

DEVELOPMENT AND VALIDATION OF A HPLC METHOD FOR DETERMINATION AND QUANTIFICATION OF RUBRAXANTHONE IN STEM BARK EXTRACT OF MANGOSTEEN

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ABSTRACT

Rubraxanthone is one of antibacterial and antioxidant compound which is isolated from stem bark of mangosteen (*Garcinia mangostana* Linn.). The precise method for analysis of plant constituents is normally a reverse-phase high performance liquid chromatographic (RP-HPLC). Development and validation of a RP-HPLC method for chromatographic separation was carried out on a reversed-phase column Shimadzu® Shimp-pack VP – ODS (4.6x250mm) using an isocratic mobile phase of 0.4% formic acid - acetonitrile at a flow rate 1mL min⁻¹, and detection was with a UV detector. The linearity of the proposed method was found in the range of 2.5-25µg/mL⁻¹ with regression coefficient 0.999. Intraday precision studies showed the relative standard deviation ≤1.58% and inter-day ≤3.20%. Accuracy of the method was determined by a recovery study conducted at 3 different levels, and the average recovery was 102.18%. The Limit of Detection Limit of Quantitation (LOQ) and were 0.47 and 1.56µg/mL⁻¹. The contents of rubraxanthone in the crude was 0.23±0.07%w/w.

Key words: High performance liquid chromatography, Rubraxanthone

INTRODUCTION

Garcinia mangostana Linn. Guttiferae, known as “queen of fruits” is cultivated in the tropical rainforest of some Southeast Asian nations like Indonesia, Malaysia, Sri Lanka, Philippines, and Thailand. The bark of *G. mangostana* is one of the sources for the extraction of rubraxanthone. Rubraxanthone is one class of xanthone compounds that have pharmacology activity as antibacterial agent, where these compounds have been shown to inhibit the growth of *Staphylococcus aureus* (Imuna, 1996), *Trichophyton mentagrophytes*, and *Microsporum gypseum*, *Staphylococcus epidermidis* (Pattalung, 1988), *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Helicobacter pylori* (Dachriyanus, 2003). In addition, rubraxanthone also been reported as antiplatelet (Jantan, 2002), and an antioxidant (Dachriyanus, 2003).

It is well accepted that for evaluating the quality of herbal medicines, it is necessary to standardize the content of the active principles. Standardization and analysis of the active principles of the herbal medicine is always difficult. Quantitative determination of active

principles in the herbal medicine preparation required optimal separation techniques by which these compounds are separated with the highest resolution and the least interferences from each other. Herbal medicine has been enjoying renaissance among the customers throughout the world. However, one of the impediments in the acceptance of herbal products worldwide is the lack of standard quality control profiles (Shinde, 2009).

Chemical and chromatographic techniques may be used to aid in the identification of an herbal material or extract. Chromatographic techniques such as HPLC, TLC, GC and capillary electrophoresis and spectroscopic methods such as IR, NMR and UV may also be used for fingerprinting (Patra, 2010). In order to control the quality of herbal drugs in a better way, we must develop new techniques and terms to the maximum extent. The development and validation of an efficient analytical method is an integral part of the quality control of the source material, to guarantee the safety and effectiveness of the resulting compound (Hefnawy, 2006).

Thus, considering the pharmacological potential of bark extract of *G. mangostana* and the lack of specifications for the quality control of this plant raw material, which is a prerequisite for the production and registration of phytomedicines. The objective of the present study was to develop and validate a method for the separation and quantitative analysis of rubraxanthone by RP-HPLC. Furthermore, no previous study described for the analysis of Rubraxanthone in stem bark extract of *G. mangostana*. So, it will provide the scientific basis for the quality control of extracts prepared from the bark of this plant.

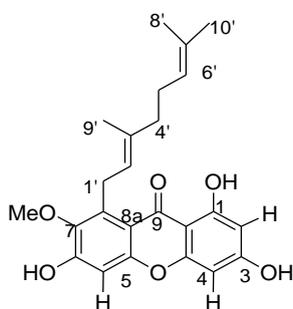


Figure 1. Rubraxanthone.

