ammonia (1:7:2) visual under 254 nm original image (A), with subtrack background (B) Hexane extracts of dried *Ardisia elliptica* fruits

The error from cutting the baseline should be concerned for image analysis (Hess, 2007). Noise removal and baseline drift correction are recommended to fix the error (Olech *et al*., 2012). Using ImageJ software, the function "Process/filters/median" was applied for a

filter with a 10 pixels. The next process was removing the baseline drift by function "Process/subtrack backgroud" and applying such a filter with a 100 pixels (figure 2).

TLC using ImageJ software as well as densitometry for quantitative analysis of embelin were validated in terms of its linearlity, range, LOD, LOQ, recovery and precision. The calibration curves were linear in range of 0.6-3.0 µg/spot with the regression coefficient (R^2) of 0.998. The LOD and LOQ were calculated using residual standard deviation of regression lines and were found to be 0.25 and 0.77 µg/spot for image analysis and 0.22 and 0.68 µg/spot for TLC scanner respectively. Spiking and recovery (3 concentrations) was determined to evaluate the accuracy of both methods and found to be within acceptable limits. The repeatability was determined by analyzing the extracts (3 concentrations/ 3 replicates) on the same day, the intermediate precision was determined in 3 different days. TLC image analysis was found to be more precise. This might be affected by the difference in peak area operating program between both methods. The validity of TLC image analysis and TLC densitometry were shown in Table 2.

The hexane extracts yielded 2.71 ± 1.13 g/100 g of dried fruits. The embelin contents in the hexane extracts determined by of TLC image analysis and TLC densitometry were 0.546 ± 0.245 and 0.581 ± 0.200/ µg/mg respectively. The amounts of embelin in *A. elliptica* dried fruits by both methods were 1.539 ± 1.102 and 1.637 ± 1.024 g/100 g respectively. It was found that the embelin contents by two methods were not statistically significantly different ($P > 0.05$). The embelin content in the fruit of *A. elliptica* in this study was agreeable with previously report of 1.24 g/100 g (Shuayprom *et al*., 2010). The embelin content in *A. elliptica* was found to be less than in *Embelia ribes* Brum. which was around 3.813 g/100g (Madhavan *et al*., 2011).

Table 2. Validity of quantitative analysis of embelin in *Ardisia elliptica* dried fruits by TLC image analysis and TLC densitometry

	TLC image analysis	TLC densitometry
Linearity	y = 16358.4x + 3168.7	y = 2415.7x + 674.0
(r^2)	(0.9975)	(0.9981)
Range	0.6-3.0 µg/spot	0.6-3.0 µg/spot
Limit of detection	0.25 µg/spot	0.22 µg/spot
Limit of quantitation	0.77 µg/spot	0.68 µg/spot
Accuracy : % Recovery	103.8 – 123.5	82.9 – 103.0
Precision: % RSD		
Repeatability	5.3 – 8.1	8.8 – 12.4
Intermediate precision	1.9 – 9.3	8.6 – 18.7

CONCLUSION

The pharmacognostic specification of *Ardisia elliptica* Thunb. (Myrsinaceae) dried fruit crude drug is established. The amount of embelin, the main chemical constituent is determined by TLC image analysis compared to TLC densitometry. TLC image analysis is valid for the quantitation of embelin in *A. elliptica* dried fruits. The embelin contents quantified by both methods are not statistically significantly different.

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