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Article

Pharmacognostic Specification and Coumarin Content of *Alyxia reinwardtii* Inner Bark

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Abstract. *Alyxia reinwardtii* Blume is used in traditional medicine for a long time. This study was carried out to investigate the standardization parameters by qualitative and quantitative analyses as well as coumarin content of *A. reinwardtii* inner bark. Loss on drying, total ash, acid-insoluble ash, water soluble extractives, ethanol soluble extractives and moisture contents were found to be 8.1 ± 0.2 , 7.6 ± 0.1 , 1.4 ± 0.1 , 16.5 ± 0.1 , 8.9 ± 0.3 and $11.9 \pm 0.2\%$ by dry weight, respectively. Coumarin in dichloromethane extract of *A. reinwardtii* inner bark was analysed by thin layer chromatography (TLC) using silica gel 60 F₂₅₄ as stationary phase, hexane and ethyl acetate (1 : 1) as mobile phase. The content was evaluated by image analysis using image J software. The regression line of method was polynomial in range of 0.075 - 1.2 mg/spot, and correlation coefficients (R^2) were 0.99. The repeatability and intermediate precisions were between 0.5 - 7.1% RSD. The percent recovery was found to be 96.8 - 107.3%. The robustness evaluated by slightly variation in mobile phase ratio was 1.3% RSD. Limit of detection (LOD) and limit of quantitation (LOQ) were 0.05 and 0.16 mg/spot respectively. The coumarin content in *A. reinwardtii* inner bark crude drug was found to be $0.75 \pm 0.01\%$ by dry weight. This study provided pharmacognostic specification toward fundamental standardization of *A. reinwardtii* inner bark. Additionally, the simple TLC with image analysis can be applied to quantitatively determine coumarin in plant material.

Keywords: Coumarin, *Alyxia reinwardtii* Blume, TLC image analysis.

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1. Introduction

Alyxia reinwardtii Blume is a brush wood that widely found in Thailand, Indonesia, Malaysia, Philippines and Vietnam. It is commonly known in Thai as “Cha-lood”. According to traditional Thai medicine, the flower has been used for treating mental confusion and hallucination associated with high fever. Moreover, the bark has been used for treatment of fainting, flatulence and fever. *A. reinwardtii* has been reported to contain three classes of secondary metabolites, i.e., coumarin and its derivatives, trimeric-iridoid diglucoside and iridolactone [1]. Coumarin has various pharmacological properties such as anti-nociceptive, bronchodilator and anti-inflammatory properties [2]. Although, *A. reinwardtii* inner bark is widely used in traditional Thai medicine but its pharmacognostic specification has not been established. Thin layer chromatography (TLC) is the method conventionally used for qualitative investigation of the active compounds in plant extracts because of the ease, simplicity and rapidity. TLC image analysis is a computerised method that used to quantitate the pixel intensity of each compound band on TLC image, transform to each chromatographic peak and calculate the peak area [3, 4]. ImageJ is a public domain Java image processing open source software which can be used for TLC image analysis due to its easily applicable [5]. This study aims to investigate the standardization parameters of *A. reinwardtii* inner bark in Thailand with the special reference to coumarin marker.

2. Materials and Methods

2.1 Plant Materials and Chemicals

The inner bark of *A. reinwardtii* was collected from 15 various sources throughout Thailand. Voucher specimens were authenticated by one of the authors (Ruangrunsi N.) and deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. Standard coumarin was purchased from Sigma-Aldrich, USA. The chemicals were analytical grade.

2.2 Crude Extract Preparation

Inner bark of *A. reinwardtii* was dried and ground to powders. The powders were exhaustively extracted with dichloromethane by Soxhlet apparatus. The extract yields were weighed and recorded.

2.3 Determination of Pharmacognostic Specification

The pharmacognostic parameters including macroscopic characters, microscopic characters, ash values (total ash and acid-insoluble ash), loss on drying, moisture content, water and ethanol extractives were carried out according to “Quality Control Methods for Medicinal Plant Materials” which was guided by WHO [6]. The parameters were evaluated as grand mean \pm pool SD. Each sample was performed in triplicate.

2.4 Determination of Coumarin Contents

Five milligrams of crude extract were dissolved with 1 ml of dichloromethane containing 10 % methanol. Five microliters of extract solution as well as coumarin standard solution were applied on the same TLC plate (silica gel 60 F₂₅₄). The TLC plate was developed in TLC chamber with a mobile phase consisted of hexane and ethyl acetate (1:1). The developed TLC plate was photographed under ultraviolet light at 254 nm and saved as tiff file. The color intensity of coumarin band was transformed to chromatographic peak area by imageJ software. The contents of coumarin in *A. reinwardtii* extracts were calculated based on external standard method. The test was done in triplicate.