There was no rubraxanthone available for standard compound, therefore in this study, rubraxanthone (Figure 1) was isolated from methanol extract of bark of *G. coma* using chromatographic techniques (column chromatography, TLC) and its structure was confirmed on the basis of spectral data.

MATERIALS AND METHODS

Chemical and reagents

All reagents and solvents were analytical and HPLC grades (merck), except formic acid. Pure isolated Rubraxanthone were used as the external standards.

Plant materials

Stem bark of *G. mangostana* was collected from Lubuk Alung, West Sumatra. The plants sample were identified by taxonomist from Herbarium ANDA Andalas University. The bark were cut into small pieces and dried in a hot oven at 50°C for 72h. The dried samples were ground into powder and passed through a sieve (20 meshes).

Isolation of rubraxanthone

Ground air-dried stem bark (2.2kg) was percolated with methanol (10L). The extract was evaporated to dryness under reduced pressure and the dark mass (113.54g) was extracted in turn with hexane (4x100mL) which on evaporation yielded a gum (5.7g), ethyl acetate (11x600mL), which on evaporation yielded a gum (43.0g) and butanol (8x150mL) which on evaporation yielded a gum (45.0g).

The ethyl acetate fraction (40g) was then subjected to column chromatography (5.0x80cm). Column chromatography (CC) was carried out using silica gel Merck 7734 and successively eluted with hexane followed by hexane : EtOAc mixture in step gradient polarity, and finally washed with methanol to give 50 fractions (50mL each) designated as fraction A (1-6, 0.25g), B (7-14, 0.80g), C (15-28, 12.5g), D (29-39, 2g), E (40-50, 11g). Fraction C was further fractionated by VLC, eluting with hexane, mixture of hexane and EtOAc in step gradient polarity manner, and finally with MeOH to afford four fractions, (C_A-C_D). fraction C_B (4g) was separated by silica gel CC, eluting with hexane-EtOAc, 9:1 to give six sub-fractions, C_{B1}-C_{B6}.

Separation of sub-fraction fraction C_{B4} (800mg) was performed by CC over silica gel, eluting with hexane-EtOAc, 9:1 to afford unpurified rubraxanthone (445mg). Purifying this fraction by recrystallisation with EtOAc and hexane, yielded 250 mg of rubraxanthone. Fraction CC (1g) was chromatographed on silica gel column (2.5x15cm) eluted with hexane and EtOAc mixture in step gradient polarity manner. The combined fractions 15-23 (450mg) was again rechromatographed on silica gel column (1.5x45) and eluted with n hexane and EtOAc mixture in step gradient polarity to give fourteen 15mL fractions of which fraction 2-8 were combined and gave 145mg of yellow solid product. Purifying this product by recrystallisation with EtOAc and hexane yielded rubraxanthone (93 mg) as yellow needles, m.p. 207-209°C. UV MeOH λ_{max} (log ϵ)nm: 203 (4.54), 241 (4.50), 312 (4.32), IR ν_{max} (KBr)cm⁻¹: 3425, 3240, 2965, 2855, 1641, 1162, 827; EIMS: m/z 410 [M]⁺ (23%), 341(100), 299(25), 69(33). High-resolution EIMS found: 410.1727 (calculated for C₂₄H₂₆O₆, m/z 410.1729).

