Determination of Rubraxanthone in the Latex of Asam Kandis (Garcinia cowa Roxb) by Reverse Phase High Performance Liquid Chromatography.

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ABSTRACT

Context: Rubraxanthone is a major compound found in Garcinia cowa Roxb which has various biological activities. This compound is likely to be responsible for the pharmacological activities of this plant. The latex of this plant was one of the source of this compound. To prevent counterfeiting, it is essential to develop a method of analysis to determine the levels of these compounds in the latex of G. cowa. Aims: To develop and validate a reverse phase-high performance liquid chromatography (RP-HPLC) technique for determination of rubraxanthone in the latex of G. cowa. Settings and Design: RP-HPLC analysis. Methods and Material: The sample was powdered and dissolve in methanol and then subjected to Reverse Phase High Performance Liquid Chromatography (RP-HPLC). Separation was carried out in a reversed-phase column Shimadzu Shimp-pack VP–ODS (4.6 x 250 mm). The elution was performed with isocratic solvent using formic acid 0.4 % v/v in methanol (15:85) with a flow rate 1 ml/minute. The solvents used for the mobile phase were filtered through membrane filter (0.45 mm pore size) and degassed before use. Total running time was 20 minutes and the sample injection volume of injection was 20 μl. While the wavelength of the UV-VIS detector was set at 243.2 nm. Results: The detection and the quantitation limits of rubraxanthone were 1.119 mg/mL and 3.731 μg/mL, respectively. A regression analysis was performed, with the observation of good linearity (r = 0.998). The values obtained for precision and accuracy determination are in agreement with ICH guidelines. It was found that rubraxanthone in dichloromethane extract of latex G. cowa was 56.56%. Conclusions: The results demonstrated that the developed method is a reliable HPLC technique for determination of rubraxanthone in the latex of G. cowa.

Key words: Latex of Garcinia cowa Roxb, Rubraxanthone, RP-HPLC, Standarization, Counterfeiting, Isocratic method.

Key Messages: The developed RP-HPLC analysis was found to be reliable method for determination of rubraxanthone in the latex of G. cowa. It was found that concentration of rubraxanthone in dichloromethane extract of the latex was 56.56%.

INTRODUCTION

Garcinia is a medicinal plant that belongs to the family guttiferae which has been widely used and traded by the Asian community as a traditional medicine to treat various diseases such as diarrhea, skin infections, wounds and as an anti-septic. One of the plants of this family which has been widely used as a traditional medicine is Garcina cowa.1 Various components of G. cowa have been used in ancient medication. The bark, latex and root have been used as an antipyretic agent, while fruit and leaves have been used as an expectorant, for indigestion and improvement of blood circulation.2 The extract of this plant was reported to have various biological activity such as cytotoxicity,3-4 antiinflammatory.5 Rubraxanthone (Figure 1) has been isolated as major component of the stem bark of G. cowa.5,6,7 This compound has antimicrobial8 and antioxidant activity.7 It also has anticancer activity against MCF-7, DU-145 and H-460 as well as can reduce levels of total cholesterol and triglyceride levels in the blood of male rats.9 This compound was also reported had anti inflammatory10 and anti platelet activity.11 The latex of this plant has been used for traditional medicine for centuries and also was one of the source of rubraxanthone. In order to maintain the quality and the quality and prevent counterfeiting, it is essential to develop a method of analysis to determine the levels of these compounds in the latex of G.cowa. The aim of this study was to develop and validated HPLC method qualitative analysis and to quantify the amount of rubraxanthone in the latex of G. cowa. This system is the first time validated HPLC method has been established for quantitative and qualitative analysis of rubraxanthone in the latex of G. cowa. Determining the amount of rubraxanthone in the latex of G. cowa has not been reported before.

MATERIALS AND METHODS

Chemicals

Standard rubraxanthone was isolated from G. cowa.8 Pure isolated rubraxanthone was used as the external standard and then dissolved in methanol. Aqueous solutions of rubraxanthone were prepared daily by weight.


standards. All reagents and solvents were analytical and HPLC grades (Merck), except formic acid. High purity bidistilled water was obtained from Otsuka Pharmaceutical Company. The mobile phase was filtered through membrane filter (0.45 μm pore size) degassed before use.

**Instrumentation condition**

HPLC method was performed on a Shimadzu (Kyoto, Japan) liquid Chromatograph system, equipped with a model LC-20 AD pump, UV-VisSPD M-20A Diode detector. Separation was carried out in a reversed-phase column Shimadzu .Shimp-pack VP–ODS (4.6 x 250mm). The elution was performed with isocratic solvent using formic acid 0.4 % v/v in methanol (15:85) with a flow rate 1 ml/minute. The solvents used for the mobile phase were filtered through membrane filter (0.45 mm pore size) and degassed before use. Total running time was 20 minutes and the sample injection volume of injection was 20 ml. While the wavelength of the UV-VIS detector was set at 243.2 nm. The compound was quantified using CLASS VP

**Plant material**

The latex of G. cowa was collected at palupua, Agam, West Sumatra in March 2016. The latex (250 g) was dried in the oven at 50°C for 72 hours. The dried latex was grinded to become a powder.