2.5 Method Validation

Accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), range and robustness were evaluated according to ICH guideline [7].

3. Results and Discussion

"*A. reinwardtii* is a ground climber; leaves opposite; inflorescence axillary or terminal, 1-3.5 cm long; corolla tube columnar, white, cream, or white with a buff coloured tube and white lobes; fruit with 1-2 articles in each string, ellipsoid or globose, maturing black" [8]. The *A. reinwardtii* fruiting branch and inflorescence drawing were exhibited in figure 1. Pharmacognostic specification is primary important tool for identification, authentication and standardization of herbal medicines. The pharmacognostic parameters of *A. reinwardtii* inner bark were shown in table 1. The histological and anatomical characteristics of *A. reinwardtii* inner bark were drawn and shown in figure 1. The data from this study could be used as standard criteria of this crude drug. Ash contents were referred to inorganic components of plant materials. Acid insoluble ash value of this plant material was not exceeding 2 % [9]. Higher ash values indicated contamination and plant material adulteration. Water soluble extractive yield is higher than ethanol soluble extractive. Extractive matters in specified solvents indicated chemical components in crude drug. Fewer extractive matters than the specified solvent indicated inferiority of crude drug. According to previous study, the coumarin content in dried stem of *A. reinwardtii* was 0.045% by weight (2.15 g of coumarin isolated from 4.8 kg of dried stem) [1]. By TLC image analysis in this study, coumarin content in dried inner bark was 0.75 ± 0.01 % by weight. The different parts of the plant might contain various coumarin contents. The method validity was demonstrated in Table 2. The accuracy was performed by recovery of spiking known three concentrations of standard coumarin in sample matrix. The recovery values were within acceptable limits (96.8-107.3%). The repeatability or intra-day precision and the intermediate precision or inter-day precision were less than 8%. The precision of coumarin quantitative analysis by TLC image analysis method was conducted by determination of 4 concentrations \times 3 replicates at the same and difference days of tests. LOD and LOQ were calculated based on the residual standard deviation of a regression line. The LOD value, regard as the lowest concentration of analyse in a sample which could be detected was found to be 0.05 mg/spot. The LOQ value, regard as the lowest concentration of analyse in a sample which could be quantitatively determined was 0.16 mg/spot. The robustness was estimated by analysis of results obtain after deliberate variation of mobile phase ratio. This study display that there were no differences (%RSD < 5) in the peak area and retardation factor of coumarin. The calibration curve was polynomial with the range of 0.075 - 1.2 mg/spot (Figure 2). The results from method validation indicated that TLC image analysis could be efficient, reliable and suitable technique for quantitative analysis of coumarin in *A. reinwardtii* inner bark.

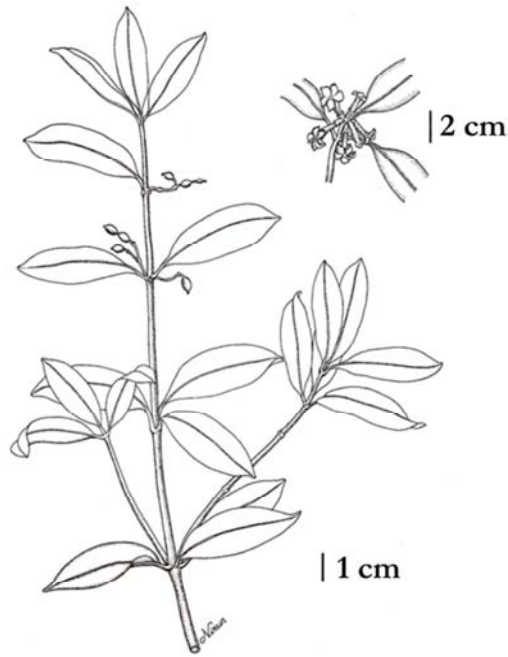
Table 1. The pharmacognostic parameters of *Alyxia reinwardtii* inner bark.

