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Original research article

Isolation and HPLC Analysis of Astilbin in *Lysiphyllum strychnifolium* (syn. *Bauhinia strychnifolia*) Stems

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ABSTRACT

Lysiphyllum strychnifolium (syn. *Bauhinia strychnifolia*), vernacularly known in Thai as "Ya-Nang-Daeng", has been traditionally used for detoxification and as an anti-fever in Thai traditional medicine. Despite published research on the plant's phytochemical constituents and their anti-cancer activity, quality assessment and pharmacological investigations have been hampered since the major bioactive compound remains unknown. Therefore, in this study, the isolation of a major compound has been explored, and its structure has been elucidated as astilbin. An RP-HPLC method was developed and validated for the determination of astilbin in *L. strychnifolium* stems. The chromatographic separation was performed on a reversed-phase C-18 column using gradient system of 2% (v/v) acetic acid and acetonitrile with flow rate of 1 mL·min^{-1}. The UV detection was set at a wavelength of 290 nm. The validated method showed good sensitivity, linearity, precision, and accuracy. The method could be used for routine analysis of astilbin in *L. strychnifolium* stems going forward. The present study provided a useful basis for the quality control of crude plant material, extract, and phytopharmaceutical products.

Keywords: *Bauhinia strychnifolia; Lysiphyllum strychnifolium;* Chemical marker; Astilbin; HPLC; Quality control

1. Introduction

A climbing herb "*Lysiphyllum strychnifolium* (Craib) A. Schmitz." (previously named *Bauhinia strychnifolia* Craib) is known in Thai as "Ya-Nang-Daeng" or "Kha-Yan" [1-2]. It is an endemic plant distributed in the north, central and eastern parts of Thailand [3].

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A decoction of the dried root and stem has been traditionally used as an anti-fever and blood detoxification medicine [4]. Pharmacological investigation revealed that the decoction of *L. strychnifolium* leaves possesses antioxidant [5-6] and anti-hyperuricemic [7] activity, while the alcoholic extract of *L. strychnifolium* stems possess anticancer [6, 8], anti-HIV-1 integrase [9], and anti-allergic [9] activities. Moreover, Influenza H5N1 neuraminidase inhibition and antibacterial activity of *L. strychnifolium* extracts have also been reported [10]. A preliminary study also showed that *L. strychnifolium* (published as *Bauhinia strychnifolia*) extract could reduce blood alcohol levels in younger age, healthy volunteers without undesirable side effects [11].

Isolated phytochemical constituents from the stems of *L. strychnifolium* revealed the presence of the flavonoids quercetin, 3,5,6,3',5'-pentahydroxy-flavanonol-3-*O*-α-*L*-rhamnopyranoside, and 3,5,7-trihydroxy-chromone-3-*O*-α-*L*-rhamnopyranoside, as well as the triterpenoids *β*-sitosterol and stigmasterol [8, 9]. Gallic acid was detected in leaves of this medicinal plant [5, 7] but it could not be detected in its stems. Despite the reported phytochemical constituents of this plant, quality control and pharmacological investigation that trace back to traditional indications have been hampered since the major bioactive compound is still unknown. Therefore, the isolation of a major compound from this plant was conducted and its structure has been successfully elucidated. Although several analytical methods have been developed for quantitative analysis of astilbin in other matrices or plant species [12-15], a simple and rapid HPLC method is required for analysis of *L. strychnifolium* extract. In this study, HPLC analysis of the plant' s major compound as a chemical marker has been validated and the present work should provide a basis for the quality control of raw materials, extracts, and phytopharmaceutical products derived from this plant.

2. Materials and Methods

2.1 Chemicals and reagents

HPLC grade methanol and acetonitrile was purchased from J.T. Baker, USA. Glacial acetic acid, analytical-reagent grade, was purchased from Merck, Germany. Water, HPLC grade, was purchased from RCI Labscan, Thailand**.** Standard astilbin was purchased from Sigma, USA. All other reagents were analytical grade if not stated otherwise.

