

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 validated 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 ml. 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%.

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INTRODUCTION

Garcinia is a medicinal plant that belongs to the family guttiferæ 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 *Garcinia 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.¹ The extract of this plant was reported to have various biological activity such as cytotoxicity,²⁻⁴ antiinflammatory.²

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.⁹ It also has anticancer activity against MCF-7, DU-145 and H-460⁶ as well as can reduce levels of total cholesterol and triglyceride levels in the blood of male

rats.¹⁰ This compound was also reported had anti-inflammatory¹¹ and anti platelet activity.¹²

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 o 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 of RP-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*.⁶ Pure isolated rubraxanthone was used as the external

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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 palupuah, 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.^{13,14} 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).

Precision and acuration

Precision was determined by analyzing three different concentrations (10.1; 20.2; 30.3 mg/mL) of standard solution rubraxanthon on the same day as much as 3 times reinjection for intraday precision and at three different concentrations (10.1; 20.2; 30.3 mg/mL) each reinjection 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 1995,¹³ 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 $y = a + bx$

$$\text{LOD} = 3 \times \sigma / S$$

$$\text{LOQ} = 10 \times \sigma / S$$

Where: σ = 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 rubraxanthon 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. Rubraxanthon 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.⁹ 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

Parameters	Rubraxanthone
Retention time (min)	15.417
Resolution	1.558
Number of theoretical plates (N)	441.772
Tailing factor (T)	1.454
Height Equivalent to a Theoretical Plate (HETP)	0.566
*Means of three injections	

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

	Concentration range ($\mu\text{g/mL}$)	Slope	Intercept	r
Rubraxanthone	10.1-30.3	103214	527433	0.998

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

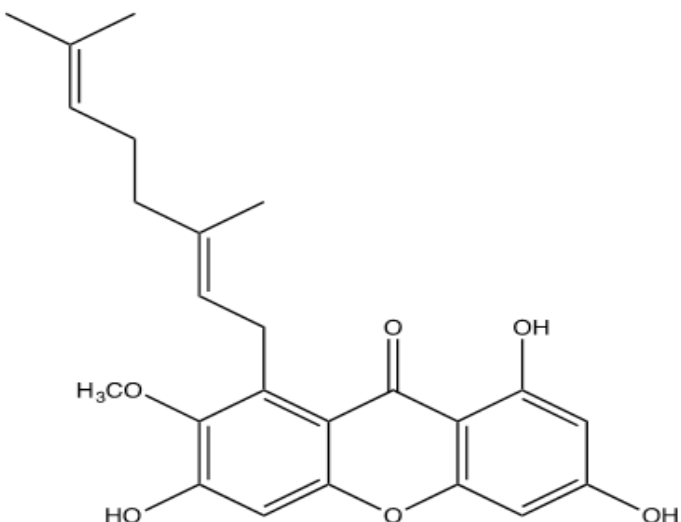
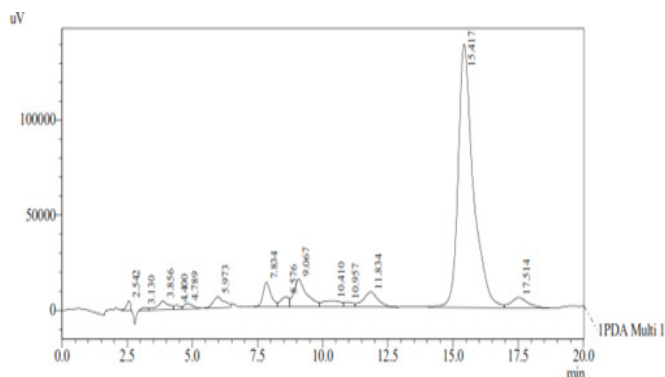
	Concentration range ($\mu\text{g/mL}$)	Slope	Standard deviation of y-intercept	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
Rubraxanthone	10.1-30.3	103214	38509,350	1,119	3,731

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

	Peak area	RSD (%)	Rubraxanthone ($\mu\text{g/mL}$)	RSD (%)
Repeatability ^a	1902049.67 \pm 0.425	1.416	29.997	1.402
Intermediate precision	2332417.78 \pm 0.590	1.926	30.610	1.947

Table 5: Accuracy of the HPLC method

	Rubraxanthone added ($\mu\text{g/mL}$)	Rubraxanthone found ($\mu\text{g/mL}$)	Recovery (%)
A	5.05	4.78	94.66
B	10.10	9.65	95.51
C	15.15	14.71	97.12

**Figure 1: Rubraxanthone****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 $\mu\text{g/mL}$ 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.¹³

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,¹³ 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

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CONFLICT OF INTERESTS

The authors have no conflict of interest

ABBREVIATIONS USED

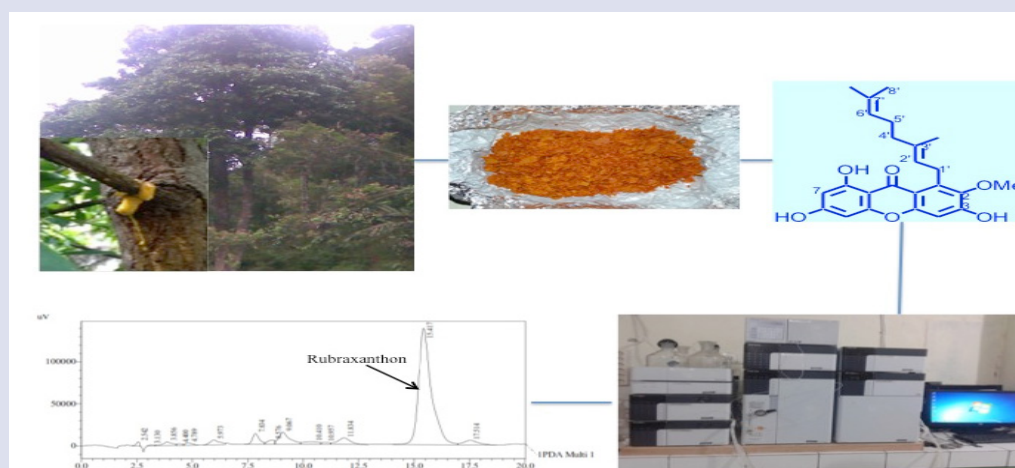
PLC: performance liquid chromatography; ICH: International Conference on Harmonization; LOD: limit of detection; LOQ: limit of quantitation; RSD: relative standard deviation; HETP: Height Equivalent to a Theoretical Plate; RP-HPLC: Reverse Phase High Performance Liquid Chromatography; ICH: International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

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PICTORIAL ABSTRACT



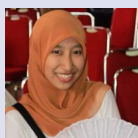
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Dachriyanus: Got his undergraduate degrees from Andalas University in 1991 and finished my PhD from University of Western Australia in 1999. He is positioned as Professor of Pharmacy at Faculty of Pharmacy, Andalas University. His research is in chemical and biological activity studies of Sumatran Plants especially Genus *Garcinia* and *Rhodomlyrtus tomentosa*.



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