HPLC Analysis of Laxative Rhein Content in *Cassia fistula* Fruits of Different Provenances in Thailand

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Abstract

The ripe fruit pulp of *Cassia fistula* Linn. (Fabaceae), which is locally called Khun or Ratchaphruek in Thailand, has long been used as a traditional laxative drug due to its anthraquinone glycosides constituent. Rhein is the major anthraquinone in the fruit pulp while minor constituents are aloin and sennosides. The aims of this study were to develop and validate a high performance liquid chromatographic method for quantitative determination of rhein content in the fruit pulp of *C. fistula* collected from different provinces in four main parts of Thailand. Chromatographic separation was performed on a BDS Hypersil C18 column (250×4.6 mm, i.d. 5 μm). The isocratic mobile phase was 0.5% aqueous acetic acid solution and methanol (40:60) with the flow rate of 1.0 mL min⁻¹. The UV detector monitored at 435 nm. This method was validated for linearity, precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ). The proposed HPLC method showed acceptable validation parameters. The correlation coefficient value was 0.999, confirming the linearity of the method. The R.S.D value was lower than 2% and the average recovery of rhein was 99.12±0.33% indicated the precision and accuracy of the method, respectively. Rhein content in the fruit pulp of *C. fistula* collected from 10 different provinces in Thailand was found in the range of 17-44 mg% w/w (average 37.6 mg% w/w). This validated HPLC method was sensitive, precise and accurate for quality assessment of raw materials of *C. fistula* fruit pulp and its products.

Keywords: anthraquinone, *Cassia fistula*, Fabaceae, laxative, rhein, high performance liquid chromatography

Introduction

*Cassia fistula* Linn. (Fabaceae) is a medium-sized, deciduous tree widely grown in tropical and subtropical areas as an ornamental plant for its beautiful, bright yellow flowers. It is commonly called “Khun” or “Ratchaphruek” in Thai (Smitinand and Larsen, 1984). In Thai traditional medicines, the ripe fruit pulp has long been used as a laxative drug (Pongboonrod, 1979) (Figure 1). The major anthraquinone constituent in the fruit pulp is rhein while minor constituents are aloin and sennosides. High concentration of soluble sugars, volatile oils, waxy and resinous substances have also been found in the fruit pulp (Rizvi et al., 2009; Bahorun et al., 2005; Barthakur et al., 1995; Gritsanapan, 1983).

Anthraquinone compounds possess well-known laxative activity. The laxative effect is caused by alteration in colonic absorption and secretion, resulting in fluid accumulation and consequently, diarrhea (Special Expert Committee of the German Federal Institute for Drugs and Medical Devices, 1998; Van Gorkom et al., 1999).

Quality assessment of the herbal preparation is necessary to ensure the efficacy and safety of the raw materials. High performance liquid chromatography (HPLC) is a widely used method for both
quantitative and qualitative analysis of plant materials. For qualitative analysis, HPLC fingerprints are used to identify and determine the components present in the herbal extracts and preparations. For quantitative analysis, it is necessary to analyze the amount of active marker compounds to assess the quality of the raw material (Ying et al., 2007; Chen et al., 2011). Therefore, the aims of this study were to develop and validate HPLC method for quantitative analysis of the rhein content in the ripe fruit pulp of *C. fistula* collected from different locations in Thailand and also to perform the qualitative analysis of their HPLC fingerprints for the authentication of *C. fistula* fruit pulp.

**Materials and Methods**

**Plant Materials**

The ripe fruits of *C. fistula* were collected from 10 provinces in four regions of Thailand: the North (Chiang Mai, Nan), the South (Nakhon Si Thammarat), the Central (Pra Nakhon Si Ayutthaya, Nakhon Pathom, Bangkok) and the Northeast (Ubon Ratchathani, Amnat Charoen, Maha Sarakham, Nakhon Ratchasima) in the summer (April, 2007). They were identified by comparing to those of the herbariums (BKF. No. 118493 and BKF. No. 114900) at The Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok. The voucher specimens (WCFP001-WCFP010) were deposited at Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University. The ripe fruits were cleaned with tap water and the fresh fruit pulp (without seed) was separated and kept in a tight container at 4°C until used.

