Development and Validation of RP-HPLC Method to Determine Anti-Allergic Compound in Thai Traditional Remedy Called Benjalokawichien

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Benjalokawichien (BLW) or Ya-Ha-Rak (HR) is a traditional remedy in the National drug list of herbal medicinal products AD 2012 of Thailand. For traditional use, BLW is used as antipyretic agent. It also has anti-allergic effect, particularly treating allergic rash. The ethanolic extract of BLW exhibited anti-allergic activity via inhibitory effect against a release of beta-hexosaminidase in RBL-2H3 cell line. Pectolinarigenin has been identified as the active compound of BLW extract. In this study, a reversed-phase high performance liquid chromatography (RP-HPLC) method was developed in order to control quality of preparation in three aspects such as chemical fingerprint, quantification and stability of the ethanolic extract. The RP-HPLC was performed with a gradient mobile phase composed of 0.1% ortho phosphoric acid and acetronitrile, and peaks were detected at 331 nm. Based on validation results, this analytical method is precise, accurate and stable for quantitative determination of pectolinarigenin. The amount of pectolinarigenin in Benjalokawichien extract determined by this method was 18.50 mg/g of extract. Therefore, this method could be considered for quality control of BLW extract.

Keywords: *Benjalokawichien, Anti-allergic activity, RP-HPLC, Pectolinarigenin*

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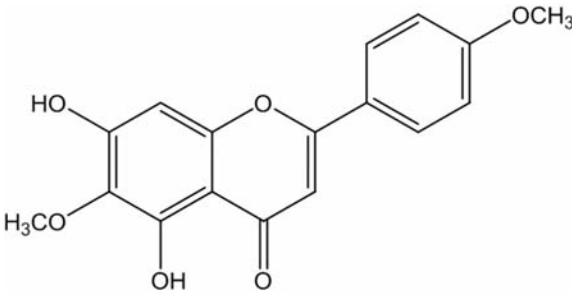
Benjalokawichien (BLW) or Ya-Ha-Rak (HR) is a traditional remedy in the National drug list of herbal medicinal products A.D. 2012 of Thailand. BLW remedy was reported to be used as antipyretic agent by Thai folk doctors. Moreover, it was used to treat allergic rash in Thai traditional medicine a long time ago. Its formula consists of five plant roots: *Ficus racemosa*, *Capparis micracantha*, *Clerodendrum petasites*, *Harrisonia perforata* and *Tiliacora triandra*. Previous studies reported pharmacological effect of these remedy as antipyretic[1,2], anti-inflammatory[2,3], and antioxidant activity[3]. In the present study, the ethanolic extract of BLW exhibited anti-allergic activity via inhibitory effect against a release of beta-hexosaminidase with IC_{50} as 39.78 µg/ml. Pectolinarigenin (Fig. 1) isolated from BLW extract showed higher anti-allergic activity than ketotifen, a positive control (IC_{50} as 6.30 and 40.39 µg/ml, respectively)[4]. Thus, pectolinarigenin was potential to be a marker for quality control of BLW remedy.

The present study was to develop a reversed-phase high performance liquid chromatography (RP-HPLC) method to control quality of BLW remedy in three aspects such as chemical fingerprint,



Pectolinarigenin

Fig. 1 Structure of Pectolinarigenin [$C_{17}H_{14}O_6$ (MW: 314.3); yellow powder].

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quantification and stability of the extract. In addition, the validation of this assay was performed in terms of specificity, limits of detection, limits of quantification, linearity, accuracy and precision.

Material and Method

Chemicals and reagents

Standard pectolinarigenin (Purity >98%) was purchased from ChemFaces (Wuhan, China). HPLC reagents such as acetronitrile, methanol, DMSO, phosphoric acid and purified water, were purchased from RCI Labscan (Bangkok, Thailand).

Plant materials and extraction

BLW remedy were collected from Dan-Chang, Suphanburi (western Thailand). The voucher specimens were deposited at the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand. It consisted of five roots: *Ficus racemosa* (SKP 117061801), *Capparis micracantha* (SKP 391031301), *Clerodendrum petasites* (SKP 202030901), *Harrisonia perforata* (SKP 178081601) and *Tiliacora triandra* (SKP 114202001). All plants were cleaned and dried at 50°C, ground and combined in equal proportion by weight, then macerated with 95% ethanol, filtered and concentrated under reduced pressure to obtain the ethanolic extracts. The percentage of yield was 3.59%.