2.2 Plant materials

The sample of *L. strychnifolium* stems was supplied by Charoensuk Pharma Supply Co., Ltd., Nakhon Pathom, Thailand. It originated from a cultivation farm in Ratchaburi, Thailand**.** The samples were identified by Ms. Pajaree Inthachub. Voucher specimens were deposited at The Drug Discovery and Development Center, Office of Advanced Science and Technology, Thammasat University, Thailand**.** The dried samples were powdered and stored in an air-tight container at room temperature in darkness.

2.3 Extraction and isolation

The dried and powdered stems (200 g) were macerated with methanol for 3 x 72 hrs**.** with occasional shaking. The extracts were pooled, filtered, and the solvent was evaporated under vacuum using a rotary evaporator, yielding 68.9 g of crude methanolic extract. The extract was separated by column chromatography (CC) (Merck silica gel 60, 70-230 mesh), eluted with ethyl acetate and methanol (95:5, v/v), and reversed-phase CC (Merck LiChroprep RP-18, 40-63 μm) eluted with methanol and water (50:50, v/v). Further purification was done by isocratic CC using Sephadex LH-20 with methanol and preparative HPLC (Hypersil C-18 column) with methanol and

water (25:75, v/v) which yielded 20 mg of astilbin.

2.4 NMR and MS measurement

The compound was dissolved in 99.8% methanol-d_4 (ca. 5 mg in 0.7 mL). All NMR spectra were recorded at room temperature on a Bruker Avance III 600 (resonance frequencies 600.25 MHz for ^{1}H and 150.95 MHz for ^{13}C) with standard Bruker pulse programs**.** Chemical shifts are given in ppm, referenced to residual solvent signals (3.31 ppm for ^{1}H, 49**.**0 ppm for ^{13}C). Mass spectra were recorded on amazon Ion trap (Bruker Daltonic) by infusion electrospray ionization (ESI) in negative mode**.** Nitrogen temperature was maintained at 200°C using a flow rate of 7.0 L min^{-1} and maintaining the nebulizer gas pressure at 2**.**0 bar.

2.4.1 Astilbin ($C_{21}H_{22}O_{11}$, M.W. 450.39 g mol^{-1})

^{1}H NMR (600 MHz, methanol-d_4), δ (ppm) = 6.97 (d, *J* = 1.9 Hz, 1H, Ar-*H*), 6.86 (dd, *J* = 8.1, 2.0 Hz, 1H, Ar−*H*), 6.83 (d, *J* = 8.1 Hz, 1H, Ar−*H*), 5.94 (d, *J* = 2.1 Hz, 1H, Ar−*H*), 5.92 (d, *J* = 2.1 Hz, 1H, Ar-*H*), 5.09 (d, *J* = 10.7 Hz, 1H, Taxifolin-*H*), 4.59 (d, *J* = 10.7 Hz, 1H, Taxifolin-*H*), 4.29-4.24 (m, 1H, Rhamnoside-*H*), 4.07 (d, *J* = 1.3 Hz, 1H, Rhamnoside-*H*), 3.68 (dd, *J* = 9.6, 3.3 Hz, 1H, Rhamnoside-*H*), 3.56 (dd, *J* = 3.2, 1.7 Hz, 1H, Rhamnoside-*H*), 3.37-3.31 (1H, Rhamnoside-*H*, overlapped with MeOD-solvent), 1.20 (d, *J* = 6.2 Hz, 3H, Rhamnoside-C*H*$_3$).

^{13}C NMR (150 MHz, methanol-d_4), δ (ppm) = 194.5 (C=O), 167.2, 164.1, 162.7, 145.9, 145.1, 127.7, 119.1, 114.9, 114.1, 101.0, 100.7, 96.0, 94.9, 82.5, 77.1, 72.4, 70.7, 70.3, 69.1, 16.4.

