HPLC method validation for determination of brazilin in *Caesalpinia sappan* L. heartwood extract

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**Introduction**

*Caesalpinia sappan* L. is a herbal medicine found in the Ayurveda and Chinese tradition medicines that have been used for a long time \(^1\). *C. sappan* is a plant in Leguminosae family, called Brazil wood or Sappan wood. There are several chemical constituents found in sappan wood such as phenolic components including xanthone, coumarin, chalcones, flavones, homoisoflavonoids, and brazilin. Brazilin (Figure 1) is the major compound in the *C. sappan* heartwood and is used as a red dye for staining in histological application \(^2\). Brazilin is reported to possess many activities i.e. antibacterial, anti-inflammatory, anti-photo aging, hypoglycemic, vasorelaxant, anti-allergic, anti-acne, antioxidant, and anti-nuclease activity \(^3\). In Thailand, it is mostly used *C. sappans* L. as a food, garment, cosmetic and colorant in refreshment \(^4\). The north of Thailand, especially in Chiang Mai, Nan and Lampang, decoction of *C. sappan* heartwood is used as an anti-inflammation for the treatment of traumatic disease and arthritis \(^5\). The screening of bioactive compounds from the herbal extract is also important to drug development. Furthermore, the determination of active compound is an important step in drug development. Therefore, the present study aimed to validate an HPLC method to determination of brazilin in *C. sappan* L. heartwood extract.

![Figure 1. Structure of brazilin](image-url)
Methods

Plant extraction

*C. sappan* heartwood was obtained from Nakhon Pathom provinces, Thailand. It was examined according to their botanical characteristics to identify the right species. *C. sappan* heartwood was pulverized and sieved. Then, it was extracted using boiling water. The extract was filtered through a Whatman No. 1 filter paper and freeze dried. The sample was kept in a bottle and stored at -20°C until use.

Preparation of standard brazilin

Stock solution of brazilin was dissolved in ultrapure water at a concentration of 1 mg/mL. The solution standard was to obtain a series ranging from 5-80 µg/ml to construct a calibration curve.

Chromatographic condition

The study was performed using an HPLC instrument (Agilent 1260 infinity) with photodiode array detector. The ZORBAX Eclipse XDB-C18 (250 x 4.6 mm, i.d., 5 µm) with an analytical guard column (12.5 x 4.6 mm, i.d., 5 µm) were used. The 10 µl injection volumes, 1 ml/min flow rate, and 25°C column temperature at were used. The quantitation wavelength was set at 280 nm. The mobile phase composed of 0.3% acetic acid in water and acetonitrile. The chromatographic separation was achieved using a gradient system (Table 1).

Table 1. The gradient system used in chromatographic separation

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0.3% Acetic acid</th>
<th>Acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>85.5</td>
<td>14.5</td>
</tr>
<tr>
<td>12</td>
<td>85.5</td>
<td>14.5</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>18</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>19</td>
<td>85.5</td>
<td>14.5</td>
</tr>
<tr>
<td>25</td>
<td>85.5</td>
<td>14.5</td>
</tr>
</tbody>
</table>

Method validation

Method validation was performed following to the ICH Harmonised Tripartite Guideline ⑥. The method was validated in five topics i.e. linearity and range, specificity, limit of detection (LOD) and limit of quantitation (LOQ), precision, and accuracy.

Linearity and range

The standard solution was studied at six concentrations, which was diluted with ultrapure water into 5, 10, 20, 40, 60, and 80 µg/mL. Each solution was injected in and the linearity was reported by the coefficient of determination ($R^2$), linear equation, and range.

Specificity

Specificity was obtained from the UV spectra of up-slope and down-slope of the interest peak. Specificity was achieved when similar UV spectra obtained.

LOD and LOQ

LOD and LOQ were determined using the calibration curve follow Eq. 1 and 2.

\[
LOD = \frac{3.3 \times \sigma}{S} \quad (1)
\]

\[
LOQ = \frac{10 \times \sigma}{S} \quad (2)
\]

\[\sigma = \text{the standard deviation of y-intercepts of the regression line}\]

\[S = \text{the slope of the calibration curve}\]
**Precision**

Three standard solution levels of brazilin; 10, 20, and 60 µg/ml were used for the precision study. Each concentration was determined in triplicate. The percent relative standard of an analysis on the same day and different three days were reported as intraday and interday precision, respectively.

**Accuracy**

The accuracy was assayed by spike method. Three standard solution levels of brazilin; 10, 20, and 60 µg/ml were added to the known amount of *C. sappan* extract. The percent recovery was reported.

**Results and Discussion**

Chromatographic system was validated. Brazilin and *C. sappan* extract were eluted at 8.7 min. The HPLC chromatogram of brazilin standard and *C. sappan* extract are shown in Figure 2.

![HPLC chromatogram](image)

Figure 2. HPLC chromatogram of (a) 20 µg/ml brazilin and (b) 1 mg/ml *C. sappan* extract

![Calibration curve](image)

Figure 3. Calibration curve of brazilin
The linear equation for brazilin was $= 19109X - 10821$ ($R^2 = 0.9997$) in the concentration range of 5-80 µg/ml, which shown in Figure 3. The up-slope and down-slope of the brazilin peak in *C. sappan* extract was similar. This result indicated that the method was specific. The LOD and LOQ were 1.30 and 3.94 µg/ml, respectively. The precision was evaluated at three different concentration. Intraday and interday precision had %RSD less than 2% and 5%, respectively. The accuracy presented as percent recovery was 99.29-104.06%. Precision and accuracy results are shown in Table 2. This validated method was used to analysis of brazilin content in *C. sappan* heartwood extract. The result showed that brazilin content was 6.10±0.38%.

### Table 2. Precision and accuracy results

<table>
<thead>
<tr>
<th>Conc. (µg/mL)</th>
<th>Precision ( %RSD)</th>
<th>Spike amount (µg/mL)</th>
<th>Accuracy Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intraday</td>
<td>Inter-day</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.56</td>
<td>3.88</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>0.33</td>
<td>4.02</td>
<td>20</td>
</tr>
<tr>
<td>60</td>
<td>0.20</td>
<td>2.70</td>
<td>60</td>
</tr>
</tbody>
</table>

**Conclusion**

This HPLC method showed good linearity, specific, precise, and accurate. The brazilin content in *C. sappan* heartwood extract was 6.10±0.38%. This HPLC method could be used for determination of brazilin in *C. sappan* heartwood extract.

**Acknowledgements**

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**References**