Original Article

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A simple high-performance liquid chromatographic method for quantitative analysis of brazilin in *Caesalpinia sappan* L. extracts

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ABSTRACT

Objective: The specific aim of the study was to develop and validate a simple reversed-phase high-performance liquid chromatographic (HPLC) method for assay of brazilin in *Caesalpinia sappan* L. (sappanwood) extracts. **Method:** Validation of the HPLC method was conducted under a gradient of methanol and 2.5% acetic acid using Halo C_{18} column. **Results:** Brazilin eluted at 6.08 min was detected at a wavelength of 280 nm. The method provided good linearity, high selectivity, specificity, accuracy and precision. **Conclusion:** The validated method was successfully applied for quantitative analysis of brazilin in various sappanwood extracts prepared in this study.

Keywords: *Caesalpinia sappan* L., sappanwood extracts, brazilin, HPLC, validation

INTRODUCTION

Sappanwood or Indian brazilwood (*Caesalpinia sappan* L.) is widely distributed in South East Asia, America, and Africa. The heartwood of sappanwood contains natural red color pigment and has been traditionally used in food, dye, and cosmetic industries. Sappanwood has various bioactivities including wound healing, treatments of blood diseases, diarrhea, skin infections, and menstrual disorder.[1-3] Brazilin [Figure 1] is one of the major phenolic constituents found in sappanwood extracts and has been shown to possess numerous pharmacological activities such as antioxidant, anti-inflammatory, anti-photoaging, antiacne, antibacteria, anti-allergic, antiplatelet aggregation, hypoglycemic, and vasorelaxant.[3-15]

The quality of herbal extracts is varied depending on the method of extraction. Ethanol has been commonly used as a solvent for extraction of sappanwood.[10,16-18] However, the crude ethanolic extract of sappanwood contains several unwanted compounds resulting in low brazilin content and poor biological activities of the extract. The partition technique or the ion-exchange chromatography has been used for further purification of sappanwood extracts to increase the brazilin content.[10,19] The reliable method for quantitative analysis of brazilin is, therefore, needed to assess the quality of various sappanwood extracts containing different amounts of brazilin.

Various methods have been reported for characterization and quantitative analysis of brazilin in sappanwood extracts or biological samples.[10,17,20-24] Although the published methods are relatively specific for brazilin analysis, they required complicated sample preparation or sophisticated analytical equipment.[21-23] Settharaksa *et al*. have proposed the high-performance liquid chromatographic (HPLC) method to determine brazilin in sappanwood extract having limit of detection (LOD) and limit of quantification (LOQ) of 1.30 and 3.49 μg/mL, respectively.[24] However, this method may not provide sufficient sensitivity for quantifying brazilin in some extracts or other samples containing low brazilin content. In addition, most of the reported methods for brazilin analysis in sappanwood extracts have not been yet validated, which is

required for routine quality control of sappanwood extract raw materials.[10,17,20]

In the present study, the gradient liquid chromatography combined with simple and fast sample preparation with adequate sensitivity was developed for quantitative analysis of brazilin in sappanwood extracts prepared by various extraction techniques. The method was completely validated in compliance with ICH Harmonized Tripartite Guideline[25] and AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals.[26]

MATERIALS AND METHODS

Materials

Heartwood of sappanwood was collected from Baan Dong Bang, Prachinburi province, Thailand in July 2014. Plant identification was performed by Prof. Dr. Boonchoo Sritularak. A voucher specimen (CS-DC-012558) is deposited at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. A brazilin standard with the purity >99%w/w was purchased from Chengdu Biopurify Phytochemical Ltd., China. HPLC grade methanol was purchased from B and J Scientific Co., Ltd., USA. AR grade glacial acetic acid was purchased from Merck Ltd., Thailand.