2.5 HPLC apparatus and conditions

The chromatography was performed on an HPLC apparatus **(**Thermo Scientific, CA, USA**)** equipped with a Spectra System pump P4000, a Spectra System auto-sampler AS3000, and a Spectra System UV6000LP diode array detector. The separation was done on a VDSpher PUR 100 C18-E column (250 x 4.6 mm i.d., 5 µm) (Chromatographie Technik GmbH, Berlin, Germany). The elution was carried out with gradient delivery systems with a flow rate of 1.0 mL min^{-1} at ambient temperature. The mobile phase consisted of 2% (v/v) acetic acid (solvent A) and acetonitrile (solvent B). Total running time was 50 min and the linear gradient program was as follows: 0% B to 15% in 5 min, 15% B for 20 min, 15% B to 18% B in 15 min, 18% B for 5 min, 18% B to 100% B in 5 min. The column was washed with acetonitrile for 10 min after each analysis and equilibrated with 2% (v/v) acetic acid for 10 min before each injection**.** The UV detection was set at a wavelength of 290 nm**.** Chromatograms were recorded and processed with ChromQuest 4.2.34 version 3.1.6 data acquisition system (ThermoFinigan, San Jose, CA, USA)chromatographic software.

2.6 Standard preparation

A standard stock solution of astilbin in methanol was prepared at a concentration of 0.5 mg mL^{-1}. Further dilution was carried out using methanol-water (60:40, v/v) as the diluting solvent to achieve the desired concentration.

2.7 Sample preparation

The dried *L. strychnifolium* stems were ground into fine powder. A mass of 0.1 g of the powder was transferred to a 100-mL volumetric flask. Then, 70 mL of methanol-water (60:40, v/v) was added. After sonication in an ultrasonic bath for 30 min, the solution was diluted to the appropriate volume with the same solvent and mixed well. The solution was then filtered through Whatman No.1 filter paper and the first 10-mL filtrate was discarded. The aliquot of the filtrate (3.0 mL) was diluted with methanol-water (60:40, v/v) in

a 10-mL volumetric flask. The solution was passed through a 0.22-µm nylon membrane filter and a 20-µL portion of each final solution was injected into the HPLC.

2.8 Method validation

Validation of the HPLC method was carried out according to the International Conference on Harmonization guidelines (ICH) [16]. The method validation parameters were specificity, accuracy, precision, linearity, limit of quantitation (LOQ), and limit of detection (LOD).

2.8.1 Specificity

Method specificity was determined by assessing peak purity of standard astilbin and test samples using UV scanning of a diode array detector.

2.8.2 Linearity

Linearity was evaluated in the concentration range of 5.55 –19.40 µg mL^{-1} with 3 replicates. The calibration curves were obtained by plotting the peak area versus the concentration of the standard solutions. The data were analyzed by the least-squares linear regression method.

2.8.3 Accuracy

Accuracy of the method was evaluated by recovery study. Standard addition was performed with pre-analyzed standard solution. Three different levels of standard mixtures were added to the sample extract. Each spike sample was prepared in triplicate. The recovery was calculated as follows: recovery(%) = 100 x (amount found – original amount)/amount added.

2.8.4 Precision

The measurement of intra- and inter-day precision was done by analyzing a sample solution. The intra-day precision was determined by analyzing 10 replications within 1 day, while the inter-day precision was examined for 6 different days. The precision was expressed as a percentage of the relative standard deviation (%RSD).

2.8.5 Limit of detection (LOD) and limit of quantitation (LOQ)

Determination of the signal-to-noise ratio was calculated under the proposed chromatographic condition. LOD was considered as 3:1 and LOQ as 10:1.

3. Results and Discussion

3.1 Isolation and structure elucidation

Dried and ground stems of *L. strychnifolium* samples were extracted with methanol and analyzed with HPLC in order to get an overview of the characteristic compound profiles. However, none of the reported constituents could be identified in major peaks according to previous studies of this plant species [8, 9]. Therefore, the isolation of the major component was undertaken. This led to detection of astilbin (Fig. 1), a flavonoid glycoside found in many plant species [17, 18].