Preparation of standard solutions and sample solutions

A stock solution of rubraxanthone reference standard was ready by dissolving an accurately weighed 10.1 mg of rubraxanthone in 10mL of methanol in a volumetric flask. The standard solution was diluted to obtain final concentrations at 10.1 μg/mL, 15.15 μg/mL, 20.2 μg/mL, 25.25 μg/mL and 30.3 μg/mL with methanol. 10.9 g dichloromethane soluble extract of the dried latex was dissolved in 100 mL methanol in a volumetric flask. Prior to analysis, the solutions were filtered through 0.45μm membrane filters.

**Quantitative analysis of rubraxanthone content**

Analysis was carried out by HPLC under the same condition as the proposed method. Rubraxanthone in the extract was calculated using its calibration curve with regarding to the dilution factor. The content of rubraxanthone in the extract was expressed as gram per 100g of the extract. Each experiment was carried out in triplicate.

**Validation of the method**

Validation of the analytical method was done according to the International Conference on Harmonization guideline. The method was validated for linearity, precision, and accuracy, limit of detection (LOD) and limit of quantitation (LOQ).

**Linearity**

Linearity was determined by using rubraxanthone standard solution to give concentration of 10.1 μg/mL, 15.15 μg/mL, 20.2 μg/mL, 25.25 μg/mL and 30.3 μg/mL in methanol, each of the standard solution was prepared (n=3). A calibration curve was made by plotting the area obtained from the analysis of the concentration of the standard solution using the linear regression equation. Linearity is determined by the price of coefficient correlation (r).

**Pression and acuration**

Precision was determined by analyzing three different concentrations (10.1; 20.2; 30.3 mg/mL) of standard solution rubraxanthone on the same day as much as 3 times reiteration for intraday precision and at three different concentrations (10.1; 20.2; 30.3 mg/mL) each reiteration 3 times per day for 3 days for interday precision. Intra- and inter-day assay precisions were determined as relative standard deviation (RSD). Intra-day precision (repeatability) involved three replicates per day and inter-day (intermediate) precision were performed on three separate days. Accuracy views of proximity compared with actual results.

**Limit of detection (LOD) and limit of quantification (LOQ)**

According to ICH 1995, technical needs for the registration of prescribe drugs for human use recommendations, the approach based on SD of the response and also the slope was used for determining the detection and quantitation limits. The limits of detection and quantitation can be calculated statistically by linear regression line of the calibration curve. The measurement value will be equal to the value of b in the linear equation $y = a + bx$

$LOD = 3 \times \sigma /S$

$LOQ = 10 \times \sigma /S$

Where: $\sigma = $ standard deviation ; $S = slope$ or linear line direction

**Recovery study**

The accuracy of this method was tested by performing recovery studies at three levels of standard rubraxanthone were added to the sample. Three different concentrations (10.1; 20.2; 30.3 mg / mL) of standard solution are added to the sample solution (21.8 mg / mL), which has been known to rubraxanthone levels in it and analyzed using HPLC method. Do three repetitions for each level of concentration. Then calculate the return of the acquisition.

**Determination of concentration of rubraxanthone by HPLC method**

The dichloromethane extract of dried latex at a concentration of 21.8 mg/mL was injected into 20 mL HPLC instrument with the same conditions as that obtained when testing the suitability of the system with three times repetition. Rubraxanthone levels in the extract was calculated using a calibration curve which has been obtained previously.

**RESULTS**

A reverse phase HPLC method was developed keeping in mind the system suitability parameters i.e. tailing factor (T), number of theoretical plates (N), runtime and the cost effectiveness (Table 1). The most optimal mobile phase for analysis rubraxanthone was methanol: 0.4% formic acid (85:15) with a flow rate of 1 mL/min. The optimized method developed resulted in the elution of rubraxanthone at 15.417. Figure 2 showed chromatogram of dichloromethane extract of the latex of G. cowa. The total run time is 20 minutes.

The described method was validated according to the ICH guidelines. The following validation characteristics were evaluated: linearity, precision, accuracy and the limits of detection and quantification (LOD and LOQ).

The linearity study by using a standard solution concentration of 5 series rubraxanthone gave regression equation $y = x$ 103 214 - 527 433 and the value of the correlation coefficient (R) = 0.998 [Table 2].

Based on the standard curve equation LOD and LOQ values obtained on the analytical methods used amounted to 1.119 mg / mL and 3.731 mg / mL [Table 3].