Preparation of Sappanwood Extracts

The dried powder (7 kg) of *Caesalpinia sappan* heartwood was macerated with 95% ethanol (3 × 28L) for 24 h at room temperature to give an ethanolic extract after removing the solvent using a rotary evaporator at 40°C.[19] The obtained crude extract was semi-purified using the partition method and ion-exchange chromatography. For the partition method, the ethanolic extract was suspended in water and then partitioned with dichloromethane, and subsequently with ethyl acetate to give the fractions of dichloromethane, ethyl acetate, and aqueous extracts, respectively. For ion-exchange chromatography, the ethanolic extract was semi-purified using Diaion® HP-20 as a stationary phase eluted with 35% ethanol to give a Diaion extract. Finally, five extracts including ethanolic, aqueous, dichloromethane, ethyl acetate, and Diaion extracts were obtained and subjected to HPLC analysis for brazilin content.

HPLC Instrumentation and Chromatographic Conditions

HPLC analysis was performed using a Shimadzu LC-20 AD system (Shimadzu, Japan) consisting of an autosampler (SIL-20 AC), a column oven (CTO-20A), and a photodiode array detector (SPD-M20A). Liquid chromatography was performed using a Halo C_{18} column (4.6 × 150 mm, 5 μm) controlled at 35°C with gradient elution at a flow rate of 1.0 mL/min. The mobile phase consisted of methanol (solvent A) and 2.5% v/v acetic acid (solvent B). The gradient was initiated with 10% solvent A and 90% solvent B for 12 min, then automatically ramped to 100% solvent A for 13 min, and automatically decreased to initial mobile phase ratio for 15 min. A detection wavelength was set at 280 nm. The injection volume was 20 µL. The total run time was 40 min with brazilin eluting at 6.08 min.

Preparation of Standard Solutions

A standard of brazilin was dissolved in methanol to obtain a stock standard solution at 2.0 mg/mL. The stock standard solution of brazilin was diluted with 2.5% acetic acid/methanol (90:10 v/v) to obtain standard solutions at 2.5, 5, 10, 15, 20, and 25 µg/mL.

Preparation of Sample Solutions

Each sappanwood extract was dissolved in methanol at concentration of 2.0 mg/mL and subsequently diluted with 2.5% acetic acid/methanol (90:10 v/v) to obtain the final concentration of 0.2 mg/mL. The solution was filtered through a 0.45-μm membrane filter before HPLC analysis.

Validation of the HPLC Method

System suitability test

System suitability was assessed by six replicate injections of the standard solution of brazilin (10 µg/mL). The percentage of relative standard deviation (%RSD) should be <2% for retention time, peak area, tailing factor, and theoretical plate.

Selectivity and specificity

Selectivity was evaluated by separate injection of the standard solution of brazilin (10 µg/mL) and the prepared sappanwood extracts including ethanolic, aqueous, dichloromethane, ethyl acetate, and Diaion extracts to determine possible interferences at the retention time of brazilin. The chromatograms of the sappanwood extracts were compared with the brazilin standard. For specificity, peak purity index of the brazilin peak was determined, and the value should be >0.99. In addition, the preliminary stability of brazilin in the ethanolic extract was investigated on acetate buffers at pH 4.5 and 5.5 under ultraviolet (UV) light exposure. Peak purity index values of brazilin before and after UV light exposure were evaluated.

Linearity

Six different concentrations of the brazilin standard solutions ranging from 2.5 to 25 µg/mL were determined. The experiment was analyzed in triplicate. The relationship between peak areas and brazilin concentrations was plotted and used as a calibration curve. The linearity was assessed by calculating the coefficient of determination (r^2), and it should be >0.999.

Linearity and Accuracy and precision

The LOD and LOQ were estimated using a linear regression equation of the calibration curve. The SD of y-intercepts and slope (s) of the regression line were used for calculating LOD and LOQ as 3.3SD/s and 10SD/s, respectively.