**HPLC Apparatus and Conditions**

HPLC was performed on a Shimadzu Technologies modular model Class VP system consisting of a SCL-10A system, a UV-vis SPD-10A detector, LC-10 AD and auto injector SIL-10A (Shimadzu, Japan). The analysis was carried out using a BDS Hypersil C18 column (250×4.6 mm, i.d. 5 μm) (Thermo Fisher Scientific Inc., USA) with a BDS Hypersil C18 guard column (10×4 mm, i.d. 5 μm) (Thermo Hypersil-Keystone, USA). The isocratic mobile phase was 0.5% aqueous acetic acid solution and methanol (40:60). The total running time was 30 min and a flow rate was 1.0 mL min⁻¹. The UV detector monitored at 435 nm while the injection volume was 20 μL.

**Standard Solution Preparation**

The rhein reference standard (Sigma, USA) was accurately weighed for preparing stock solution (1 mg mL⁻¹). Standard working solution of rhein was prepared by diluting the stock solution with 60% methanol in the range of 1.25-20 μg mL⁻¹.

**Sample Preparation**

Each sample of *C. fistula* fruit pulp (0.2 g) was accurately weighed, dissolved in 60% methanol and adjusted to 10 mL in a volumetric flask. Each solution was filtered through a 0.45 μm nylon membrane filter and analyzed in triplicate.

**Method Validation**

The HPLC method was validated by evaluation of linearity, precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ) according to the International Conference on Harmonization guideline (ICH, 1996).

**Linearity**

Five concentrations of rhein reference standard were prepared in 60% methanol. Each concentration was performed in triplicate. The calibration curve was obtained by plotting the peak areas from HPLC analysis of each concentration versus the concentrations of the standard. The regression equation and correlation coefficient ($r^2$) were then obtained.
Precision

Intraday precision was determined by analyzing 20 µg mL\(^{-1}\) of rhein reference standard solution (n=6) on the same day and interday precision was determined by analyzing 4, 15, 20 µg mL\(^{-1}\) of rhein reference standard solution (n=3) on 3 different days. The precision was expressed as relative standard deviation (RSD).

Accuracy

Accuracy of the method was confirmed by determining the percent recovery. The recovery of rhein was performed on sample spiked with three concentration levels of rhein reference standard solution (approximately 50%, 100% and 150% of the determined content of the C. fistula fruit pulp) (n=3).

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were calculated base on standard deviation (SD) of the response and the slope of the calibration curve.

Results and Discussion

The proposed HPLC method for analyzing the content of rhein in the fruit pulp of C. fistula showed acceptable validation parameters. The correlation coefficient (\(r^2\)) value of standard rhein was 0.999, confirming the linearity of the method. The average R.S.D. value lower than 2% and the average recovery of rhein of 99.12% indicated the precision and accuracy of the method, respectively. The LOD and LOQ were 0.112 and 0.340 µg mL\(^{-1}\), respectively indicating a high sensitivity of the method (Table 1). Rhein content in the fresh fruit pulp determined by the validated HPLC condition was in the range of 0.0170-0.0440% w/w calculated as rhein (Figure 2 and Table 2). The average content of rhein anthraquinone in the fruit pulp of C. fistula was 0.0376% w/w.

