Quality assessment and liriodenine quantification of *Nelumbo nucifera* dried leaf in Thailand

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ABSTRACT

Introduction: *Nelumbo nucifera* Gaertn. dried leaves are used as crude drug in various Thai traditional recipe. Previous studies found that the leaf extracts exerted potent antioxidant effects, antispasm, direct vasodilatation action and moderate hypotensive effect on experimental animals. Liriodenine is an alkaloid prominent found in *Nelumbonaceae*. Its anticancer potential has been previously reported. Materials and Methods: *N. nucifera* leaves from 15 different locations throughout Thailand were examined for pharmacognostic specification. Liriodenine quantification by Thin Layer Chromatography (TLC) image analysis was developed and compared to High Performance Liquid Chromatography (HPLC). Results: Anatomical and histological observation showed air chamber, trichosclereid and calcium oxalate. Pharmacognostic parameters revealed that the acid-insoluble ash, total ash, loss on drying and water content should be not more than 2.61, 9.62, 7.69 and 7.06% of dry weight respectively; while ethanol and water-soluble extractive should be not less than 6.24 and 9.51% of dry weight respectively. TLC image demonstrated clearly fluorescent spot of liriodenine at \( R_f \) 0.75 under UV 365 nm. Liriodenine content was 4.8 mg% by both methods. Conclusion: This study provided pharmacognostic specification toward fundamental standardization of *N. nucifera* leaf crude drug in Thailand. Additionally, the simple TLC with image analysis was shown as HPLC alternative to quantify the content of medicinal potential liriodenine alkaloid in this crude drug.

Keywords: Liriodenine, Medicinal plant, Quality control, Standardization

INTRODUCTION

Medicinal plants have been used extensively for treatment since ancient time and became the principle source of medicines at present. Meanwhile Thailand has strong potential for therapeutic application; since 2004 the government has been implemented and developed Thai herbal products to meet international standard. However, adulteration of crude drugs still occurs. Thus to control the quality of medicinal plants, establishing the standard specification is needed.

*Nelumbo nucifera* Gaertn. (Nelumbonaceae), commonly known in Thai as Bua-luang, is native to tropical Asia and Queensland, Australia. For centuries, sacred lotus is particularly valued for Thai medicinal properties and Thais treat lotus plants as part of their repertoire of medicinal herbs. Virtually all parts of versatile plant including stamens, pollens, flowers, stems, tubers and leaves are used.

The leaf part of *N. nucifera* has been long used as traditional medicine in Asia, including Thailand. The leaf is bitter, sweet and neutral.⁹ Previous studies revealed that the leaf extract showed direct vaso-dilatation action, anti-inflammation, antioxidation and moderate hypotensive effect on experimental animals.¹²–¹⁴ Alkaloid are the main products in *N. nucifera* leaf and have been used as effective drugs for hematemesis, epistaxis and hemoptysis.¹⁵ Moreover, recent studies revealed the effect of alkaloids in the leaf can lower the body weight, the lee's index, adipose tissue weight, and plasma lipid levels in high
fat diet-induced obese rats. Otherwise, large circular leaves are used for food preparation, sometimes applied to be food containers and umbrella. In traditional medicine, it has been combined with any other herbs to treat sunstroke, fever, diarrhea, dizziness and stomach problems.

Liriodenine (Figure 1), an aporphine isoquinoline alkaloid constituted in lotus leaves, was selected as a marker in present study according to its interesting properties and many biological activities found including anti-platelet, anti-fungal and anti-microbial actions. Additionally recent studies revealed that it had potent cytotoxicity against a number of cancer cell lines.

The present study aimed to provide pharmacognostic parameters available for standardization of *N. nucifera* leaf crude drug in Thailand as well as estimate the liriodenine content of *N. nucifera* crude drug using TLC image analysis by Scion Image software compared to HPLC. All measurements were done in triplicate.

### MATERIALS AND METHODS

#### Plant Collection and Extraction

*N. nucifera* leaves were collected from 15 different locations throughout Thailand. Plant specimens were authenticated by one of the authors (N.R.). The voucher and numbers of specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. Macroscopic, microscopic and quality evaluations of air-dried specimens were examined according to World Health Organization (WHO) quality control methods for medicinal plant materials as briefly described below:

Crude extracts were performed with 95% ethanol in a Soxhlet apparatus.