Accuracy and precision

Accuracy and precision of the HPLC method were determined at three different concentrations using the standard addition method. The samples were prepared by spiking the sappanwood ethyl acetate extract solution (25 µg/mL) with the stock standard solution (2.0 mg/mL) to give additional brazilin concentrations of 2.5, 10, and 20 µg/mL. The ethyl acetate extract solution (20 mg/mL) was prepared by dissolving

the ethyl acetate extract in methanol and then diluted with 2.5% acetic acid/methanol (90:10 v/v) to obtain the final concentration of 25 μg/mL. Intraday and interday accuracy and precision of three different concentrations were analyzed in triplicate. Interday accuracy and precision were performed on three different days. The percentage of recovery and percentage RSD were calculated to evaluate the accuracy and precision of the method, respectively. The percentage recovery should be in the range of 85–110% and the percentage RSD values should be <2.

RESULTS AND DISCUSSION

The HPLC method was developed and optimized to obtain a suitable chromatographic condition for the determination of brazilin in sappanwood extracts. The HPLC chromatogram monitored for 40 min could completely resolve brazilin at 6.08 min from any other impurities presented in the sappanwood extracts [Figure 2]. The experiment demonstrated that the washing process could start at minute 8. However, a peak of around 10 min was observed. Therefore, the washing process was set to begin at minute 12 to show the peak at around 10 min as a part of the extract. However, this peak was not identified in this study. At a retention time of 13–25 min, the linear gradient elution up to 100% v/v methanol for 13 min was sufficient to elute non-polar impurities from the chromatographic column. This step was, therefore, useful for the prevention of carry-over from highly-retained impurities to the next injection. In addition, this step could extend the lifetime of the column. After the washing period, the mobile phase was reverted to the initial mobile phase ratio to equilibrate the column for 15 min (at a retention time of 26–40 min) before next injection. Thus, the developed method was chromatographically suitable for brazilin analysis in the sappanwood extracts.

Validation of the HPLC Method

Validation of the developed HPLC method was performed in accordance with ICH and AOAC Guidelines. System suitability, selectivity, specificity, linearity, LOD, LOQ, accuracy, and precision were investigated.

System suitability

The brazilin standard solution at 10 μg/mL was injected with six replicates. The percentage RSD values of retention times and peak areas were <2%, indicating the low variation of repeated injection [Table 1]. Furthermore, tailing factor (T) was <2, indicating the symmetry of the analyte peak. The theoretical plate was found to be >1500, demonstrating high efficiency of a column during analysis. Overall, the chromatographic suitability parameters were complied with ICH and AOAC guidelines. The results indicate that the HPLC system and conditions were suitable to use for further validation and sample analysis.

Selectivity and specificity

Regarding selectivity, the peak of brazilin in sappanwood extracts showed the retention time identical to that of the brazilin standard solution [Figure 3]. The result indicates that any impurities presented in the sappanwood extracts have no significant impact on the retention time and characteristics of the brazilin peak on the HPLC chromatogram.

The peak purity index values of the brazilin peak from the sappanwood extracts were in the range of 0.9976–0.9979, indicating the specificity of the HPLC method. Furthermore, the preliminary stability of brazilin in the ethanolic extract was evaluated on acidic pH conditions under UV light exposure. The results show that peak areas of brazilin in the extract solution at pH 4.5 and 5.5 were decreased after UV light exposure. Peak purity index values of brazilin before and after UV light exposure at pH 4.5 were 0.9987 and 0.9981, respectively, while those at pH 5.5 were 0.9988 and 0.9984, respectively. All experiments gave values of peak purity index >0.99, demonstrating that no coelution was observed at the same retention time of the brazilin peak. The results indicate that the developed method was spectrally selective and specific.