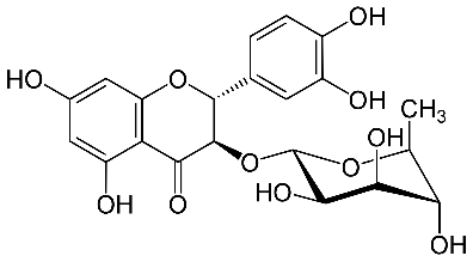

Fig. 1. Chemical structure of astilbin.

Isolated astilbin appears as a brownish-white amorphous powder. Its structure was elucidated by comparing its ^{1}H and ^{13}C NMR spectra with those reported in literature [19]. The mass spectral data (Fig. 2) revealing a $[M-H]^-$ peak at m/z = 449 in negative mode also confirmed its structure.

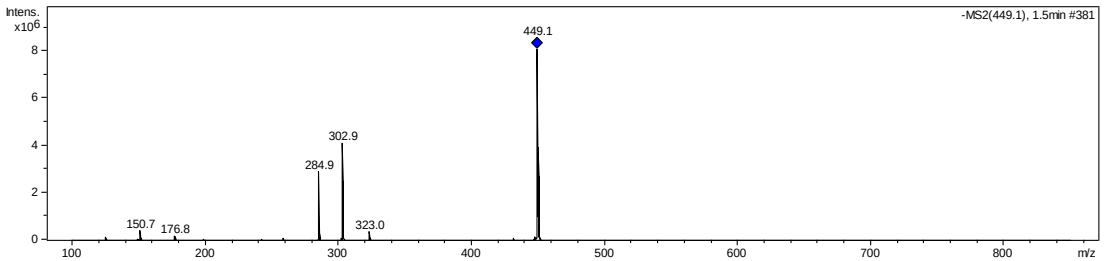

Fig. 2. The mass spectra of astilbin acquired in negative mode.

Identification of astilbin was confirmed by comparing the co-chromatography of standard astilbin, crude plant extract, and spiked sample. A comparison of the astilbin UV spectra from an *L. strychnifolium* sample and standard astilbin was done for verification (Fig. 3).

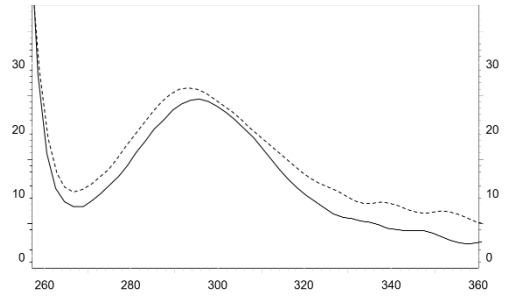

Fig. 3. Overlapped UV spectra of standard astilbin (bold line) and crude *L. strychnifolium* stems (dashed line).

Astilbin was identified from this plant for the first time. It is also a major component in *L. strychnifolium* stem extract which possesses various biological activities [18] relevant to its traditional uses. Therefore, it is suitable to use as chemical marker for performing quality control.

3.2 HPLC method development

The HPLC method was developed for determination of astilbin in *L. strychnifolium* stems. Optimization of the mobile phase composition was done using a reversed-phase C-18 column which is broadly used in pharmaceutical separation. The gradient system was developed in order to separate a wide range of compounds in sample solution. The analysis time was 50 min and the astilbin peak was found at the retention time of 34. 06 min. The chromatograms of optimized conditions are shown in Fig. 4. The detection was set at a wavelength 290 nm, which is the maximum absorbance of astilbin.