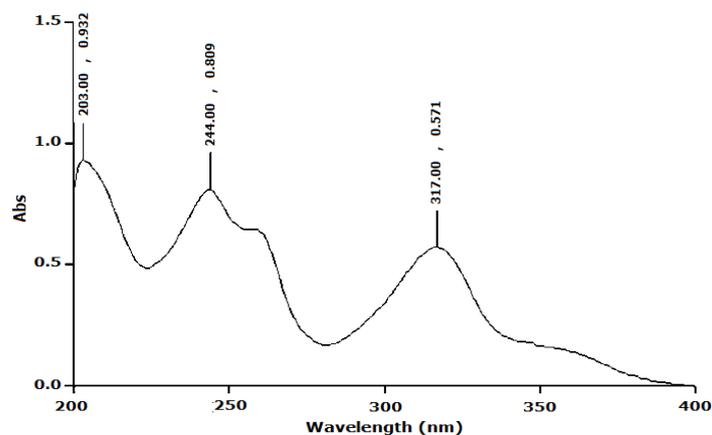


Figure 2. UV spectrum of rubraxanthone in MeOH

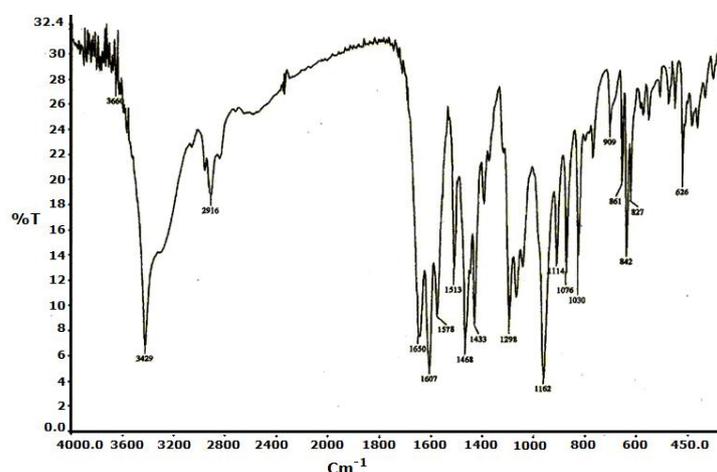


Figure 3. FTIR spectrum of rubraxanthone in KBr plate

^1H NMR (500 MHz, CD_3OD): δ 6.72 (1H, *s*, H-5), 6.21 (1H, *d*, $J=2.0$ Hz, H-4), 6.18 (1H, *d*, $J=2.0$ Hz, H-2), 5.20 (1H, *t*, $J=7.0$ Hz, H-6'), 5.01 (1H, *t*, $J=7.0$ Hz, H-2'), 4.07 (2H, *d*, $J=7.0$ Hz, H-1'), 3.4 (3H, *s*, 7-OMe), 2.05 (2H, *t*, $J=7.0$ Hz, H-5'), 1.98 (2H, *t*, $J=7.0$ Hz, H-4'), 1.81 (3H, *s*, H-8'), 1.55 (3H, *s*, H-10'), 1.52 (3H, *s*, H-9').

^{13}C NMR (125 MHz, CD_3OD): 182.00 (C-9), 164.8 (C-1), 163.6 (C-3), 157.3 (C-6), 156.9 (C-5a), 155.7 (C-4a), 143.8 (C-7), 137.5 (C-8), 134.3 (C-3'), 130.8 (C-7'), 124.3 (C-2'), 124.0 (C-6'), 111.0 (C-8a), 102.8 (C-9a), 101.7 (C-5), 97.6 (C-2), 92.9 (C-4), 60.2 (7-OMe), 39.6 (C-4'), 26.4 (C-5'), 25.8 (C-1'), 24.6 (C-9'), 16.5 (C-10'), 15.5 (C-8').

Instrumentation and chromatographic condition

HPLC method was performed on a Shimadzu (Kyoto, Japan) liquid chromatograph system, equipped with a model LC-20 AD pump, UV-Vis SPD M-20A Diode detector. Separation was performed in a reversed-phase column Shimadzu® Shimp-pack VP-ODS (4.6x250mm). The elution was carried out with isocratic solvent using 0.4% v/v formic acid - acetonitrile with a flow rate 1 mL min^{-1} . The mobile phase was prepared daily, filtered through a $0.45\mu\text{m}$ and sonicated before use. Total running time was 20min and the sample injection volume was $10\mu\text{L}$ while the wavelength of the UV-VIS detector was set at 243nm. The compound was quantified using CLASS VP software.