Linearity

The calibration curve was constructed by plotting the concentrations of brazilin standard against the peak areas. The slope, y-intercept, and coefficient of determination (r^2)

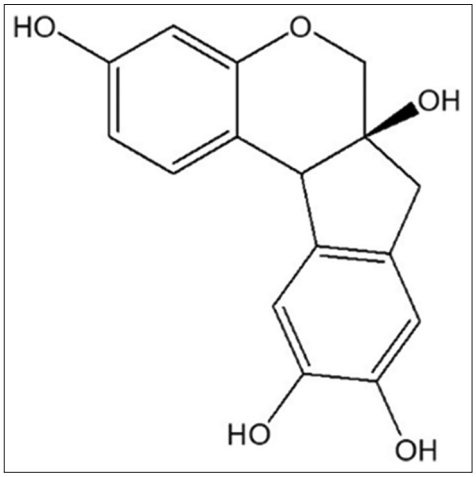

Figure 1: The chemical structure of brazilin

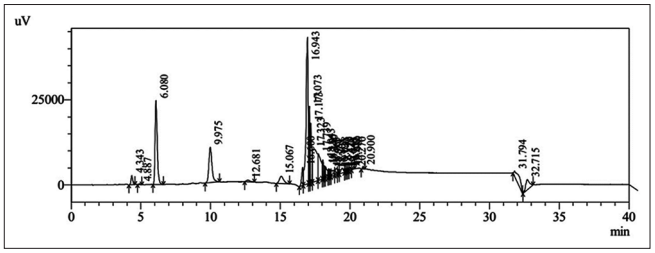

Figure 2: The typical HPLC chromatogram of the ethanolic extract of sappanwood monitored for 40 min

Table 1: System suitability parameters of the developed HPLC method ($n=6$)

Parameters	Retention time (min)	Peak area	Tailing factor	Theoretical plate
Mean	6.11	227810	1.32	47329
%RSD	0.04	0.24	0.08	0.19

HPLC: High-performance liquid chromatographic, %RSD: Percentage of relative standard deviation

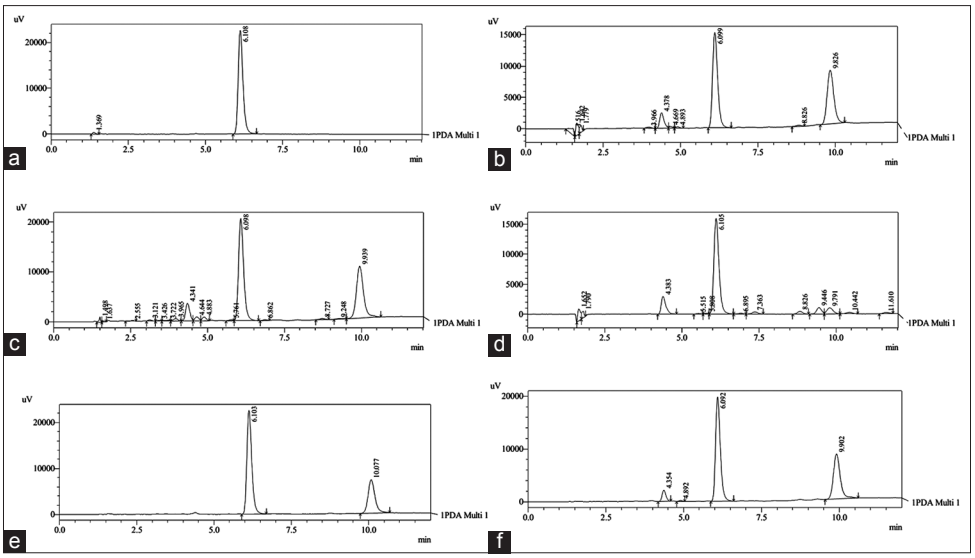

Figure 3: Typical HPLC chromatograms of brazilin standard (a) and 5 sappanwood extracts (b-f) including ethanolic, aqueous, dichloromethane, ethyl acetate, and Diaion extracts, presenting the brazilin peaks at the retention time of 6.108, 6.099, 6.098, 6.105, 6.103, and 6.092 min, respectively