Specification	Content (% dry weight) *
Loss on drying	8.1 \pm 0.2
Total ash	7.6 \pm 0.1
Acid-insoluble ash	1.4 \pm 0.1
Ethanol soluble extractives	8.9 \pm 0.3
Water soluble extractives	16.5 \pm 0.1
Moisture	11.9 \pm 0.2

* Grand mean \pm Pool SD

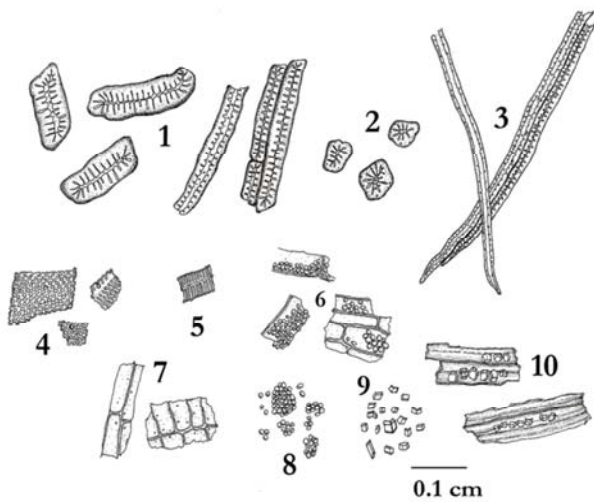
Table 2. Method validation of TLC image analysis of coumarin in *Alyxia reinwardtii* inner bark.

Parameter	Validity
Accuracy	96.8-107.3 % recovery
Precision	0.5-7.1 % RSD
Robustness	1.27 % RSD
Limit of detection	0.05 mg/spot
Limit of quantitation	0.16 mg/spot



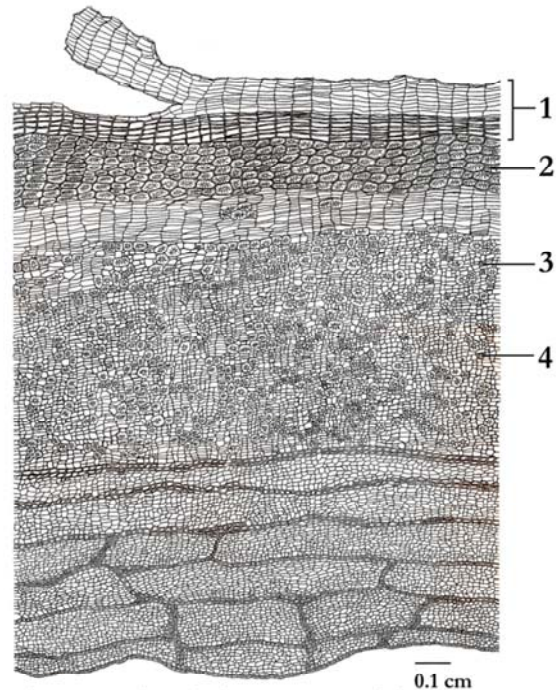
Crude drug

Fruiting branch and Inflorescence



**Histological character of inner bark
power**

1. Sclereid, longitudinal view, 2. Sclereid, transverse view, 3. Fragment of fiber,
4. Fragment of pitted vessel, 5. Fragment of reticulated vessel, 6. Parenchyma containing starch granule, 7. Fragment of parenchyma, 8. Starch granule, 9. Prism crystals of calcium oxalate, 10. Calcium oxalate prism sheath



**Anatomical character of inner
bark transverse section**

1. Periderm, 2. Sclereid, 3. Parenchyma containing starch granule, 4. Parenchyma containing prism crystal of calcium oxalate

Fig. 1. Macroscopic and microscopic characteristics of *Alyxia reinwardtii*.

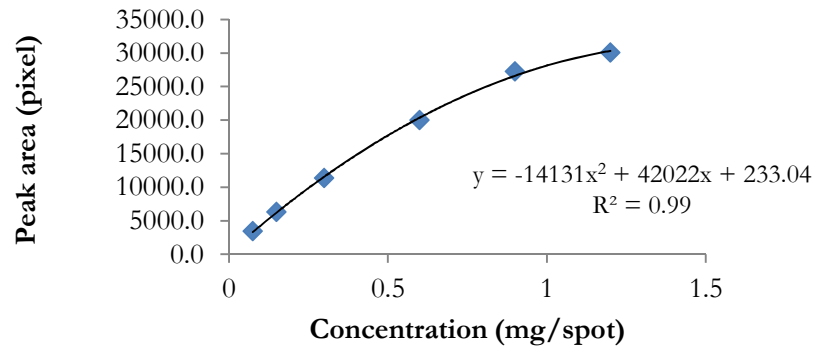


Fig. 2. The calibration curve of coumarin standard.