Preparation of sample for HPLC analysis

Ten milligrams of BLW extract was dissolved in 1 ml methanol, then sonicated for 15 minutes. This solution was filtered through a membrane filter (pore size 0.45 µm) prior to analysis.

Preparation of standard solutions

A stock solution of pectolinarigenin was prepared at a concentration of 1.0 mg/ml with DMSO and stored at -20°C until use.

RP-HPLC analysis

Studies on chemical fingerprint and quantification of active constituents were carried out using a High performance liquid chromatography (HPLC) system (Agilent® LC 1100/1200 system), with photodiode array (PDA) detector (model G1315D) and automatic injector (model G1329A). A reversed-phase column was ZORBAX Eclipse XDB-C18 column (4.6x250 mm, 5 micron) protected by Eclipse XDB-C18 analytical guard cartridge (4.6x12.5 mm, 5 micron). The mobile phase was a mixture of 0.1% ortho phosphoric acid (A) and acetronitrile (B). Initial conditions were started with a gradient elution of 95-5% A and 5-95% B for 30 min. This was followed by a gradient elution of 5-95% A and 95-5% B until t = 35 min, after which the column was eluted with an isocratic elution for 5 min. The flow rate was 1 ml/minute with detection at UV 331 nm. The operating temperature was maintained at room temperature (25°C). Data were analyzed by ChemStation® software.

Validation of RP-HPLC method

The validation of the analytical method for pectolinarigenin from BLW extract was examined in terms of specificity, linearity, accuracy, precision as well as the limit of detection (LOD) and limit of quantitation (LOQ).

Specificity

Peak identification was carried out using the standard pectolinarigenin and PDA detector. The UV spectra of standard spectra were taken at various points of the peaks and compared with BLW sample to check peak homogeneity.

LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) were determined by means of serial dilutions of pectolinarigenin based on signal-to-noise ratio of 3:1 and 10:1, respectively.

Linearity

The linearity was validated by preparing the standard pectolinarigenin solutions at least five concentrations. A volume of 10 µl of each concentration was injected into the HPLC column. Triplicate analyses were performed in three different days. The standard curve was analyzed using the linear least-squares regression equation derived from the peak area.

Precision

The precision was validated by preparing the standard pectolinarigenin solutions of at least three concentrations. A volume of 10 µl of each concentration was injected into the HPLC column. Concentrations of standard compound from the experiments were calculated with a linear equation of the standard curve. The precision of the analysis method was examined by the intermediate evaluation method using measurements of the intra- and inter-day variability.

Intra-day variability was determined by

analyzing the sample solution during one of the study days (24 h), while inter-day variability was performed on three different days. Standard deviation (SD) to the mean was used as coefficient of variation (CV), values obtained from the results of triplicate testing. The calculated CV should not greater than 2%.

Accuracy

The standard of pectolinarigenin with the known amount was spiked to BLW sample solution, of which the contents of pectolinarigenin were determined before adding the standard compounds. The three injections for each concentration were performed per day for different three days and calculated percentage recovery of standard pectolinarigenin.

Results and Discussion

The optimal conditions to determine quantitatively pectolinarigenin in BLW extract was the use of gradient RP-HPLC system. Pectolinarigenin has absorption at 331 nm, this wavelength was used for quantification. Mixture of 0.1% ortho phosphoric acid and acetronitrile were examined as the mobile phase and optimized to obtaining a good resolution. Based on the HPLC analysis, pectolinarigenin was a minor compound with a content of 0.18% w/w.

Defining the specificity, linearity, LOD, LOQ, precision and accuracy validated the RP-HPLC method. Specificity was evaluated using UV-absorption spectra produced by PDA. The spectra were taken at three points of the peaks. When they were compared with standard pectolinarigenin, homogeneity of spectra of peak was found, with a retention time of 21.49 min (Fig. 2).