Table 2: Accuracy and precision for the HPLC determination of brazilin

Brazilin	Amount added (μg/ml)	Amount found (μg/ml)	%Recovery	%RSD
Repeatability (intraday) n=3				
	2.50	2.21±0.02	88.54±0.60	0.68
	10.54	10.48±0.04	99.40±0.40	0.41
	21.90	22.03±0.17	100.60±0.76	0.76
Intermediate precision (interday, 3 days) n=3				
Day 1	2.50	2.21±0.02	88.54±0.60	0.68
	10.54	10.48±0.04	99.40±0.40	0.41
	21.90	22.03±0.17	100.60±0.76	0.76
Day 2	2.50	2.29±0.02	91.60±0.89	0.97
	10.40	10.40±0.02	99.98±0.19	0.19
	21.59	21.27±0.13	98.50±0.59	0.59
Day 3	2.50	2.22±0.002	88.89±0.10	0.11
	10.61	10.58±0.09	99.72±0.82	0.85
	22.06	21.87±0.23	99.13±1.03	1.04

HPLC: High-performance liquid chromatographic, %RSD: Percentage of relative standard deviation

of the calibration curve were obtained from the regression analysis. The residual sum of square was found to be 1.22 × 10^8. The regression equation of the brazilin standard was y = 20827x + 14459 (r^2 = 0.9993), indicating a high degree of correlation and good linearity of the method. The results meet the acceptance criteria according to the ICH and AOAC guidelines, which stated that the coefficient of determination should be >0.999.

LOD and LOQ

The LOD and LOQ values for the HPLC determination of brazilin were found at 0.68 and 2.06 μg/mL, respectively. These results indicate that the method provided adequate sensitivity for brazilin analysis in the extracts.

Accuracy and precision

The accuracy and precision of the HPLC method for brazilin determination are summarized in Table 2. The percentage recovery values were between 88.5 and 100.6% and the percentage RSD values were 0.11–1.04. The results indicate the good accuracy and precision of the method.

Quantitative analysis of brazilin in the sappanwood extracts

The validated HPLC method was applied for quantitative analysis of brazilin in the various sappanwood extracts [Table 3]. Brazilin contents found in the extracts were consistent with our previous results determined using thin-layer chromatographic densitometric

Table 3: Brazilin contents in various sappanwood extracts (n=3, mean±SD)

Sappanwood extracts	Brazilin content (%w/w)
Ethanolic extract	7.70±0.21
Aqueous extract	11.14±0.27*
Dichloromethane extract	7.98±0.17
Ethyl acetate extract	11.38±0.15*
Diaion extract	13.24±0.25*

*Significantly different when compared to the ethanolic extract (P<0.05). SD: Standard deviation

method.[19] The order of brazilin content in all extracts were as follows: Diaion extract > ethyl acetate extract ≈ aqueous extract > dichloromethane extract ≈ ethanolic extract (crude extract). The results show that the further purification of the ethanolic extract through the partition with ethyl acetate or ion-exchange chromatographic methods could significantly increase the content of brazilin (P < 0.05) in the extracts. In addition, the brazilin contents found in the ethyl acetate and aqueous extracts were not significantly different (P > 0.05) because brazilin can dissolve in both ethyl acetate and water.[3,27] The dichloromethane extract has no significant difference in the brazilin content comparing to the ethanolic extract (P > 0.05). The dichloromethane extract had the lowest brazilin content for the partition method because dichloromethane is less polar than water and ethyl acetate and therefore dichloromethane demonstrated less extraction efficiency. The Diaion extract had significantly higher brazilin content than other extracts because of the adsorbent property of Diaion® HP-20 resin which could remove unwanted compounds or impurities from the ethanolic extract.[10,28]

CONCLUSIONS

In the present study, a simple sample preparation coupled with a gradient HPLC method for analysis of brazilin in the sappanwood extracts was developed and validated according to ICH and AOAC guidelines. All validation parameters meet the acceptance criteria with good linearity, high selectivity, specificity, accuracy, and precision. In addition, the validated method could be successfully applied for analysis of brazilin in the sappanwood extracts prepared by different extraction methods. The validated HPLC method is, therefore, recommended for routine monitoring the quality of various sappanwood extracts and could be further applied for analysis of brazilin in formulations.

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