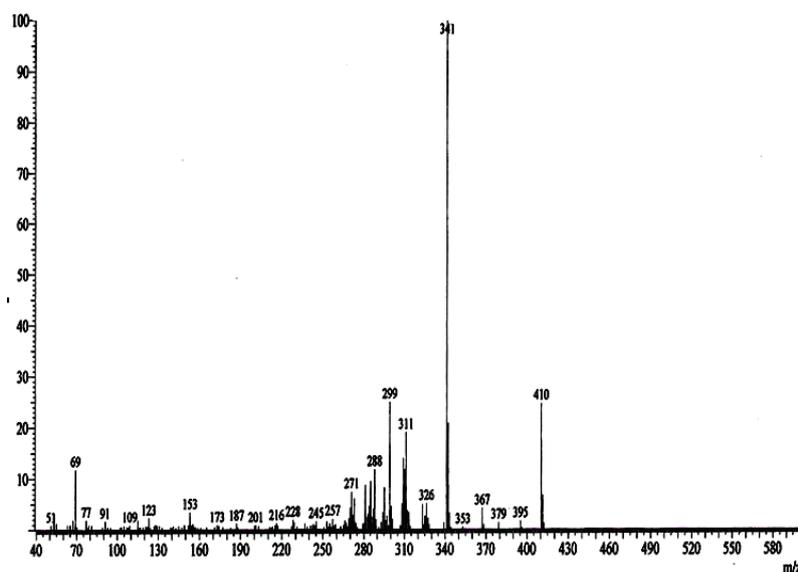


Figure 4. EI-MS spectrum of rubraxanthone

Preparation of standard solutions

A stock solution of rubraxanthone reference standard was prepared by dissolving an accurately weighed 10mg of rubraxanthone in 10mL of methanol in a volumetric flask. Various concentrations of the standard solution were diluted to obtain final concentrations at 2.5: 5: 10: 12.5: and 25 $\mu\text{g}/\text{mL}^{-1}$ with methanol.

Preparation of sample solutions

The powder bark of *G. mangostana* (100g) was separately placed into a thimble and was extracted with 1000mL of 95% ethanol at room temperature for 18h. Each extract was filtered through a Whatman no. 1 filter paper by suction. The filtrate was concentrated under reduced pressure at 50°C using a rotary vacuum evaporator. The final weight of the crude extract was weighed and calculated for the yield. The extraction of each sample was done in triplicate.

Each dried extract (10mg) was accurately weighed and transferred to a 10mL volumetric flask. Methanol was added to volume (final concentration 1.000 $\mu\text{g}/\text{mL}^{-1}$). Aliquot of the solution (2.5mL) was diluted with methanol in a 10mL volumetric flask to make a concentration of 250 $\mu\text{g}/\text{mL}^{-1}$. Prior to analysis, the solutions were filtered through 0.45 μm membrane filters.

Quantitative analysis of rubraxanthone content

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

Validation of the method

Validation of the analytical method was done according to the International Conference on Harmonization guideline (ICH, 1995). 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 in the concentration range were of 2.5–25 $\mu\text{g}/\text{mL}^{-1}$ in methanol, each of the standard solution was prepared (n=3). The calibration curves were obtained by plotting the peak area versus the concentration of the standard solutions.