The LOD represents the lowest concentration of pectolinarigenin that can be detected by the instrument and the analytical method, whereas the LOQ represents the lowest concentration of pectolinarigenin that can be determined with acceptable precision and accuracy by the instrument and method. It was founded that the RP-HPLC method was very sensitive to pectolinarigenin with LOD and LOQ of 0.55 and 1.66 µg/ml, respectively (Table 1).

Linearity was evaluated using standard samples over five calibration points with six measurements for each calibration points. Three separate calibration curves of each standard obtained on different days by plotting the peak area versus concentration were found to be linear when evaluated by linear regression analysis. Pectolinarigenin exhibited good linearity over the evaluated range with correlation

Table 1. Linear ranges, correlation coefficients (r^2), LOD and LOQ of calibration curves of pectolinarigenin

Parameters	Pectolinarigenin
Concentration (µg/ml)	25-400
Linear equation (y = ax + b)	y = 46.213x+27.344*
Linearity (r^2)	0.9998
LOD (µg/ml)	0.5480
LOQ (µg/ml)	1.6610

*Y is peak area, X is the concentration of the analyzed sample

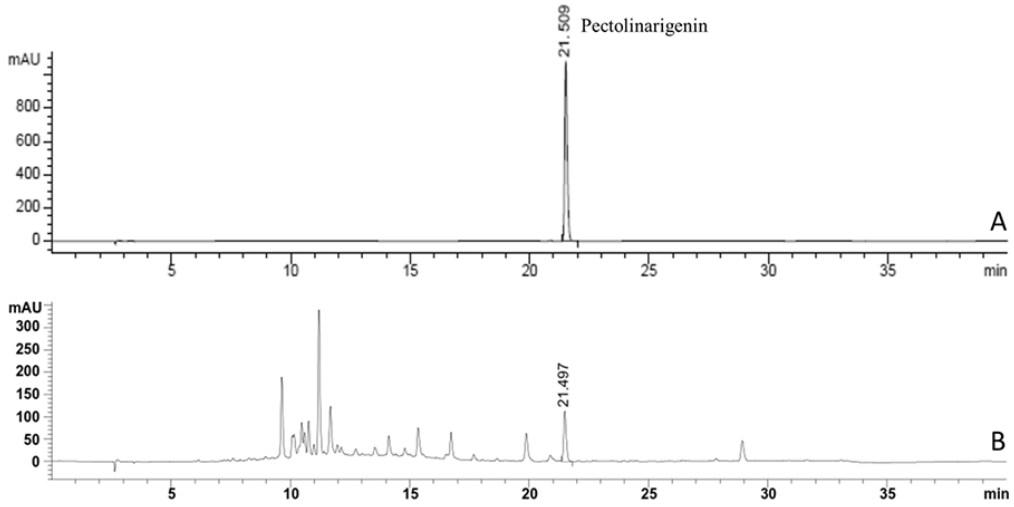

Fig. 2 HPLC chromatograms of standard pectolinarigenin (A) and BLW extract (B).

Table 2. Precision validation of the analytical method for pectolinarigenin

Concentration (µg/ml)	Intra-day[a] (n = 3)		Inter-day[b] (n = 9)	
	Measured Conc. (µg/ml)	% CV	Measured Conc. (µg/ml)	% CV
25	24.76±0.14	0.57	24.85±0.28	1.14
50	50.09±0.36	0.72	50.95±0.69	1.35
100	100.46±0.15	0.15	102.03±1.21	1.19
200	199.59±0.16	0.08	201.24±1.77	0.88
400	400.10±0.30	0.07	398.79±1.41	0.35

[a] All values are mean ± SD as obtained by triplicate analyses in a day
[b] All values are mean ± SD, obtained by triplicate analyses per day over 3 days

Table 3. Accuracy validation of the analytical method for pectolinarigenin

Spiked level (µg/ml)	Recovery (%)[a]			Mean (%)	CV (%)
	1	2	3		
25	102.16	102.49	101.57	102.07±0.47	0.46
50	99.04	98.08	98.31	98.48±0.50	0.51
100	99.53	98.16	98.35	98.68±0.74	0.75

[a] All values are mean ± SD as obtained by triplicate analyses in a day

coefficients (r^2) of 0.9998 (Table 1).