#### Pharmacognostic Investigation

Macroscopic examination was identified visually for size, color, and other inspections. Free hand sections of the leaf and crude drug powders were performed with a magnification of 4x, 10x, 20x and 40x under microscopy and compared the scale with the 0.01 mm micrometer.

Five grams of sample were heated till constantly weight for determination of loss on drying. Then ignited gradually increasing the heat to 500–600°C until it was white, cooled and weighted to calculate total ash. The ash was boiled with 25.0 ml of hydrochloric acid (70 g/l); insoluble matter was ignited and cooled again to constantly yield acid-insoluble ash. Water content was conducted by azetropic distillation. Determination of extractive values was carried out with 95% ethanol and distilled water as solvents. Clevenger apparatus was applied to determine volatile oil content in ground sample. TLC fingerprinting was performed as follow: the extracted sample was dissolved in methanol; then applied 5 µl to a pre-coated silica gel 60 F plate (Merck, Germany; 0.25 mm thickness, 20 x 20 cm). The chromatogram was developed in the saturated TLC chamber with the specified solvent as chloroform and methanol (95:5). Removed the plate; allowed it to dry in air and observed the produced spots in daylight, under short wave and long wave ultraviolet light (λ 254 and 365 nm respectively) and sprayed the spots with anisaldehyde staining reagent.

#### Liriodenine Quantification

Standard liriodenine (LC/MS grade) was purchased from Specs, The Netherlands. Its purity was reconfirmed by NMR spectroscopy.

#### TLC Image Analysis

TLC separation was performed as aforementioned with some modification. After applying 2 µL of each solution onto TLC plates, they were firstly developed using methanol to a distance of 6 mm to expand the band length. Ensuring air-drying, the plates were developed with mobile phase using chloroform and methanol (95:5) to a distance of 80 mm, then dried and visualized under UV 365 nm. The image was taken by digital camera (Canon PowerShot A650). TLC images saved as TIFF files were analyzed by Scion Image program for Windows (version Alpha 4.0.3.2, Scion Corporation, Maryland, USA). After importing images, they were resized with scale to fit windows mode and modified grayscale selection with smoothing menu to reduce noises of image. Using the rectangular selection tool and load macros command to create the plot profile, then the areas and gray value of the selection were measured as square pixels.

#### HPLC Analysis

Instrumentation was performed with a SHIMADZU gradient system (Kyoto, Japan) equipped with LC-20AD pumps, a CTO-20AC column oven, DGU-20A3 degasser and a

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Figure 1. Chemical structure of liriodenine.
SPD-M20A diode array detector (DAD) set λ at 407 nm. Separation was carried out with an Inersil ® ODS-3, C-18 column (particle size of the packing 5 μm, 4.6 × 250 mm) and HPLC guard column (5 μm, 4.0 × 10 mm). The mobile phase consisted of A (formate buffer consisting of 1% formic acid, adjusted to pH 4.5 with diethylamine) and B (100% acetonitrile) from 60:40 to 40:60 over 35 minutes, filtered and degassed with ultrasonic bath prior to use. Each solution was filtered through 0.45 μm syringe filter. Aliquot (20 μL) of each sample was injected into the system at flow rate of 1 mL/min with column temperature set at 25°C.

**Calibration Curve and Method Validation**

According to TLC image analysis, 2 μL of 5–200 μg/mL standard liriodenine dissolved in methanol was applied on TLC plate to generate calibration curve. The standard curve was analyzed using the least square regression equation derived from peak area. In addition standard solutions for HPLC analysis were also prepared in methanol to provide serial concentrations within range 5–200 μg/mL. Linear regression calculated from the peak area was used to construct the calibration curve. Precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ) were determined using spike method.

**RESULTS AND DISCUSSION**

*N. nucifera* is an aquatic perennial herb that grows in ponds, pools, rivers and lakes. The plant grows from a rhizome constricted at its nodes and it's somewhat pinkish. The petiole is to 2 feet long, terete, fistulous, and glabrous. The blade is 25–90 cm in diameter, round, thin, glabrous, and entire at the margin. It floats on the top of water surface. The flowers are conspicuous, 10–25 cm in diameter, pink or white, the petals oblong-elliptic to obovate, 5 cm–11 cm × 2.5 cm–5 cm. The fruits are conical, green, and up to 15 cm long[16] (Figure 2). Figure 3 showed greenish brown to brown color in dried leaves. The anatomical and histological investigation of *N. nucifera* dried leaves demonstrated in Figures 4, 5. Dominant characters from microscopic study demonstrated air chambers, calcium oxalate and trichosclereid on transverse section of leaf and powders.