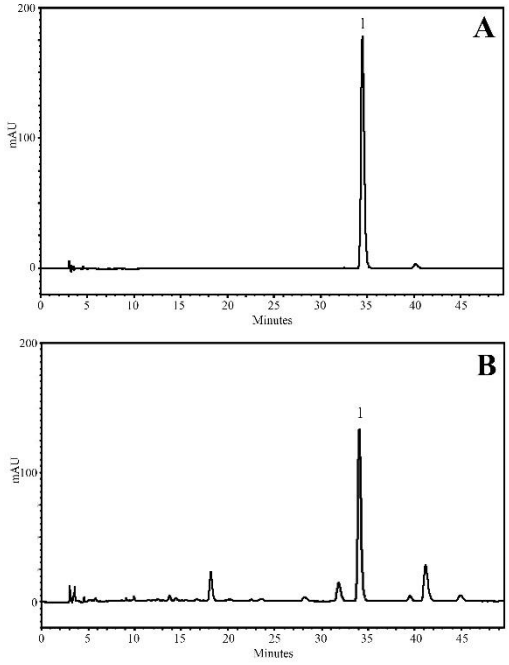

Fig. 4. Chromatograms of (A) 1…standard astilbin 13.89 µg mL^{-1} and (B) crude *L. strychnifolium* stems containing 1…astilbin 3.2 % (w/w). Stationary phase, VDSpher PUR 100 C18-E, 250 x 4.6 mm i.d., 5 µm; mobile phase, A: 2 % (v/v) acetic acid in water, B: acetonitrile; gradient: 0% B to 15% in 5 min, 15% B for 20 min, 15% B to 18% B in 15 min, 18% B for 5 min, 18% B to 100% B in 5 min; flow rate, 1 mL min^{-1}; detection, DAD, 290 nm; injection volume, 20 µL. Peak 1= astilbin.

3.3 Method validation

The HPLC method was validated for analysis of astilbin in *L. strychnifolium* stems. Linearity, precision, accuracy, LOD, and LOQ were analyzed for method validation parameters [16]. Linearity was assessed across a concentration range of 5.55 – 19.40 μg mL^{-1}. The plot of the peak area versus concentration provided good linearity for this method, with a determination coefficient (r^2) of 0.9994 (Table 1). The LOD and LOQ were found to be 0.23 and 0.69 μg mL^{-1} respectively, which indicate a high sensitivity of the method. The intra-day (n = 10), and inter-day (n = 6) precision as indicated by %RSD were 0.85 and 1.99 %, respectively. The results showed acceptable precision of the method, with RSD values lower than 2 %. Accuracy was evaluated by recovery study. The recoveries at 3 different levels of astilbin were 100.52, 100.77, and 100.92 %, with an average of 100.74 % (Table 2). These values indicate the accuracy of the method. The content of astilbin in crude *L. strychnifolium* stem samples was 3.20 ± 0.06 % (w/w).

Table 1. Method validation parameters for the determination of astilbin by the proposed HPLC method.

Parameters	Result
Regression equation[1]	Y = 322322 X – 211451
Determination coefficient (r^2)	0.9994
Precision (%RSD)	
Intra-day	0.85
Inter-day	1.99
LOD (μg mL^{-1})	0.23
LOQ (μg mL^{-1})	0.69

[1]X is the concentration of astilbin in **μ**g mL^{-1}, Y is the peak area at 290 nm

Table 2. Recovery study of astilbin by the proposed HPLC method.

Serial No.	Theoretical amount (μg mL^{-1})	Experimental amount[1] (μg mL^{-1})	Recovery[1] (%)[1]
1	8.88	8.93 ± 0.05	100.52 ± 0.60
2	11.11	11.20 ± 0.11	100.77 ± 0.10
3	13.33	13.45 ± 0.04	100.92 ± 0.30
	Average		100.74

[1]Expressed as mean ± standard deviation (SD; n = 3)

4. Conclusion

Astilbin was isolated and identified from *L. strychnifolium* stems for the first time. It is suggested to use this compound as a chemical marker for quality control of this medicinal plant due to its status as a major active component. The RP HPLC method used was developed and validated for analysis of astilbin in crude stem. Validation was successfully performed to ensure the linearity, precision, accuracy, and sensitivity of the method. The developed method could be used for routine analysis of the plant material, extract, and phytopharmaceutical products in practical application. The present study provides a useful basis for quality control and further development of this medicinal plant.

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