The repeatability and intermediate precision were 3.03% and 2.84%, respectively. The mean of peak area, rubraxanthone content and the relative standard deviation are reported in Table 4.

The accuracy of the method was determined by adding known amount of rubraxanthone standard in known latex of G. cowa extract at three levels of concentration. The mean values of the percentage analytical re-
Table 1: System suitability data of the proposed method

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Rubraxanthone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (min)</td>
<td>15.417</td>
</tr>
<tr>
<td>Resolution</td>
<td>1.558</td>
</tr>
<tr>
<td>Number of theoretical plates (N)</td>
<td>441.772</td>
</tr>
<tr>
<td>Tailing factor (T)</td>
<td>1.454</td>
</tr>
<tr>
<td>Height Equivalent to a Theoretical Plate (HETP)</td>
<td>0.566</td>
</tr>
</tbody>
</table>

*Means of three injections

Table 2: Linearity of Rubraxanthone in the RP-HPLC analysis

<table>
<thead>
<tr>
<th>Concentration range (μg/mL)</th>
<th>Slope</th>
<th>Intercept</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubraxanthone</td>
<td>103214</td>
<td>527433</td>
<td>0.998</td>
</tr>
</tbody>
</table>

Table 3: Limit of Detection (LOD) and Limit of Quantification (LOQ)

<table>
<thead>
<tr>
<th>Concentration range (μg/mL)</th>
<th>Slope</th>
<th>Standard deviation of y-intercept</th>
<th>LOD (μg/mL)</th>
<th>LOQ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubraxanthone</td>
<td>103214</td>
<td>38509.350</td>
<td>1,119</td>
<td>3,731</td>
</tr>
</tbody>
</table>

Table 4: Precision of HPLC method for quantitation of rubraxanthone in the latex of G. cowa Roxb

<table>
<thead>
<tr>
<th>Peak area</th>
<th>RSD (%)</th>
<th>Rubraxanthone (μg/mL)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeatability</td>
<td>1902049.67±0.425</td>
<td>1.416</td>
<td>29.997</td>
</tr>
<tr>
<td>Intermediate precision</td>
<td>2332417.78±0.590</td>
<td>1.926</td>
<td>30.610</td>
</tr>
</tbody>
</table>

Table 5: Accuracy of the HPLC method

<table>
<thead>
<tr>
<th>Rubraxanthone added (µg/mL)</th>
<th>Rubraxanthone found (µg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5.05</td>
<td>4.78</td>
</tr>
<tr>
<td>B</td>
<td>10.10</td>
<td>9.65</td>
</tr>
<tr>
<td>C</td>
<td>15.15</td>
<td>14.71</td>
</tr>
</tbody>
</table>

Figure 2: Typical chromatogram of dichloromethane extract of the latex of G. cowa with mobile phase methanol: formic acid 0.4% (85:15) with flow rate 1 mL/min.

coveries for the concentration of 5.05, 10.10 and 15.15 μg/mL of rubraxanthone were 94.66%, 95.51% and 97.12%, respectively Table 5.

**DISCUSSION**

HPLC method with isocratic elution was developed for the quantification of rubraxanthone in soluble dichloromethane extracts of latex of G. cowa. The chromatographic analysis developed for quantitative determination of this xanthone was validated according to ICH guidelines to obtain reproducible analyses with high degree of accuracy and precision. A regression analysis was performed, with the observation of good linearity in a wide concentration range. The values obtained for precision and accuracy determination are in agreement with ICH guidelines, which indicated that the chromatographic conditions used are reliable to quantify the xanthone. From the test results obtained by the average level of rubraxanthone in plant extract G.cowa is 56.56%.

**CONCLUSION**

Concentration of rubraxanthone in dichloromethane extract of the latex of kandis acid G. cowa was 56.56%. This exudate can be used as source of rubraxanthone.

**ACKNOWLEDGMENTS**

This research was funded by Faculty of Pharmacy, Andalas University no 23/UN.16/HKRGB/LPPM/2016.

**CONFLICT OF INTERESTS**

The authors have no conflict of interest.

**ABBREVIATIONS USED**

PLC: performance liquid chromatography; ICH: International Conference on Harmonization; LOD: limit of detection; LOQ: limit of quantitation; RSD: relative standard deviation; HETP: Height Equivalent to a Theoretical Plate; RP-HPLC: Reverse Phase High Performance Liquid Chromatography; ICH: International Conference on Harmonization; LOD: limit of detection; LOQ: limit of quantitation; RSD: relative standard deviation; HETP: Height Equivalent to a Theoretical Plate.

**REFERENCES**

3. Wahyuni FS, Triastuti DH, Arifin H. Cytotoxicity Study of Ethanol Extract of the