Both intra- and inter-day precisions of the analytical method were evaluated by triplicate analysis. The RP-HPLC method showed to be reproducible and reliable with both intra-day and inter-day precision being lower than 2 %CV (Table 2).

The accuracy of the method was determined by investigating the recovery of samples of spiking standard pectolinarigenin into BLW extract and comparing the measured value to the true value. The percentage of recovery that being close to 100% indicated good accuracy of the method obtained. The RP-HPLC method exhibited good recovery in the range of 92-103% (Table 3).

The ethanolic extracts of Benjalokawichien were determined content of pectolinarigenin by development RP-HPLC method. The result found the amount of pectolinarigenin was 18.50 mg/g of extract.

Conclusion

In the present study, a gradient RP-HPLC method was developed and validated. The method showed specificity, high sensitivity, good linearity, precision and accuracy. This RP-HPLC method could be considered for quantitative determination of pectolinarigenin in the BLW extract.

What is already known on this topic?

Benjalokawichien is Thai traditional remedy in the National drug list of herbal medicinal products of Thailand that is indicated for use as antipyretic drug. It was the subject of a previous study related to the mechanism of these remedies as antipyretic[1,2], anti-inflammatory[2,3] and antioxidant activity[3]. Pectolinarigenin was isolated from its extract and showed high anti-allergic activity[4]. There was no method for quality control such as chemical fingerprinting, quantification and stability of these extracts.

What the present study adds?

In the present study, a gradient RP-HPLC method was developed and validated. The method showed specificity, high sensitivity, good linearity, precision and accuracy. This RP-HPLC method could be considered for quality control of Benjalokawichien extract.

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Potential conflicts of interest

None.

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การพัฒนาและการตรวจสอบความใช้ได้ของวิธีการวิเคราะห์หาปริมาณสารต้านภูมิแพ้ในตำรับยาไทยชื่อเบญจโลกวิเชียร โดยใช้เทคนิค RP-HPLC

อินทัช ศักดิ์ภักดีเจริญ, ธนา จักษ์เมธา, อรุณพร อิฐรัตน์

ตำรับยาเบญจโลกวิเชียรหรือยาห้ารากเป็นตำรับยาแผนไทยที่อยู่ในบัญชียาหลักแห่งชาติ (บัญชียาจากสมุนไพร) พ.ศ. 2555 ใช้บรรเทาอาการไข้ นอกจากนั้นยังมีการนำมาใช้ในการรักษาผื่นแพ้อีกด้วย จากการศึกษาพบว่าสารสกัดเอทานอลของตำรับยาเบญจโลกวิเชียรมีฤทธิ์ต้านภูมิแพ้ โดยสามารถยับยั้งการหลั่งเอนไซม์ beta-hexosaminidase ในเซลล์แมสต์ของหนู (RBL-2H3 cell line) สารสำคัญที่ออกฤทธิ์ คือ pectolinarigenin การศึกษาครั้งนี้เป็นการพัฒนาเทคนิค reversed-phase high-performance liquid chromatography (RP-HPLC) เพื่อใช้ในการควบคุมคุณภาพ ได้แก่ การดูลายพิมพ์นิ้วมือ การหาปริมาณสารสำคัญ และการศึกษาความคงตัวของสารสกัดเอทานอลของตำรับยาเบญจโลกวิเชียร โดยใช้ตัวทำละลายเคลื่อนที่เป็น 0.1% ortho phosphoric acid และ acetronitrile ตรวจวัดภายใต้ความยาวคลื่นแสงที่ 331 นาโนเมตร จากการตรวจสอบความใช้ของวิธีวิเคราะห์ พบว่าวิธีวิเคราะห์มีความเที่ยงตรง ความแม่นยำ และมีความเสถียรในการใช้หาปริมาณของสาร pectolinarigenin เมื่อนำวิธีที่พัฒนามาวิเคราะห์หาสาร pectolinarigenin ในสารสกัดพบว่ามีปริมาณ 18.50 มิลลิกรัม/กรัมสารสกัด ดังนั้นวิธีการที่พัฒนาขึ้นมานี้จึงเหมาะที่จะนำมาใช้ในการควบคุมคุณภาพของสารสกัดเอทานอลของตำรับยาเบญจโลกวิเชียรต่อไป