From the results, the content of rhein in the fresh fruit pulp of C. fistula from 4 regions of Thailand was in order of southern > northeastern > central > northern. The fruit pulp of C. fistula from the South contained the highest content of rhein (0.0440% w/w) while the lowest content was found in northern samples (0.0170% w/w). These results correlate to the former study of rhein content in the fruit pulp of C. fistula from different parts of Thailand determined by a TLC-densitometry (Nualkaew, 1999). This variability could be due to differences in the climate and soil condition of C. fistula plantation. The South of Thailand, a narrow peninsula proximate to the sea, has tropical monsoon climate which has high rainfall and humidity, whereas the climate in the North of Thailand is cool and mountainous. The northeastern part of Thailand consists mainly of the dry plateau and the weather is mostly dry all year, while the weather in the central part is generally hot and humid. Therefore, the South of Thailand has suitable weather and soil condition for C. fistula plantation to promote higher rhein-containing fruit pulp. However, the content of rhein in C. fistula fruit pulp was not statistically significantly different determined by one way ANOVA.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression equation(^1)</td>
<td>(Y = 138,888X - 23,774)</td>
</tr>
<tr>
<td>Correlation coefficient ((r^2))</td>
<td>0.9997</td>
</tr>
<tr>
<td>LOD (µg mL(^{-1}))</td>
<td>0.112</td>
</tr>
<tr>
<td>LOQ (µg mL(^{-1}))</td>
<td>0.340</td>
</tr>
<tr>
<td>Range (µg mL(^{-1}))</td>
<td>1.25-20.00 µg mL(^{-1})</td>
</tr>
<tr>
<td>%Recovery(^2)</td>
<td>99.12±0.33</td>
</tr>
<tr>
<td>%RSD of repeatability</td>
<td>1.47</td>
</tr>
<tr>
<td>%RSD of intermediate precision</td>
<td>1.93</td>
</tr>
</tbody>
</table>

\(^1\) X is the concentration of rhein in µg mL\(^{-1}\), Y is the peak area at 435 nm  
\(^2\) expressed as mean SD (n=3)
Table 2 Rhein content determined by the proposed HPLC method in C. fistula fruit pulp from different provinces in Thailand.

<table>
<thead>
<tr>
<th>Part</th>
<th>Province</th>
<th>Content of rhein using HPLC method(^1) (%w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>North</td>
<td>Nan</td>
<td>0.0170±0.002</td>
</tr>
<tr>
<td></td>
<td>Chiang Mai</td>
<td>0.0181±0.001</td>
</tr>
<tr>
<td>Northeast</td>
<td>Ubon Ratchathani</td>
<td>0.0308±0.002</td>
</tr>
<tr>
<td></td>
<td>Amnat Charoen</td>
<td>0.0378±0.003</td>
</tr>
<tr>
<td></td>
<td>Maha Sarakham</td>
<td>0.0384±0.003</td>
</tr>
<tr>
<td></td>
<td>Nakhon Rachasima</td>
<td>0.0271±0.002</td>
</tr>
<tr>
<td>Central</td>
<td>Prat Nakhon Si Ayutthaya</td>
<td>0.0388±0.004</td>
</tr>
<tr>
<td></td>
<td>Nakhon Pathom</td>
<td>0.0175±0.002</td>
</tr>
<tr>
<td></td>
<td>Bangkok</td>
<td>0.0381±0.003</td>
</tr>
<tr>
<td>South</td>
<td>Nakhon Si Thammarat</td>
<td>0.0440±0.003</td>
</tr>
<tr>
<td></td>
<td>average</td>
<td>0.0376±0.010</td>
</tr>
</tbody>
</table>

\(^1\) expressed as mean SD (n=3).

According to Thai traditional usage of C. fistula (Pongboonrod, 1979), the laxative pill was obtained by boiling the fresh fruit pulp with water, filtered through muslin cloth then evaporated to yield soft extract and made as laxative pills. The recommended dose is 4-8 g per day of fresh fruit pulp equivalent to 1.5-2.2 mg of rhein. Since the fruit pulp of C. fistula has long been used as a safe traditional laxative drug and it is abundantly found as wastes in all parts of Thailand and many other countries, it would be economically and pharmaceutically beneficial to develop laxative preparations from the standardized fruit pulp of C. fistula, such Senna’s products. This information would be useful as a guideline for quality control of C. fistula fruit pulp to be used as a herbal laxative raw material.

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References


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