**Plant description:** The plant grows from a rhizome constricted at its nodes. The petiole is to 2 feet long, terete, fistulous, and glabrous. The blade is 25–90 cm in diameter, round, thin, glabrous, and entire at the margin. It floats on the top of water surface. The flowers are conspicuous, 10–25 cm in diameter, pink or white, the petals oblong-elliptic to obovate, 5 cm–11 cm × 2.5 cm–5 cm. The fruits are conical, green, and up to 15 cm long[16]
Figure 5. Powdered *Nelumbo nucifera* leaves: 1. reticulate vessels 2. trichosclereids 3. epidermal cells 4. fragments of parenchyma, longitudinal view 5. vein 6. fibers 7. collenchyma 8. vessels 9. calcium oxalate crystals.

Table 1. Physicochemical Characteristics of *N. nucifera* Leaves

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean ± SD*</th>
<th>Min – Max</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid-insoluble ashes</td>
<td>2.61 ± 0.24</td>
<td>1.62 – 3.77</td>
<td>g/100 g</td>
</tr>
<tr>
<td>Total ash</td>
<td>9.62 ± 0.07</td>
<td>7.94 – 11.64</td>
<td>g/100 g</td>
</tr>
<tr>
<td>Loss on drying</td>
<td>7.69 ± 0.07</td>
<td>4.22 – 10.02</td>
<td>g/100 g</td>
</tr>
<tr>
<td>Water content</td>
<td>7.06 ± 0.11</td>
<td>4.60 – 9.02</td>
<td>g/100 g</td>
</tr>
<tr>
<td>Ethanol-soluble extractive</td>
<td>6.24 ± 0.22</td>
<td>3.37 – 13.33</td>
<td>g/100 g</td>
</tr>
<tr>
<td>Water-soluble extractive</td>
<td>9.51 ± 0.24</td>
<td>5.24 – 12.92</td>
<td>g/100 g</td>
</tr>
<tr>
<td>Liriodenine content (by Scion)</td>
<td>4.88 ± 2.98</td>
<td>0.93 – 10.35</td>
<td>mg/100 g</td>
</tr>
<tr>
<td>Liriodenine content (by HPLC)</td>
<td>4.85 ± 3.10</td>
<td>0.92 – 10.97</td>
<td>mg/100 g</td>
</tr>
</tbody>
</table>

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

Figure 6. TLC fingerprinting of a methanolic extract of dried *Nelumbo nucifera* leaf:  
I) Detection under UV light 254  
II) Detection under UV light 365  
III) Detection with anisaldehyde spraying and heat under visible light  
IV) Standard liriodenine under UV light 365.

Figure 7. HPLC chromatogram of *N. nucifera* leaf (source no.15); sample concentration was 0.1 g/ml of crude material. Liriodenine peak was at 11 min of retention time, analyzed using SHIMADZU gradient system with Inersil ODS-3, C-18 column (4.6 × 250 mm), DAD detector at 407 nm. Mobile phase consisted of A (formate buffer consisting 1% formic acid, adjusted to pH 4.5 diethylamine) and B (100% acetonitrile) from 60:40 to 40:60 over 35 min. at 25ºc, flow rate 1 ml/min.
indicated that the content of alkaloids, including liriodenine, by HPLC-UV analysis in *Zanthoxylum nitidum* varied significantly from habitat to habitat. Therefore, variation of liriodenine content may be due to various factors such as effect of growth period and environment, maturity of plant and storage time.

Calibration curve of standard compound showed good linearity relationship for both methods ($R^2 > 0.995$) over the range 5–200 µg/mL. According to TLC image analysis developed, method validation was conducted indicating polynomial regression equation $y = -0.1573x^2 + 61.392x + 151.38$ where $y$ is AUC and $x$ is concentration. Method gave adequate precision estimated as %RSD for within-day and day-to-day. Limit of detection and quantitation were 0.03 and 0.1 µg/mL, respectively. Accuracy was indicated by the percentage mean of recovery values were 101.12, 101.97, 103.90% of 3 levels of liriodenine addition.

CONCLUSION

These pharmacognostic investigations could be set as the standard parameter to be useful for quality control and authentication of the *N. nucifera* leaves crude drug in Thailand. TLC visualization under UV 365 nm with image analysis software and visible detector HPLC method can be applied to quantitatively determine liriodenine containing in plant materials.

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