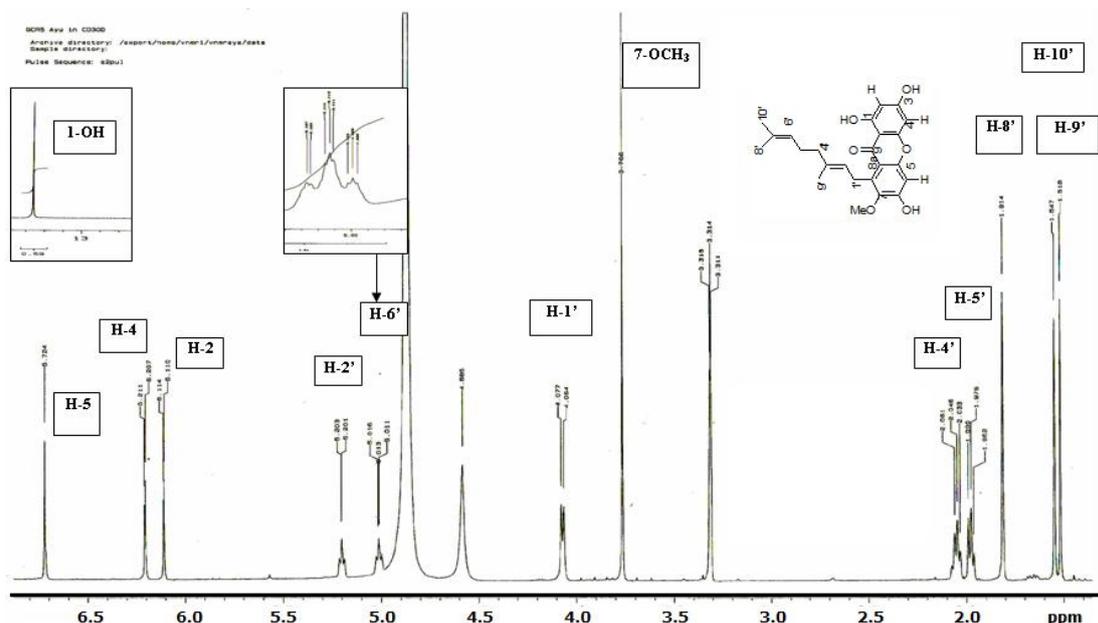


Figure 5. ¹H-NMR spectrum of rubraxanthone in CD₃OD

Precision

The precision was determined by analyzing 2.5:10 and 25 μg/mL⁻¹ of standard solution of rubraxanthone (n=3). Intra- and inter-day assay precision were determined as relative standard deviation (RSD). Intra-day precision (repeatability) involved here replicates per day and inter-day (intermediate) precision were performed on three separate days.

Accuracy

The accuracy of the method was tested by performing recovery studies. Three different levels concentration (2.5:10 and 25 μg/mL⁻¹) of the standard solution in methanol were added to the sample solution (10.43 μg/mL⁻¹) and analyzed by the proposed RP HPLC method. The recovery and average recovery were calculated. Three determinations were performed for each concentration level.

LOD and LOQ

According to ICH (1995), technical requirements for the registration of pharmaceuticals for human use recommendations, the approach based on SD of the response and the slope were used for determining the detection and quantitation limits.

RESULTS AND DISCUSSION

HPLC method with isocratic elution was developed for the quantification of rubraxanthone in stem bark extracts of *G. mangostana*. Optimization of mobile phase was performed based on asymmetric factor and peak area obtained. Different mobile phases were used but satisfactory separation, well resolved and good symmetrical peaks were obtained with the mobile phase 0.4% formic acid and acetonitrile (25:75%v/v). The retention time of rubraxanthone was found to be 12.121 min in relation to asymmetry, the peaks showed an asymmetric factor was at 0.815 (<2.5), which indicates a good baseline (Figure 6). The number of theoretical plates was found to be 14296 (>2500), which indicates efficient performance of the column. The UV spectra of Rubraxanthone showed the maximum absorption at 243nm. Thus, it was chosen as detection wave length in liquid chromatography.

The calibration curve for rubraxanthone was obtained by plotting the peak area ratio versus the concentration of rubraxanthone over the range of 2.5-25 μg/mL⁻¹, and it was found to be linear with r=0.999.