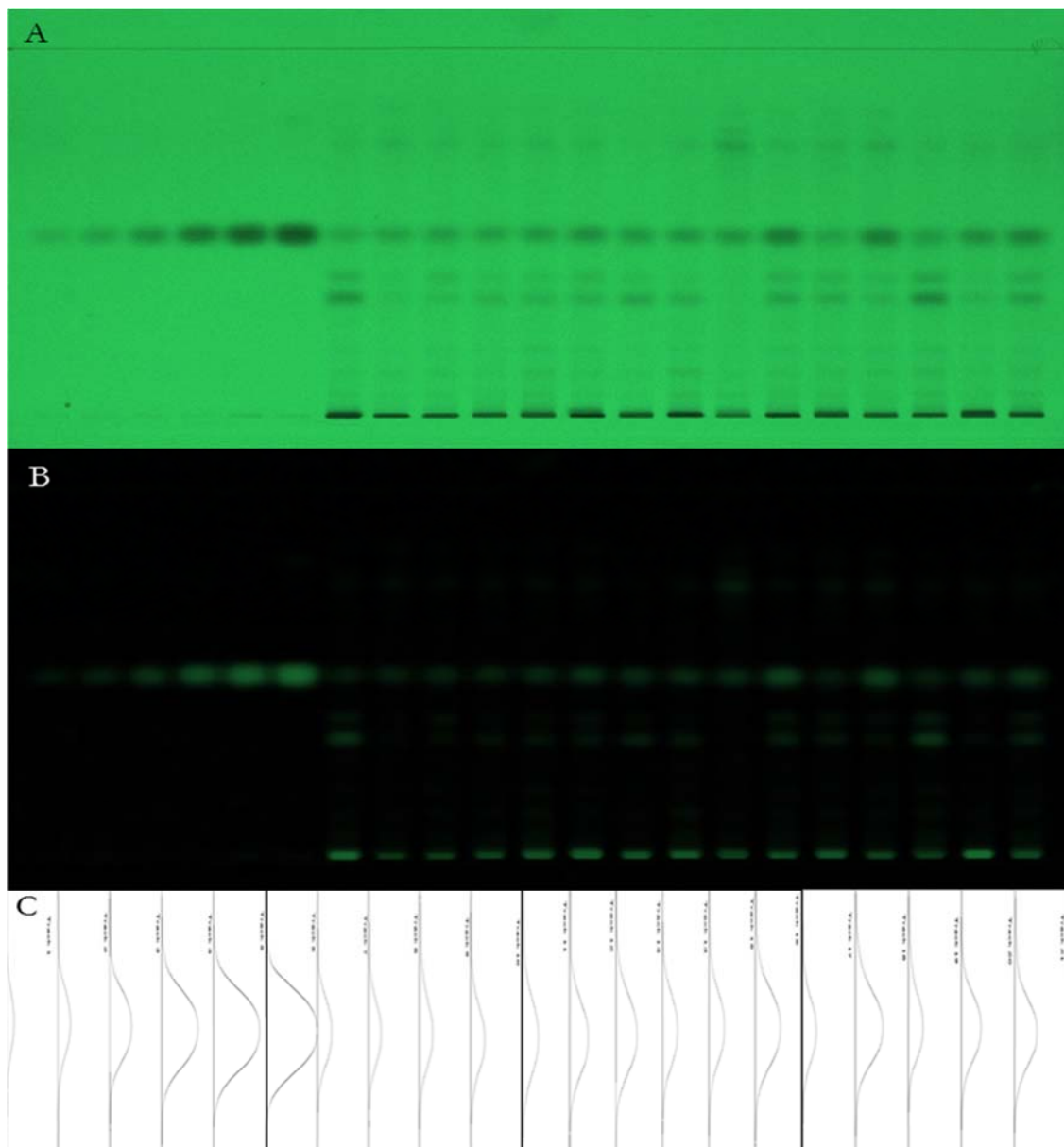


Fig 3. The TLC plate developed with hexane: ethyl acetate (1:1) photographed under UV 254 nm (A), with subtract background (B), chromatographic peak area by image] software (C); Track 1-6 were standard coumarin, Track 7-21 were sample from 15 different sources throughout Thailand.

4. Conclusion

Quality control and standardization of herbal crude drug are important for quality herbal medicine. In this study, the pharmacognostic specifications of *A. reimwardtii* inner bark in Thailand were established. Furthermore, conventional TLC combined with image analysis software could be used for quantification of coumarin in this crude drug.

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