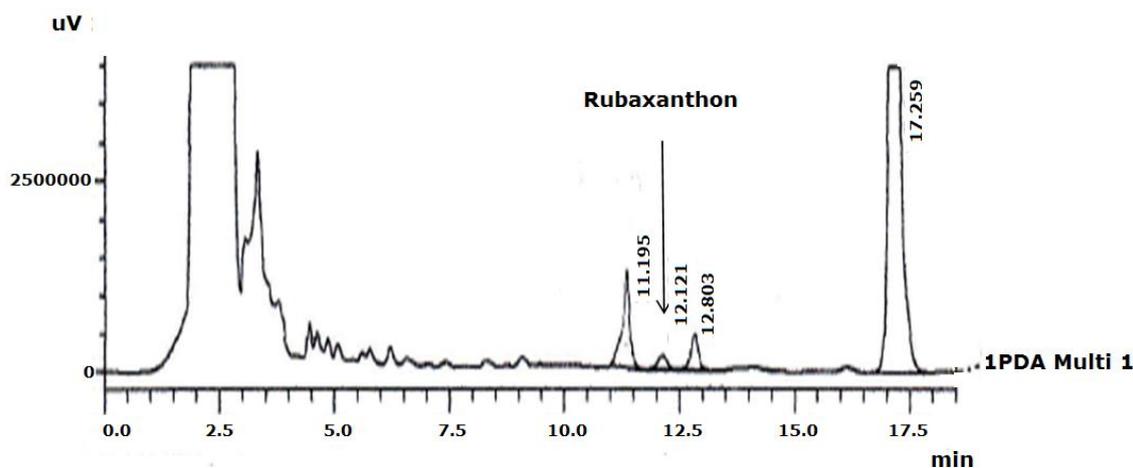


Figure 7. HPLC fingerprint of *G. mangostana* bark extract (showing a chemical structure of identified chemicals)

Table I Method validation parameters for quantification of rubraxanthon by the proposed HPLC method

| Parameters | Results |
|---|-----------------------|
| Linear range ($\mu\text{g}/\text{mL}^{-1}$) | 2.5 -25 |
| Regression equation* | $y = 156554 x - 6185$ |
| Correlation coefficient | 0.999 |
| LOD ($\mu\text{g}/\text{mL}$) | 0.47 |
| LOQ ($\mu\text{g}/\text{mL}$) | 1.56 |

* x is the concentration of rubraxanthon in $\mu\text{g mL}^{-1}$; y is the peak area at 243 nm

Table II. Precision study of rubraxanthone by the proposal HPLC method

| | Peak area | RSD (%) | Rubraxanthone ($\mu\text{g}/\text{mL}^{-1}$) | RSD (%) |
|-----------------------|-------------------|---------|--|---------|
| Intra-day precision * | 390207 ± 6247 | 1.6 | 2.532 ± 0.040 | 1.58 |
| Inter-day precision** | 383567 ± 7237 | 4.5 | 2.489 ± 0.039 | 3.20 |

The results are mean \pm SD of 9 determination. * 3 replicates were assayed on the same day; **3 replicates were assayed on 3 different days

Table III. Recovery study of rubraxanthone by the proposed HPLC method

| | Rubraxanthone added ($\mu\text{g}/\text{mL}$) | Rubraxanthone found ($\mu\text{g}/\text{mL}^{-1}$) | Recovery (%) |
|---|---|--|-------------------|
| A | 2.5 | 2.48 ± 0.07 | 99.39 ± 2.79 |
| B | 10 | 10.47 ± 0.22 | 104.47 ± 2.19 |
| C | 25 | 25.67 ± 0.28 | 102.69 ± 1.13 |

A - Low concentration, B - intermediate concentration and C - high concentration for range calibration. The result are mean \pm SD of 3 experiments

CONCLUSIONS

The proposed HPLC method promoted high precision, sensitivity and accuracy for quality control of extract of bark *G. mangostana*. This proposed method will be useful for quantitative analysis in standardization and quality assessment of extract of bark *G. mangostana* for pharmaceutical uses.

ACKNOWLEDGMENTS

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